# CARACTERIZACION DE PROTEINAS DE LA MEMBRANA LINFOCITARIA EN EL RATON.

TESIS para aspirar al grado de
DOCTOR EN BIOQUIMICA

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# I N T R O D U C C I O N

El fundamento de la bioquímica moderna, reside en el concento de la célula. Una característica importante de todas las células, procarióticas y eucarióticas, es una membrana - externa limítrofe. En las células eucarióticas, esta membrana de la superficie de la célula, se llama membrana plasmática. En años recientes, ésta se ha convertido más y más en el objeto de estudio intenso y conjeturas, desempeñando, como claramente lo hace, una parte crítica en muchas de las actividades necesarias para mantener la vida. Por ejemplo, la membrana - plasmática, no solamente controla el "influjo" y "aflujo" - (entrada y salida) de iones y nutrientes, sino que, además - contiene una variedad de sistemas de reconocimiento (receptores) para señales externas como : drogas, hormonas, antígenos y otras células. Aunque la membrana de diferentes tipos de - células varía respecto a la naturaleza y las cantidades relativas de estos receptores y moléculas que controlan la permeabilidad de la membrana, la estructura de todas las membranas plasmáticas animales de explica por el modelo del mosaico fluído de Singer y Nicolson (1972). En este modelo, las proteínas de la membrana no se encuentran fijas, sino libres - para moverse en el plano de la bicapa de lípidos.

Sin embargo, una de las preguntas clave en biología actual, es explicar la regulación de la expresión génica, que sigue a la interacción de los receptores de la superficie celular con los tipos de moléculas mencionados arriba. En la - mayoría de los casos, el evento crítico es la combinación de los efectores biológicamente activos y las moléculas de recep

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tores específicos en la membrana celular. Es por eso de suma importancia que dichas moléculas receptoras sean aisladas e identificadas, siendo el propósito a largo plazo, explicar en términos moleculares, la alteración del fenotipo celular, que sucede a las interacciones receptor-ligando.

Aunque los estudios que se esbozarán enseguida se limitan a las células de las series linfoides, la metodología es de aplicación general. (Parkhouse y Abney, 1974*).

La membrana de los linfocitos contiene marcadores antigénicos bien definidos (p. el antígeno Θ, Raff, 1971), receptores para inmunoglobulina agregada (Basten et al, 1972), receptores para el componente C3 activado del complemento (Nussenzweig y Pincus, 1972), moléculas codificadas por el complejo mayor de histocompatibilidad, y que controla las interacciones celulares en la respuesta inmune (Munro y Bright, 1976), y receptores para antígenos extraños (Parkhouse y Abney, 1976), para lectinas (Greaves et al, 1973), para insulina (Hadden et al., 1972), para hormona del crecimiento (Lesniak et al., 1973) y para histamina (Melmon et al., 1973).

Así pues, los linfocitos no son solamente células que estén relativamente bien caracterizadas, sino que también desempeñan una función definida : responden a los antígenos extraños. Por estas razones son una elección atractiva como material inicial para trabajar en la caracterización bioquímica de las moléculas de la membrana plasmática.

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* Las referencias de mi propia bibliografía se encuentran subrayadas para distinguirlas de las demás.

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Como el evento crucial en las respuestas inmunes es el reconocimiento del antígeno por los linfocitos, la mayor parte del trabajo descrito aquí, fué dirigido hacia la caracterización estructural y funcional de los receptores para el antígeno.

Sin embargo, un factor de complicación en el estudio de los linfocitos es su heterogeneidad, expresada en su forma más básica en dos subpoblaciones mayores : Linfocitos-B y linfocitos-T (Parkhouse y Abney, 1976, para revisión). Aunque se tiene la certeza de que ambos tipos de células pueden reconocer al antígeno de una manera muy específica, los sucesos - que resultan de este evento de transducción (supuestamente - localizado en la membrana) son muy diferentes para los dos - tipos celulares. Los linfocitos-B se desarrollan a células - plasmáticas, las cuales son células especializadas que secretan grandes cantidades de anticuerpo específicamente dirigido hacia los determinantes antigénicos del antígeno estimulante original. Los linfocitos-T, por el otro lado, nunca secretan anticuerpos, pero pueden matar a las células que contienen a los antígenos relevantes, a esto se debe que sean factores - tan importantes en el transplante de órganos y, se cree la - vigilancia inmune y el rechazo de células tumorales que surgen de manera espontánea.

Al inicio del trabajo que se describirá más adelante, existía la idea generalizada de que el receptor para el antígeno en los linfocitos-B y -T era exactamente el mismo, es - decir, inmunoglobulina (Marchalonis y Cone, 1973).

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Más aún, para ambos tipos de células, la inmunoglobulina receptor, se pensaba que estaba restringida a la clase IgM. - Como se demostrará más adelante este no es el caso. Los linfocitos-B no solamente poseen IgM; con frecuencia tienen además una molécula de inmunoglobulina que no se encuentra en - el suero. Esta inmunoglobulina, a la que hemos llamado IgD,- está, por lo tanto, especializada para funcionar en el contexto de la membrana linfocitaria y por consiguiente en el control de la estimulación por el antígeno. Se presentarán los - datos positivos y negativos para los linfocitos-T.

Un tropiezo para demostrar inmunoglobulina en la superficie de estas células será informado, pero la aplicación general de nuestra metodología será válida debido a la caracterización bioquímica de otras moléculas localizadas en la superficie de los linfocitos-T.

Más aún, es posible que dos de las estructuras que serán descritas pudieran haber sido identificadas erróneamente como inmunoglobulina por otros (p.e. Marchalonis y Cone - 1973). La razón para decir ésto, es que, si bien no son inmunoglobulina ciertamente, pueden ser confundidas como tal, si no hay suficiente cuidado en la ejecución de los experimentos.

## II. METODOLOGIA

La metodología está descrita en detalle en las publicaciones relacionadas con el trabajo hecho y citadas en contexto, por lo que me limitaré a dar los comentarios aclaratorios.

El reactivo crucial utilizado para la identificación y caracterización de moléculas de la superficie celular, fué anticuerpo.

La reactividad entre un anticuerpo dado y las membranas plasmáticas se toma generalmente como evidencia de la presencia del antígeno relevante en la superficie celular. Cualquiera que sea el sistema usado para detectar la reactividad, es obviamente crucial que los anticuerpos empleados en la investigación sean caracterizados estrictamente. En principio, los sueros deberán ser verificados con respecto a la presencia de especificidades indeseadas, por medio de técnicas por lo menos tan sensibles como la metodología seguida en la investigación. Por ejemplo, la ausencia de actividad de un anticuerpo contra cadenas ligeras, juzgando por análisis de difusión doble (Ouchterlony), no puede garantizar que la muestra del anticuerpo no detecte cadenas ligeras con procedimientos más sensibles, p.e. fluorescencia o radioinmuno-precipitación.

El procedimiento acostumbrado para hacer un suero anti-inmunoglobulina específico para un isotipo (clase o subclase), es pasarlo a través de inmunoabsorbentes en fase sólida, conteniendo todos los demás isotipos. Después

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de dicho tratamiento, sin embargo, aún hay posibilidades de que el antisuero tenga especificidades no deseadas. - Estas pueden ser contra componentes no buscados de manera rutinaria, como $\alpha$-2-macroglobulina, que de hecho se encuentra en la superficie de los linfocitos-B (McCormick et al., 1973). Alternativamente, un suero anti-inmunoglobulina podría tener anticuerpos dirigidos contra los determinantes de la región variable, compartidos por algunas, pero no todas, las representantes de las clases de-inmunoglobulina. Frecuentemente esas especificidades pueden escapar a la detección en muchos sistemas de prueba. Surge la posibilidad de que haya anticuerpos contra inmunoglobulina que reaccionen de forma cruzada con $\beta$-2-microglobulina debido a que se ha encontrado que hay homología en la secuencia entre estas dos entidades (Peterson et al., 1972). Como la $\beta$-2-microglobulina se encuentra-asociada con los antígenos de histocompatibilidad en la-mayoría de las células, la presencia de estos anticuerpos crearía problemas obvios pero tal vez no previstos. La - remoción de las especificidades no deseadas se debe hacer en inmunoabsorbentes con fase sólida para evadir artefactos que surjan de la presencia de complejos antígeno-anticuerpo en el suero. Un problema mayúsculo son los anticuerpos - heterófilos contra las superficies celulares. Mientras - muchos de estos se puedan remover por absorción con membranas de los órganos convenientes (p.e. hígado y riñón para anti-inmunoglobulina), en ocasiones no es posible llevar a cabo la absorción apropiada.

La última prueba de especificidad es, de hecho, -

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aislar la molécula en la membrana celular con la que se - combine el anticuerpo, y llevar a cabo la caracterización bioquímica. Esto es posible si se marca de antemano la superficie celular con iodo radiactivo, utilizando la enzima lactoperoxidasa (Philips y Morrison, 1970). Después puede disolverse la célula en disolventes disociantes (Marchalonis, Cone y Atwell, 1972) o detergentes (Vitetta, Baur y Uhr, 1971). La mezcla total de las moléculas radiactivas de la superficie celular se hacen reaccionar con el anticuerpo de prueba y el precipitado resultante es caracterizado, generalmente por electroforesis en geles de poliacrilamida conteniendo reactivos disociantes (dodecil sulfato de sodio, SDS). Al hacer esto, uno no solamente está registrando el hecho de que un antisuero reacciona con una célula, sino también se define con qué reacciona el antisuero.

En el sistema más simple, los anticuerpos están unidos con moléculas fluorescentes o radiactivas, mezclados con linfocitos vivos, que después son observados en su superficie por microscopía, p.e. la presencia de inmunoglobulina en la superficie de los linfocitos-B es demostrada rápidamente utilizando anti-inmunoglobulina conjugada con un fluorocromo.

La técnica menos ambigua para identificar proteínas de la superficie celular con anticuerpos es el radiomarcaje de las células. En principio las células marcadas son solubilizadas y los componentes de la superficie celular son purificados selectivamente por la adición del anticuerpo apropiado. El anticuerpo se combina con cualquiera de - las estructuras por las que tenga especificidad, y la precipitación de estos complejos se puede llevar a cabo con

la adición de antígeno no radiactivo (precipitación directa), o de anticuerpo contra el primer anticuerpo (precipitación indirecta). Por ejemplo, cuando es utilizado suero de conejo anti-inmunoglobulina de ratón con linfocitos-B de ratón marcados, la precipitación puede efectuarse agregando inmunoglobulina de ratón no marcada o suero de cabra anti-inmunoglobulina de conejo. Los precipitados son lavados y la cantidad de radiactividad que contienen se expresa como una fracción de la radiactividad total que se puso al inicio en forma de material macromolecular (determinado por precipitación con ácido tricloroacético). Como estos precipitados inmunológicos específicos, tienen invariablemente radiactividad atrapada o absorbida de manera inespecífica, la radiactividad precipitada deberá siempre ser caracterizada por análisis en geles disociantes.

Hay dos formas de marcar radiactivamente a las células, externamente o internamente. En el primer procedimiento (Philips y Morrison, 1970; Marchalonis, Cone y Atwell 1972; Vitetta, Baur y Uhr, 1971), las proteínas externas de la membrana celular, son marcadas con $^{125}I$, utilizando lactoperoxidasa como catalizador. En consecuencia, las células pueden ser solubilizadas simplemente y el extracto analizado por coprecipitación con los anticuerpos apropiados. La desventaja es, que una identificación positiva no garantiza necesariamente síntesis endógena por la célula. En principio, el método está sujeto a los mismos problemas que la inmunofluorescencia, pero da la caracterización molecular cuando se lleva a cabo el análisis en geles.

Para tener una prueba directa de síntesis endógena,

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las células se deben marcar internamente "in vitro" con aminoácidos (o azúcares) radiactivos. Aquí, el problema estriba en que la mayoría de la célula se marca, y que la membrana plasmática, que tiene el receptor para el antígeno, representa un porcentaje muy pequeño de la proteína celular total; aproximadamente el 5% de los linfocitos de cerdo (Allan y Crumpton, 1971; Crumpton y Snary, 1974) o de ratón (Abney y Parkhouse, datos sin publicar). Si asumimos que el receptor para el antígeno no es superior al 5% de la membrana celular, entonces el rendimiento teórico para las células marcadas interiormente , debe ser muy bajo.

Sin embargo, es posible preparar la fracción de membrana plasmática de las células, y utilizar esto como el material inicial para la coprecipitación (Parkhouse y Abney, 1974). La desventaja obvia de este procedimiento es que toma largo tiempo, aunque vale la pena llevarlo a cabo porque quita gran cantidad de "ruido" antes de llevar a cabo la coprecipitación.

Un buen ejemplo de los problemas que se pueden presentar cuando se tiñen células vivas con antisueros fluorescentes pobremente caracterizados, está dado en algo de nuestro propio trabajo. Se había publicado (Santana et al., 1974) que un suero anti-inmunoglobulina de ratón podía teñir timocitos de ratón. Los autores concluyeron, por lo tanto, que estas células tenían inmunoglobulina de superficie. Cuando sometimos su antisuero a las pruebas bioquímicas rigurosas esbozadas anteriormente, se aclaró que el componente de los timocitos que reaccionaba con el antisuero, no era inmunoglobulina (Santana et al., 1976). En lugar de ello, surgió que el antisuero contenía por lo menos dos anticuerpos :

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uno que reaccionaba con inmunoglobulina de ratón (ausente en los timocitos), y otro, responsable de la fluorescencia positiva, que reaccionaba con un componente de la membrana de los timocitos, de naturaleza diferente a la inmunoglobulina. Nosotros creemos que este material debía haber estado presente en el suero de ratón y fué copurificado subsecuentemente en la preparación de inmunoglobulina usada para inmunizar al conejo que dió el antisuero en cuestión.

La lección que se puede aprender de esto es que en el estudio de las superficies celulares los procedimientos inmunofluorescentes, solamente pueden ser totalmente válidos cuando el antisuero está bien caracterizado y esto quiere decir, caracterización bioquímica, de la discutida con anterioridad.

Un punto que vale la pena mencionar es que, el análisis en geles del material aislado de las superficies celulares, debería hacerse siempre con reducción química previa y sin ella; esto tiene el objeto de determinar la presencia o ausencia de subunidades unidas por disulfuros, en la molécula que se está estudiando. Otra vez, la importancia de esta precaución, se puede ilustrar con nuestro trabajo. Está claro que los timocitos pueden tener en la superficie una molécula que, en los geles con dodecil sulfato de sodio (SDS), co-emigra con inmunoglobulina no reducida (Parkhouse y Abney, 1974). Lo que es más, es precipitada por ciertos antisueros contra inmunoglobulina de ratón (Santana et al., 1976). Sin embargo, estaríamos muy equivocados al concluir que esta molécula es inmunoglobulina. De hecho, no es reducible a cadenas pesadas

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(H) y ligeras (L), las cuales, por medio de puentes de disulfuro, dan como resultado la estructura $H_2L_2$, característicamente de inmunoglobulina.

Así pues, se encontró que esta molécula de los timocitos muestra el mismo peso molecular alto (aproximadamente 200,000) cuando es analizada con reducción química, o sin ella. Por tanto tiene una sola cadena polipeptídica en su molécula y no una estructura con subunidades.

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## II. RESULTADOS

### a) Linfocitos-T

No hemos sido capaces de demostrar la presencia de inmunoglobulina en la superficie de los timocitos o los linfocitos-T periféricos (Julius et al., 1973), cuando las células estaban marcadas externamente con $^{125}I$. Los extractos de células se prepararon con Nonidate P-40 al 1% (p/v), ó - deoxicolato de sodio al 1% (p/v) o con Urea 9 M-ácido acético 1.5M. En todos los casos, se recuperó esencialmente toda la radiactividad incorporada a las células, en forma soluble, después de añadir el agente extractor. La remoción por diálisis, del material de peso molecular bajo, dió - excelente rendimiento de material precipitable con ácido tricloro acético (TCA) en los lisados con detergente, sin embargo en los lisados con urea-ácido acético hubo mucha - agregación, que causó rendimientos variables (10-80%), al ser dializados. La adición de una gran variedad de reactivos anti-inmunoglobulina de ratón (con las especificidades para todas las clases conocidas de cadenas pesadas y ligeras) a los lisados dializados, fué incapaz de precipitar cantidades detectables de inmunoglobulina de ratón.

También estuvo ausente cuando se añadieron inhibidores de enzimas proteolíticas (Trasylol, fluoruro de fenilmetil-sulfonilo, iodoacetamida y ácido-$\varepsilon$-amino caproico) en el momento de hacer la lisis y durante los procesos de aislamiento. Estos resultados contrastan con los que - obtuvimos con linfocitos esplénicos, donde la inmunoglobulina era rápidamente detectable. El Nonidate P-40 dió los mejores resultados, el desoxicolato de sodio los dió satisfactorios y la urea-ácido acético fué errática. Como -

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Marchalonis y Cone han dicho y publicado enfáticamente que - la inmunoglobulina de timocitos no se solubiliza con Nonidate P-40, la perla obtenida después de centrifugar los extractos de NP-40 de los timocitos marcados, se extrajo con urea-ácido acético, pero de nuevo, los resultados fueron negativos.

La controversia de que los timocitos tengan inmunoglobulina de superficie o no, ha girado alrededór de aspectos metodológicos, en particular, la elección del disolvente extractor, elección del anticuerpo usado para la purificación de la inmunoglobulina de superficie y la posibilidad de proteólisis de la misma. Teniendo en cuenta que la inmunoglobulina de las células T se ha caracterizado como una molécula $\mu_2L_2$ unida - por disulfuros (Marchalonis y Cone, 1973), diseñamos un experimento en el cual todas estas variables metodológicas pudieran ser ignoradas (Parkhouse y Abney, 1974). Las células marcadas con $^{125}I$ se extrajeron por 3 minutos a 100°C con dodecil sulfato de sodio (SDS) al 4%-iodoacetamida 0.10M-fosfato de - sodio 0.05M, pH 7.0. El extracto se dializó por 2 horas (SDS-2%-iodoacetamida 50 mM-fosfato de sodio 0.05M, pH 7.0), se - mezcló con $^{131}I$-IgM parcialmente reducida y así, directamente se aplicó a un gel de poliacrilamida-SDS. Se examinaron tres poblaciones celulares : células de bazo normal, células de - bazo "B" (provenientes de animales timectomizados a las dos semanas, irradiados letalmente y reconstituídos con hígado - fetal) y timocitos. Con el marcador interno de $^{131}I$ como referencia, la región correspondiente a $\mu_2L_2$ de cada gel fué eluída con SDS al 2% ditiotreitol 2 mM-fosfato de sodio 50 mM, pH 7.0. El eluado se calentó (15 min. a 100°C) para asegurar la reducción completa, se alquiló (con iodoacetamida - 10 mM) y se volvió a someter a electroforesis. Así fué posible determinar, por lo tanto, si el material de la superficie

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celular marcado con $^{125}I$ con la movilidad de $\mu_2L_2$, se podía resolver en cadenas pesadas y ligeras.

Cuando se analizaron los segundos geles, estaba claro que el marcador interno ($^{131}I$- $\mu_2L_2$) se había reducido a cadenas $\mu$ y L. Una porción considerable de material de la superficie celular ($^{125}I$) del "bazo B" que co-emigró con la $^{131}I$-$\mu_2L_2$ era sensible a la reducción, dando un pico en la parte correspondiente a las cadenas $\mu$. La fracción comparable obtenida de los timocitos no fué sensible a la reducción. Por lo tanto los timocitos no tienen inmunoglobulina de superficie, similar a $\mu_2L_2$ unidas por disulfuros, que pueda ser revelada por marcaje externo. El material presente en la superficie de los timocitos de ratón, que co-emigra con $\mu_2L_2$, y que se encuentra presente en cantidades relativamente altas, no tiene su contraparte en las células de bazo y por lo tanto puede ser una proteína de superficie particular de los timocitos.

Si la inmunoglobulina estuviera presente en los timocitos, pero inaccesible a la iodación, entonces esto se debería revelar con el marcaje interno. De acuerdo con esto, los timocitos fueron marcados "in vitro" con $^{3}H$-Leucina de 4 a 24 horas y se prepararon sus membranas. Sin embargo, fuimos incapaces de detectar inmunoglobulina en esas muestras de membranas plasmáticas (Límite de detección : 0.1% de la radiactividad total), aunque las membranas de células de bazo preparadas de igual manera, contenían inmunoglobulina fácilmente demostrable (3% de la radiactividad total). Cuando se agregaron células de bazo internamente marcadas a un gran exceso de células de bazo o timocitos no marcados, las membranas plasmáticas preparadas en ambos casos, rindieron can

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tidades semejantes de inmunoglobulina. Por lo tanto tenemos confianza de que nuestros datos negativos para los timocitos no se deben a proteólisis (Parkhouse y Abney, 1974).

Los experimentos arriba descritos, arguyen en contra de la presencia de inmunoglobulina en la superficie de los timocitos. Para complementar nuestro estudio, cambiamos al sistema de "liberación metabólica" (metabolic release) -- (Abney y Parkhouse, 1976).

Aquí, los timocitos marcados con $^{125}I$, son incubados "in vitro" por 2 a 4 horas y el medio es usado como una - fuente de proteínas de la superficie celular. Se ha dicho - que, utilizando este sistema (Cone et al., 1974), la inmuno globulina es liberada de los timocitos y es citofílica para los macrófagos. La inmunoglobulina fué caracterizada como - un polipéptido con la movilidad de cadena -$\mu$ en geles de - SDS. En nuestros experimentos, los timocitos marcados en la superficie se cultivaron "in vitro" por 4 horas. El medio de cultivo se dializó contra solución de fosfatos (0.05 M, -- pH 7.0)- Na Cl 0.15M (PBS) fría y se dividió en tres porcio nes : (a) Control, no recibió ningún tratamiento, (b) Preci pitación no-específica, IgG normal de conejo con suero de - cabra anti-IgG de conejo, (c) Precipitación con anti-inmuno globulina, conejo anti- (IgM ($\lambda_1$), IgA ($\lambda_2$), $IgG_1$ (K), - $IgG_{2a}$ (K) y cadenas K libres, todas de ratón) más suero de cabra anti-IgG de conejo. Los precipitados se removieron y los sobrenadantes se incubaron con macrófagos (células del exhudado peritoneal de ratones inyectados con 1 ml de pro- teosa peptona al 2%, por vía intraperitoneal 4 días antes - de sacrificarlos), por 90 minutos a 0° . Las condiciones de incubación fueron tales que, 2.5 X $10^5$ macrófagos se mezcla ron con el material liberado de 2 X $10^6$ timocitos.

Las células se centrifugaron y lavaron con PBS, se disolvieron con SDS y se aplicaron en geles de poliacrilamida con reducción (con ditiotreitol) o sin ella.

Cuando las tres muestras fueron probadas, se observó que habían absorbido cantidades muy semejantes de radiactividad (aproximadamente 4% de la cantidad inicial). En el análisis en gel de las muestras reducidas, la mayoría del material absorbido se encontró que migraba en la misma posición que la cadena -$\mu$; sin embargo, este componente se encontró cuando las células fueron incubadas con las tres diferentes fuentes de material de superficie. Como una de las muestras fué absorbida con suero poliespecífico anti-inmunoglobulina de ratón, el material radiactivo en la posición de la cadena $\mu$ no puede ser inmunoglobulina. Más aún, cuando las muestras no fueron reducidas antes del análisis en gel, el pico en la posición de la cadena-$\mu$ persistió, en ausencia de un componente radiactivo en la posición de IgM monomérica ($\mu_2L_2$). - Es posible, aunque no es una prueba concluyente, que esta molécula haya sido confundida anteriormente y considerada inmunoglobulina.

Otra causa posible de identificación positiva de inmunoglobulina en los linfocitos-T es el antígeno de peso molecular alto (200,000) que se detectó en esas células por impureza de los antisueros anti-inmunoglobulina de ratón (Santana et al., 1976; ya discutido en la sección II). Como esta molécula era estable a la acción de los agentes reductores y no reaccionaba con anticuerpos anti-inmunoglobulina de ratón, - cuidadosamente preparados (Parkhouse y Abney, 1974; Abney y Parkhouse, 1976; Santana et al., 1976), no era inmunoglobulina, sin embargo, en vista de que pudimos demostrar la pre-

sencia de esta proteína, los aspectos técnicos del trabajo no eran responsables de la incapacidad para demostrar inmunoglobulina. En otras palabras, fuímos capaces de demostrar un componente de la superficie de las células-T, a pesar de que este no era inmunoglobulina. Este punto fué enfatizado posteriormente con la caracterización exitosa de la nucleótido pirofosfatasa de la superficie de ambos tipos celulares : linfocitos-B y -T del ratón (Abney et al. 1976).

En trabajo previo se mostró que la nucleótido pirofosfatasa era una ecto-enzima de los hepatocitos del ratón (Evans et al., 1974). Después del aislamiento de la enzima, se preparó un antisuero en conejos y se usó como reactivo para una molécula semejante en la superficie de los linfocitos.

Los linfocitos aislados de bazo de ratón, hidrolizaron UDP-galactosa añadida al medio. La fracción de membranas plasmáticas de linfocitos, se encontró enriquecida de la actividad de nucleótido pirofosfatasa (responsable de esta hidrólisis) y en forma semejante, la fosfodiesterasa alcalina y la 5' nucleotidasa. Las superficies celulares de los linfocitos de bazo y del timo de ratón se radiomarcaron con $^{125}I$ utilizando el método catalizando con lactoperoxidasa. Los extractos de detergentes de las células se mezclaron con suero anti-nucleótido pirofosfatasa de membrana plasmática de hepatocitos de ratón, purificada, y los inmunoprecipitados se analizaron por electroforesis en geles de poliacrilamida. Solamente se observó un componente radiactivo predominante, de tamaño similar a la enzima del hígado (P.M. aparente 110,000 - 130,000). La electroforesis de una fracción de membrana plasmática de bazo marcada con $^{125}I$ mostró varios picos. Cuando se pasaron extractos de

detergentes de linfocitos de bazo por columnas de sefarosa conteniendo antisuero contra nucleótido pisofosfatasa unido convalentemente, la actividad quedó retenida en las esferas, mientras que se eluyeron otras proteínas y la actividad de leucina-naftilamidasa. Los resultados indican que las actividades de nucleótido pirofosfatasa y la fosfodiesterasa alcalina, se deben a la localización de las mismas o enzimas similares en la cara externa de la membrana plasmática de los linfocitos.

No se comprende totalmente la función de una nucleótido pirofosfatasa que se encuentra en la superficie de los linfocitos y otras células de mamífero. La enzima puede servir para restringir la entrada de algunos nucleótidos a la célula, p.e. productos de degradación de RNA, o puede servir para conservar los nucleótidos celulares, asegurando su hidrólisis a nuceósidos en la membrana plasmática, en conjunción con las nucleótido fosfohidrolasas (p.e. 5' nucleotidasa). Existen mecanismos específicos para el transporte de los nucleósidos hacia el interior de las células (Berlin y Oliver, 1975); la liberación de adenosina por los linfocitos al suero, puede causar vaso-dilatación (Dobson et al., 1971), asegurando así el movimiento de estas células a través de vasos sanguíneos muy estrechos. Es claro que el conocimiento de la topografía de las enzimas de la membrana plasmática es el primer paso dirigido hacia la determinación de sus posibles implicaciones en el transporte y en el metabolismo de los nucleótidos en las células de mamífero.

De cualquier manera, la presencia de esta actividad enzimática en la superficie de los linfocitos, ciertamente

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hace que la idea de intercambio de información entre linfo citos a través de moléculas de RNA, sea muy poco probable ya que la actividad enzimática descrita, se esperaría que degradara a las moléculas de RNA.

En resumen, en una serie de experimentos muy riguro sos, hubo falla total en demostrar inmunoglobulina en la - superficie de los linfocitos-T, en condiciones en que la presencia de inmunoglobulina de superficie era fácilmente demostrable en los linfocitos-B (ver sección III-b, adelan te).

Se presentaron dos posibles candidatos que erróneamente pudieran haber sido identificados como inmunoglobulina. Esto, unido a la identificación positiva de la nucleó tido pirafosfatasa en la superficie de las células-T (y - células-B) arguye en contra de "problemas metodológicos" como causa de los datos negativos.

Experimentos recientes (Binz y Wigzell, 1977) confirman nuestras conclusiones, ya que identifican como el recep tor para el antígeno de las células-T, a una molécula de peso molecular 150,000, compuesta de dos cadenas polipeptídicas de peso molecular semejante, unidas por puentes di sulfuro. Así pues, esta molécula no tiene cadenas ligeras como la inmunoglobulina. Más aún, los antisueros contra - todas las clases conocidas de cadena pesada, no reaccionan con preparaciones de receptor de células-T purificado.

Resultados semejantes han sido publicados por Krawinkel y Rajewski (1976).

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b) Linfocitos-B

La mayoría del trabajo en las células-B ha sido publicado (ver referencias) y revisado también (Parkhouse et al., 1976). Esta sección será breve y solamente haciendo resaltar algunos puntos importantes.

Los resultados que obtuvimos en el ratón (Abney y Parkhouse, 1974) estuvieron estrictamente coincidentes con el trabajo de otros (Vitetta et al., 1975; Melcher et al., 1974) y se puede resumir como sigue : los linfocitos de bazo o de nódulos linfáticos se marcaron externamente con -- icdo radiactivo, usando lactoperoxidasa, la inmunoglobulina marcada se removió selectivamente por precipitación con anticuerpo específico anti-inmunoglobulina de ratón. Al ser caracterizados por eletroforesis en geles disociantes, la inmunoglobulina de superficie era predominantemente de -- estructura $H_2L_2$, unidas con puentes disulfuro; aunque se encontraron presentes algunas subunidades HL. No se encontró inmunoglobulina 19S. Utilizando antisueros específicos para las cadenas pesadas del ratón, no pudimos detectar IgG ó IgA. La precipitación con anti-$\mu$ removió parte, pero no toda la inmunoglobulina de superficie. La inmunoglobulina remanente, después de precipitar con anti cadena-$\mu$ (y remover el precipitado), se aisló con la adición de anti-cadena K, y después de haber sido sometida a reducción, rindió cadenas pesadas con tamaño intermedio entre $\mu$ y $\gamma^1$, y también cadenas ligeras.

Podemos concluir que hay dos clases de cadenas pesadas, expresadas en la superficie de los linfocitos de ratón. Una es IgM, y la otra, como no reacciona con anti-$\mu$ , anti-$\alpha$ ó anti-$\gamma^1$, parece ser un homólogo de IgD. Este candidato pa

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ra IgD en el ratón, semeja a su contraparte humana, en el tamaño de la cadena pesada, en la susceptibilidad tan marcada a la proteólisis y en su presencia en la superficie de los linfocitos. Es demasiado pequeña para ser IgE. No consideramos ni como la posibilidad que la presencia de las células-T sea la causante de esta heterogeneidad, ya que obtenemos los mismos resultados con células de bazo de ratones desnudos (atímicos congénitos). Además las células-T periféricas, purificadas por paso a través de columnas de nylón (Julius et al., 1973), no contenían inmunoglobulina, usando la misma metodología.

Utilizando células de bazo de ratones CBA de seis semanas de edad, o ratones desnudos, encontramos que el 60% de la inmunoglobulina de superficie es IgM; el resto IgD. Por lo tanto, la expresión de IgD en la superficie celular, no depende de la presencia de los linfocitos-T. Sin embargo, en el hígado fetal, y en hígado o bazo de ratón neonato, solamente se detecta IgM, indicando que en el ratón la expresión de IgM antecede a la de IgD en el desarrollo embrionario. Este hallazgo está de acuerdo con resultados en el humano (Vossen y Hijmans, 1976). Anteriormente se había pensado que la IgD antecedía a la IgM en el humano, y los resultados estaban basados en comparaciones hechas entre sangre periférica de adulto y de cordón umbilical (Knapp et al., 1973; Rowe et al., 1973), sin embargo, estos resultados al parecer son incorrectos. Es interesante señalar que la secuencia de aminoácidos en la IgD humana es más homóloga con la IgE que con la IgG ó IgM, (Spiegelberg, 1975), lo que sugiere que la IgD evolucionó algún tiempo después de la formación del gene para cadenas $\mu$.

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Así pues, la IgD aparece de manera subsecuente a la IgM en evolución y en ontogenia.

Una observación curiosa fué la diferencia marcada entre los linfocitos-B del bazo y los de los nódulos linfáticos, ya que, aunque solo se encontraron IgM e IgD en ambas poblaciones, la relación IgM/IgD fué de 1.5 en el bazo y 0.3 en los nódulos. Considerando esto paralelamente al hecho de que la IgD aparece después de la IgM en la ontogenia, se sugiere que la población de linfocitos-B en los nódulos linfáticos sea más madura que la población presente en el bazo. Podemos hacer notar que las células de bazo responden a la estimulación con lipopolisacárido de E.coli, mientras que las de nódulos linfáticos, no (G. Janossy y R.M.E. Parkhouse, sin publicar) y que las células de bazo secretan básicamente IgM, mientras que el mayor producto de las células de los nódulos linfáticos es IgG (Parkhouse 1973).

El procedimiento de marcaje externo puede dar la cantidad total de IgM e IgD, pero no su distribución en células individuales. Basados en la rareza de que se presenten otras inmunoglobulinas en la superficie de los linfocitos-B, aparte de IgM e IgD, se utilizó tinción inmunofluorescente para indicar que en el ratón, como en el humano, hay células de bazo con IgM e IgD en la superficie y otras que tienen ambas (Parkhouse y Abney, 1975, Parkhouse et al., 1976). En esos experimentos, la IgM fué la primera en ser cubierta con anti-cadena $\mu$ marcada con rodamina (formación de casquete), y después una segunda tinción con anti Fab-K marcado con fluoresceína, en presencia de azida de sodio, para obtener una tinción en forma de anillo (inhibir la for

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mación del casquete), que se tomó como evidencia de la presencia de IgD.

Recientemente se ha obtenido un antisuero específico para la IgD de ratón, que ha confirmado el resultado anterior (Abney et al., 1976b). Utilizando este antisuero se pudo mostrar que la mayoría de los linfocitos-B en los nódulos linfáticos y en las placas de Peyer expresan solamente IgD, aunque algunos expresan a ambas (IgM e IgD) y se vieron ocasionalmente células con IgM solamente. Sin embargo, en el bazo, las tres clases de células estuvieron presentes en proporciones semejantes. Al observar con más atención a las células con tinción doble, se notó una variación considerable en las intensidades relativas de ambos fluorocromos, lo que sugiere una variación en la relación de IgM e IgD de célula a célula, es decir hay toda una gama de células, que van, desde las que tienen mucha IgM y poca IgD, hasta las que tienen la relación contraria : poca IgM y mucha IgD. Esto, junto con la observación de los tres tipos de células (i.e. IgM, IgD e IgM-IgD), y el hecho de que la expresión de la IgM antecede a la expresión de la IgD en la ontogenia, sugiere fuertemente una secuencia en el desarrollo de la expresión de la inmunoglobulina en los linfocitos-B; que va de IgM a IgD con el paso a través de células intermedias con ambos isotipos. Así pues, los linfocitos-B de los nódulos linfáticos y de las placas de Peyer estarían constituídas por células más maduras. Es interesante mencionar dentro de este contexto, el hallazgo de que, los esplenocitos (células de bazo) grandes e inmaduros en el ratón, están enriquecidos con IgM, mientras que los

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más pequeños, y se piensa, más maduros, están enriquecidos de IgD (Goodman et al., 1975).

Estas consideraciones nos llevaron a examinar la posibilidad de que estuviera especializada a actuar como receptor para el antígeno en las células de memoria (Abney et al., 1976c). Sin embargo, encontramos que las células de memoria de dos clonas de células formadoras de anticuerpo, tenían receptores de la clase IgM, a pesar de que las clonas habían estado produciendo anticuerpos anti-DNP de $IgG_1$ ó $IgG_{2a}$, a lo largo de 9 a 15 meses, con estimulación antigénica. Así pues, hay evidencia de que hubo un cambio fenotípico en la región constante de la cadena pesada, después de re-exponer al antígeno a estas células de memoria. Se llegó a estas conclusiones porque la remoción de la IgM de la población de células de memoria inhibió el reconocimiento del antígeno y la diferenciación subsiguiente a células secretoras de IgG; sin embargo esto no sucedió al remover IgG ó IgD de la superficie celular. Así pues, la IgM de superficie no puede ser responsable solamente de la inducción de tolerancia inmune, así como tampoco se puede pensar que la IgD sea el único receptor para el antígeno para disparo de respuesta en las células B de memoria. En lugar de ello, puede haber heterogeneidad en los receptores de inmunoglobulina en los linfocitos-B de memoria(o de otro tipo), que refleja la presencia de subpoblaciones que van surgiendo en la presencia de la selección antigénica, o en su ausencia. Tenemos cierta evidencia preliminar (Cooper et al., 1976), que sugiere que los precursores inmediatos de las células con IgG en la superficie, pueden expresar, IgM ó IgD. Así pues, la expresión de lgG puede llevarse a cabo de manera independiente de IgD, lo que estaría de

acuerdo, con nuestros datos, de que, por lo menos algunas células-B de memoria reconocen al antígeno a través de - IgM.

Los detalles en cuanto al control y la evolución - de la expresión de inmunoglobulina, quedan aún pendientes de aclarar, así como el papel que desempeña la IgD, de una manera definida. Las células que llevan IgD, ciertamente derivan de linfocitos precursores IgM positivos y la alta frecuencia de linfocitos que expresan dos clases de inmuno globulina tienen implicaciones profundas para el mecanismo de integración génica V-C.

Es un hecho extraordinario que la IgD no se hubiera detectado antes en los linfocitos-B; sin embargo no se puede detectar en el suero normal de ratón (Abney et al., 1976b), por lo que su abundancia en las células-B debe implicar un papel como receptor para el antígeno. Esto, tan fácil de - decir, no es particularmente explícito; de momento podemos pensar en posibles funciones para IgD. Como se discutió - antes, puede haber una secuencia de desarrollo, y de acuerdo con esto los linfocitos inmaduros con IgM se desarrollan a linfocitos maduros con IgD pasando por un estado intermedio en que tiene a ambos isotipos. ¿Será posible que cada uno de estos tipos celulares desempeñe funciones inmunológicas diferentes? ¿Será que todos los linfocitos-B pasan por estos tres diferentes y bien distinguibles estadios, o será que algunos son diversificados en otras direcciones (p.e. IgG) en cualquier estadio de la ruta de diferenciación? La respuesta será dada probablemente de una forma definitiva con tiempo y experimentación utilizando la "sorteadora de células activadas por fluorescencia". Una pregunta de lo más

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intrigante es, si IgM e IgD dan diferentes señales al - estar simultáneamente en un linfocito. En cierta manera se podría pensar que no, ya que ambas comparten la misma región variable. Sin embargo, una pregunta clave, podría ser la forma exacta de inserción de estas dos inmunoglobulinas en la membrana, p.e. ¿Estarán unidas o - asociadas al mismo componente de la superficie celular o a diferentes? Otro aspecto importante, es ¿cuando surgió IgD exactamente en la evolución? Si este isotipo no se encuentra p.e. en peces elasmobranquios, pueden tenerse algunas pistas sobre su función. La susceptibilidad tan marcada de la IgD a la proteólisis, se puede concebir como una pista; de hecho, se ha sugerido un modelo para la estimulación del linfocito-B, que considera crucial la ruptura de IgD, siguiendo un estudio sistemático en el efecto de la tripsina sobre la inmunoglobulina de superficie (Bourgois et al., 1977).

Las dos inmunoglobulinas de superficie (IgM e IgD), fueron dirigidas por tripsina individualmente, y sus productos fueron caracterizados por análisis en geles. Su - susceptibilidad a la tripsina, y los productos que liberaron, son muy semejantes a los correspondientes en el - humano. Así pues hay evidencia de homología estructural entre la IgD humana y de ratón, dado que los perfiles de los fragmentos resultantes de la digestión fueron notablemente semejantes. La susceptibilidad extrema a la proteólisis, de la IgD de superficie, contrastó con la resistencia de la IgM de superficie. A temperatura ambiente y a tiempos tan largos como cinco horas de incubación con - tripsina hay muy poca conversión de IgM a los fragmentos Fc y Fab. Por otro lado, la IgD de superficie fué marca-

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damente susceptible. Hubo una conversión de 80% a Fab y Fc en 10 minutos a 0°C. Es razonable pues, sugerir que las propiedades bioquímicas de las inmunoglobulinas M y D de superficie están relacionadas con sus funciones respectivas. Se ha propuesto (Vitetta y Uhr, 1975) que la proteólisis de IgD en la célula, se lleva a cabo - después de que ha habido interacción con el antígeno, lo que produce un cambio en la molécula, la cual expo ne el sitio para ruptura (en la región de la bisagra) por enzimas proteolíticas, que hace que en el Fc se - exponga un sitio necesario para el disparo de la célula. Una hipótesis alternativa sería que el complejo antíge no-Fab (anticuerpo) es liberado de la célula por lo - que la ruptura jugaría un papel fundamental. Así pues, nuestra proposición es que, el papel fundamental de la IgD de superficie, es el liberar un fragmento (Fab $\delta$) después de exponerse al antígeno, con la proteólisis subsiguiente. Este fragmento, que por supuesto tiene a las porciones variables (esto es, determinantes - idiotípicos), sirve para permitir una respuesta regula dora anti-idiotipo, que actúa a través del reconocimien to del idiotipo de IgM; resistente a la proteasa, y que persiste en la superficie de la célula. Esta proposi- ción, que es enteramente hipotética, se apoya en el he cho de que muchas células-B sensibles al antígeno, -- tienen IgM e IgD en la superficie. Cuando esto sucede, ambas clases de inmunoglobulina se dirigen al mismo de terminante antigénico, ya que comparten las mismas re- giones variables (y por lo tanto idiotipos) (Parkhouse y Abney, 1976, revisión). Aunque la sugerencia está ba sada en datos puede resultar correcta o incorrecta, pe

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ro tiene la ventaja de que se puede probar experimentalmente.

En resumen, la caracterización de la inmunoglobulina de la superficie de los linfocitos-B, mostró que - además de IgM, había una cantidad vasta de una inmunoglobulina que llamamos IgD, no detectable en el suero. Se - piensa pues, que esta última clase de inmunoglobulina sirve para una función especializada, que aún está por definirse. Se llevaron a cabo experimentos que excluyeron la posibilidad de una función exclusiva en las células-B de memoria para esta molécula. En otros experimentos se encontró que la IgD de superficie es extremadamente sensible a la proteólisis, lo que es contrario para la IgM de superficie. Como consecuencia se propuso una función hipotética (pero probable) para la IgD, que promueva una respuesta reguladora anti-idiotipo.

El trabajo descrito que se llevó a cabo para definir el papel biológico de la IgD de la superficie de los linfocitos, no se habría podido hacer, sin haber diseñado los procedimientos para el aislamiento de la IgD de - superficie (Abney et al., 1976b), así como preparar un - antisuero específico para IgD, para utilizarlo como un - auxilio metodológico.

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# CARACTERIZACION DE LA INMUNOGLOBULINA DE SUPERFICIE DE LOS LINFOCITOS-B DE RATON.

TESIS para aspirar al grado de
DOCTOR EN BIOQUIMICA
Presenta :

REBECA ABNEY SOTO

# I N T R O D U C C I O N

Los experimentos clásicos de Gowans y sus colaboradores (1) establecieron que el linfocito era la célula - clave que al reaccionar con el antígeno, por contacto, se transforma en la célula efectora de las respuestas inmunes, (humoral y celular) como resultado de una serie de eventos diferenciativos complejos y poco entendidos.

Un aspecto de la respuesta inmune, es su especificidad, y se explica por su selectividad hacia el antígeno, como lo predicen los autores que apoyan las hipótesis selectivas de la inmunidad (2-4). Asi pues, los receptores para el antígeno en un linfocito individual son de una so la especificidad, pero la especificidad varía de célula a célula en la población total de linfocitos de un organismo. En consecuencia, cuando un determinante inmunogénico se introduce en un animal vertebrado, sólo una pequeña - porción de células con las estructuras adecuadas en los - receptores son capaces de interactuar con el antígeno y - por lo tanto son seleccionadas. Enseguida de la interacción con el antígeno dichas células se dividen y diferencian a células efectoras. Durante este proceso, algunas células se mantienen como una colección aparte, como células de - memoria, las cuales forman una población expandida que, - con una exposición subsecuente al mismo antígeno, provoca la bien documentada respuesta inmune secundaria.

Sin embargo, a pesar de que los linfocitos se puedan ver muy similares bajo el microscopio de luz, son poblaciones heterogéneas de células.

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La división más simple y más amplia que se puede hacer es : Linfocitos-B y linfocitos-T, aunque es cierto que hay una proporción pequeña de células que tienen las características morfológicas de los linfocitos y no pueden entrar en ninguna de estas dos categorías. Estos posiblemente sean los precursores de las células-B o las células que funcionan en las reacciones citotóxicas mediadas por anticuerpos. El fundamento de esta división se apoya en los experimentos hechos en pollos (5,6). Con la remoción del timo se encontró el menoscabo de la respuesta celular, mientras que con la remoción de la bursa de Fabricio se deprime profundamente el desarrollo de la respuesta inmune humoral. En los ratones, de una manera similar, la remoción del timo conlleva una depresión de la respuesta inmune celular (7). Hasta la fecha no ha sido posible señalar en el mamífero el equivalente de la bursa en el pollo, y la evidencia reciente sugiere que es multifocal (8). Sin embargo, fué posible mostrar que en el ratón, los precursores de las células secretoras de anticuerpos son linfocitos-B y que su maduración depende frecuentemente de la interacción con linfocitos-T (9). Este concepto de una interacción entre linfocitos-B y -T, tuvo origen en los experimentos en que la mezcla de ambos tipos de células eran considerablemente más eficientes en transferir las respuestas inmunes, que cualquier tipo celular sólo (10). También estaba claro que el linfocito-T, aunque prolifera cuando se le presenta el antígeno, no se diferencia en célula secretora de anticuerpos (11-13).

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El esquema se completó con el advenimiento de los marcadores de la superficie celular para los linfocitos-B y -T (14) y la demostración de que en muchas respuestas - inmunes, mientras los linfocitos-B pueden sintetizar el - anticuerpo, el linfocito-T es necesario para la inducción ("ayuda") (15). Los marcadores de superficie celular más populares son : inmunoglobulina y Teta-1 para los linfocitos-B y -T respectivamente. Así pues, los linfocitos-B llevan inmunoglobulina en su superficie y se diferencian en - células-B de memoria y células secretoras de anticuerpos - cuando son expuestas al antígeno, comúnmente con la influencia colaboradora de los linfocitos-T. Los linfocitos-T por si mismos son responsables de los fenómenos variados de la inmunidad mediada por células. Sobrepuestos a este esquema ya complejo, están : los posibles papeles desempeñados por los macrófagos en la inducción inmune, y los efectos positivos (ayuda) y negativos (supresión) de los linfocitos-T sobre las respuestas de los linfocitos-T y -B (16,17).

Asi pues una pregunta clave en la Biología moderna es la regulación de la expresión génica que sigue a la interacción de las células con hormonas, compuestas farmacologicamente activos, antígenos y otros tipos de células.

En la mayoría de los casos, el suceso crítico es - la combinación entre los efectores biológicamente activos y las moléculas receptoras específicas localizadas en la - membrana celular. Por lo tanto es de gran importancia que dichas moléculas sean aisladas e identificadas, y el proyecto a largo plazo será explicar en términos moleculares, las alteraciones del fenotipo celular que sigue a las interacciones del receptor con el "ligando".

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Aunque en el presente trabajo se restringe el estudio a las células linfocéticas, la metodología es de aplicación general a otros sistemas.

En la actualidad hay evidencia experimental de que los linfocitos-B de una gran variedad de especies animales tienen inmunoglobulina asociada con la membrana celular y que funciona como receptor para el antígeno (18).

La clase predominante en los conejos (19) y en los ratones (20) es la IgM, sin embargo la IgG asociada a la - membrana de los linfocitos, está presente, pero hasta cierto punto depende de la especie animal, fuente de las células (tejido) y método de detección (20,21,22).

En los estudios hechos sobre la secreción de la IgM de tejidos linfoides del ratón, se ha encontrado que la polimerización de las subunidades 7S a la forma pentamérica-19S, se lleva a cabo a un tiempo muy cercano a la secreción (23,24). En este momento algunos residuos de cisteína dejan de estar bloqueados (25), tal vez por la acción de una enzima "rearreglasa de disulfuros" (26) y cadena "J", se incorporan la fucosa y la galactosa a la molécula (27,28). - Así pues, el proceso de polimerización requiere de la integración de varios sucesos bioquímicos definidos.

De aqui la idea de que los determinantes de IgM - presentes en la superficie de las células linfoides exista como subunidades 7S deficientes en carbohidrato(29). Esta predicción ha estado apoyada por el aislamiento de IgM 7S de linfocitos marcados "in vitro" con $^{3}$H-Leucina (30) y - por el radiomarcaje de la superficie de los linfocitos - (31,32). Mientras hay acuerdo en que este es el caso para

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los linfocitos-B, la presencia o ausencia de inmunoglobulina identificable en la superficie de los linfocitos-T, está en controversia (31,32).

En vista de los paneles desempeñados por los linfocitos-T en la inmunidad celular y tal vez en la vigilancia inmune, es de importancia crucial que se demuestre la naturaleza del receptor de la superficie celular de una manera satisfactoria, y esta es la intención inmediata de este trabajo.

# M E T O D O S

## ESBOZO DEL ENFOQUE EXPERIMENTAL :

Todos los experimentos se llevaron a cabo en ratones.

## MARCAJE ENZIMATICO DE LA SUPERFICIE CELULAR :

Las susnensiones de células fueron marcadas utilizando lactoperoxidasa y $^{125}I$. El material radiactivo se localiza en la membrana plasmática (31,32).

La objeción principal de este sistema, es que las proteínas del suero absorbidas de forma inespecífica sobre la superficie celular (p.e. anticuerpo citofílico) se pueden marcar y dar resultados espureos. De hecho se ha demostrado que los linfocitos-T tienen gran capacidad para absorber inmunoglobulina (33,35).

Otra objeción, es que las proteínas que estén muy enterradas en la membrana no se marquen eficientemente.

Sin embargo estas objeciones se pueden subsanar facilmente. En el primer caso, es sabido que el anti-- cuerpo citofílico se desprende muy fácilmente de la superficie celular, con solamente incubar las células a - 37° por 30 minutos a una hora (34), de tal manera que se pueden pre-incubar las células antes de marcarlas, y - comparar los resultados con las células marcadas sin - éste paso previo. Los resultados indicaron que no se - observa diferencia. De hecho, es muy difícil marcar el anticuerpo citofílico, ya que se pierde con las lavadas

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de las células al preparar las suspensiones.

En el caso de la segunda objeción, tampoco es seria, ya que si una proteína se encuentra fungiendo como receptor, es difícil pensar que se encuentre "escondida" en el interior de la membrana.

ELECCION DE CELULAS :

Timocitos como fuente de células-T no activadas- y que llevan en la superficie los antígenos TL y Teta (14).

Timocitos de animales tratados con cortisona, como fuente de células que tienen el antígeno teta, pero han perdido el antígeno TL. Células de bazo y nódulos linfáticos, como fuente de células-B y -T periféricas. Células de bazo de animales timectomizados, letalmente irradiados y reconstituídos con células de hígado fetal tratadas con anti-teta y complemento, como fuente de -linfocitos-B.

Células de bazo de ratones atímicos congénitos -(nu/nu), como fuente de células-B.

AISLAMIENTO DEL MATERIAL RADIO-MARCADO DE LA SUPERFICIE CELULAR :

Como la radiactividad está limitada a la superficie celular, las suspensiones de células se extrajeron con detergente no iónico (Nonidato-P40) que no interfiriere con la inmuno-precipitación , ya que los componentes pudieron ser precipitados selectivamente con los -

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antisueros específicos adecuados y caracterizados en electroforesis en geles de poliacrilamida con detergente (dodecil sulfato de sodio).

USO DE PROTEINAS DE DIVERSOS MIELOMAS :

La purificación de las diferentes clases y subclases de inmunoglobulinas a partir del suero de animales - normales, es muy deficiente, ya que hay proteínas como - la ferritina que permanece con las inmunoglobulinas, a - pesar de utilizar técnicas cromatográficas con sesadex y con resinas de intercambio iónico.

Además, cuando se separan eventualmente las proteínas que no son inmunoglobulina, todavía queda el problema de separar y purificar las diferentes clases y subclases.

Otro problema que surge, son las cantidades relativas de cada una de las diferentes inmunoglobulinas.

Por tales motivos, es mejor partir de proteínas - de mielomas, ya que poseen la gran ventaja de tener a una sola clase o subclase de inmunoglobulina predominante, - lo que significa mayor pureza y mayor cantidad, sobretodo si se van a utilizar como inmunógenos y como patrones de comparación en los geles de poliacrilamida o en el radio-inmuno-ensayo, para la titulación de anticuerpos clase-específicos.

Si el objeto de este trabajo es el aislar y caracterizar las inmunoglobulinas de superficie, es aconsejable el uso de patrones de comparación en los geles, y para evitar errores de interpretación, estos patrones se -

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utilizaron marcados con un radioisótopo diferente ($^{131}I$) y se mezclaron con las muestras por analizar, permitiendo así conocer la posición exacta del patrón y del problema.

Como la energía de emisión del $^{131}I$ tiene cierta "derrama" en el canal de $^{125}I$, se contaron ambos radioisótopos, separados primero y mezclados después. Para hacer los cálculos de la derrama del $^{131}I$ en el canal de $^{125}I$, se encontró que ésta equivalió al 25,9%, así que este porcentaje se tuvo que sustraer para tener las lecturas reales.

Otro de los motivos por los cuales se utilizaron proteínas de mieloma purificadas, como inmunógenos, fué el tener la seguridad de que solamente la proteína deseada produjera anticuerpos, y no tener otro tipo de contaminante desconocido (o conocido). Esto es particularmente importante cuando se utilizan técnicas de inmunofluorescencia, ya que si los antisueros no están perfectamente caracterizados, pueden llevar a conclusiones totalmente erróneas (p.e. se estaría observando la reacción del antisuero con algún otro componente de la membrana celular, y se inferiría que es inmunoglobulina).

ANIMALES :

a) Ratones : Hembras LPE (Libres de patógenos - específicos : SPF) de las cepas ingénitas Balb/c, CBA y $C_3H/E$, de 3 a 6 semanas de edad; fetos - y ratones neonatos Balb/c; ratonas adultas de 3 a 13 meses de edad, de las mismas cepas.

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b) Conejos : Animales crecidos en el bioterio, de 1,5 a 2 Kg. de peso, hembras y machos.

c) Cabras : Animales adultos mantenidos en la granja.

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INMUNOGLOBULINAS DE RATON :

| Inmunoglobulina | Tumor | Fuente | Preparación* |
|---|---|---|---|
| 1. IgM ($\mu$, K) | TEPC 183 | Suero | Gradiente de sacarosa, geles de acrilamida preparativos (1) |
| 2. IgM ($\mu$, $\lambda_1$) | MOPC 104E | Suero | Gradiente de sacarosa, geles de acrilamida preparativos (1) |
| 3. IgA ($\alpha$, $\lambda_2$) | MOPC 315 | Suero | -DNP-Lisil-Sefarosa (2) |
| 4. $IgG_1$ ($\gamma_1$, K) | MOPC 21 | Suero | Sulfato de Amonio, DEAE (3) |
| 5. $IgG_{2a}$ ($\gamma_{2a}$, K) | Adj PC5 | Suero | Sulfato deAmonio, DEAE (3) |
| 6. $IgG_{2b}$ ($\gamma_{2b}$, K) | MOPC 195 | Suero | Sulfato de Amonio, DEAE (3) |
| 7. $IgG_3$ ($\gamma_3$, K) | FLOPC 21 | Suero | Sulfato de Amonio, DEAE (3) |
| 8. K | MOPC 47A | Orina | Sulfato de Amonio, DEAE (3) |
| 9. $\lambda$ | MOPC 104E(O) | Orina | Sulfato de Amonio, DEAE (3) |
| 10. Fab $\gamma_{2a}$ (K) | Adj PC5 | Suero | Sulfato de Amonio, DEAE (4) |

* Explicación de la preparación de Inmunoglobulinas de plasmacitomas.

(1) Un mililitro de suero aplicado a un gradiente lineal de sacarosa (25 ml.) de 10-30% (p/v) en TRIS-glicina pH 8.7. Centrifugado 16 h. a 100,000 g. - El pico recogido, formado por IgM y $\alpha_2$ macroglobulina, fué sometido a electroforesis preparativa en acrilamida al 3%. Después de algunas impurezas menores se colectó el primer pico de $\alpha_2$ - macroglobulina, seguido de la IgM (30).

(2) Sefarosa conjugada con DNP-lisina por el método de bromuro de cianógeno, en una columna. Se aplica el suero, y la proteína se eluye con DNP-glicina (26).

(3) Un volumen de suero, diluido con tres volúmenes de amortiguador de fosfatos-salina y precipitado con cuatro volúmenes de solución saturada de sulfato de amonio. Dializado contra amortiguador de fosfatos de potasio 10 mM, pH 8 y aplicado a una columna de DEAE-celulosa, equilibrada con amortiguador de fosfatos de potasio 10 mM, pH 8; después de aplicada la proteína y lavada con la misma solución hasta que la lectura de D.O. 280 sea de cero, fué eluída con diez volúmenes de columna de un gradiente de fosfatos de potasio de 10 mM a 30 mM, pH 8. El pico eluído, fué precipitado con sulfato de amonio al 50% de saturación.

(4) La purificación se lleva a cabo como en (3), y - posteriormente se prepara el Fab por digestión -

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con papaína de acuerdo con el método de Knopf, - Parkhouse y Lennox (36).

PREPARACION DE ANTISUEROS.

1. Antisueros de conejo : 1 mg. de proteína de ratón en adyuvante completo de Freund en tres inyecciones a lo largo de seis semanas. Los animales fueron sangrados de siete a catorce días después de la última inyección.

2. Antisueros de cabra : Se obtuvieron de la misma manera que los de conejo.

ESPECIFICIDAD DE LOS ANTISUEROS :

a) Antisueros poliespecíficos : i.e. Anti-Fab :

Probado como anticuerpo precipitante contra todos los isotipos de la variedad K, utilizando placas de doble difusión en agarosa (Ouchterlony). Solamente se usaron los anticuerpos que precipitaron todas las clases.

b) Antisueros monoespecíficos.

Hechos específicos por paso en columnas apropiadas de inmunoglobulina conjugada con sefarosa. La especificidad se probó por coprecipitación en radio-inmuno ensayo, utilizando proteína radiactiva de todas las clases.

Radio-inmuno ensayo : Antisuero de prueba 10 $\mu$l, proteína de mieloma radiactiva (aproximadamente -

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10 ng con $10^4$ c.p.m. de $^{131}I$), a temperatura ambiente 30 minutos. Agregar la cantidad adecuada de - suero de cabra anti-gama globulina de conejo. Esperar a que se forme el precipitado, centrifugar - y contar el sobrenadante y el precipitado. Se estandariza con referencia a controles positivos (anti-Fab) y negativos (inmunoglobulina normal de conejo). Las proteínas de mieloma se marcan con $^{131}I$ usando la técnica de la cloramina-T, descrita por Hunter- y Greenwood (37).

3. Antisueros fluorescentes de cabra anti-gamma globulina de conejo :

Se utilizó el método de Goldstein y Cebra (1965) - (38) y que esencialmente consiste en hacer una purificación parcial de las inmunoglobulinas del suero por precipitación con sulfato de amonio; seleccionar grupos de inmunoglobulinas con punto isoeléctrico restringido usando cromatografía en DEAE-celulosa equilibrada con solucción amortiguadora de fosfatos de sodio 0.01 M, pH 7.5 y eluyendo con - gradiente escalonado de concentración de sales - (NaCl 0, 0.05, 0.1, 0.2 M).Concentración de los - picos de proteína a 10-20 mg/ml, y diálisis contra NaCl 0.15 M. Las fracciones obtenidas individualmente se conjugan con isotiocianato de tetrametilrodamina o bien con isotiocianato de fluoresceína- (30 $\mu$g de TRITC ó 12.5 $\mu$g de FITC/mg de proteína). La conjugación se lleva a cabo a 0°C por dos horas a pH 9. 0-9.5, mantenido por la adición de Na OH -

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diluída (0.1 - 0.01 N) se deja a 4° C durante la noche para que el pH decrezca espontáneamente, ajustarlo a 7.6. Remover el fluorocromo no unido en sephadex - G-50 equilibrado con fosfatos 0.01 M pH 7.5 y fraccionar la inmunoglobulina conjugada en DEAE-celulosa, con el gradiente escalonado descrito arriba. A medida que aumenta la conjugación, disminuye el punto isoeléctrico de la proteína conjugada. Así pues si se empieza con fracciones de inmunoglobulina que tengan un punto isoeléctrico homogéneo, la columna de DEAE-celulosa permite seleccionar a las moléculas de inmunoglobulina que tengan unidas cantidades similares de fluorocromo por molécula. Esto permite excluir al anticuerpo poco conjugado, que se une a los sitios antigénicos y que no se puede detectar, y también al anticuerpo conjugado en exceso, que puede estar inactivo o bien que se puede adherir en forma inespecífica a las membranas celulares.

Las inmunoglobulinas que tienen una relación de densidad óptica de 2-3 (FITC= $\frac{D.0.280^{nm}}{D.0.495^{nm}}$ ó TRITC=$\frac{D.0.280^{nm}}{D.0.515^{nm}}$) son las utilizadas.

PRUEBA CON CELULAS VIVAS :

Se utiliza el anticuerpo fluorocromo a varias diluciones, notando dos aspectos fundamentales a) Brillantez y b) Número de células teñidas; se utiliza a la dilución necesaria en que se tenga el máximo de ambas, ya que sigue una curva semejante a la de Gauss.

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a) Tinción de células vivas :

Se hace siguiendo el método descrito por Raff (39). Es fundamental que las células estén realmente vivas (es decir, que no se encuentren células muertas), para evitar la absorción inespecífica del anticuerpo, ó el paso inespecífico del mismo al interior de las células muertas.

$2-4x10^7$ células/ml. 20$\mu$l, se dejan a temperatura ambiente con el anticuerpo 30 minutos, se lavan 2 veces y se incuban otros 30 minutos con el anticuerpo de cabra anti-conejo conjugado con el fluorocromo, para que se forme el casquete se lavan 2 veces y se montan en un portaobjetos con cubreobjetos, sellado con parafina líquida (a 50° C). Se ven al microscopio de fluorescencia, obteniendo el porcentaje de células teñidas con respecto a las no teñidas, utilizando filtro de interferencia de 510 nm para fluoresceína ó de 570 nm para rodamina.

b) Tinción doble de células vivas :

Se promueve la formación de casquete con el primer anticuerpo de conejo y cabra anti-conejo conjugado con un fluorocromo (p.e. rodamina); después se ponen las células en baño de hielo y se tratan con el segundo anticuerpo de conejo seguido de cabra-anti-conejo conjugado con el otro fluorocromo (p.e. fluoresceína), en presencia de azida de sodio al 0.03% para evitar la formación de casquete.

Así se puede observar el número de células con tinción sencilla (uno u otro fluorocromo; casque-

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te o anillo) y las células con tinción doble (ambos fluorocromos; casquete y anillo) y calcular el porcentaje, respecto a las no teñidas.

MARCAJE DE LAS MEMBRANAS CELULARES CON $^{125}I$.

Se utilizó el método descrito por Phillips y Morrison (40) donde la reacción de incorporación del yodo a las proteínas de la membrana celular se lleva a cabo por la catálisis de la enzima peroxidasa sobre el peróxido de hidrógeno, siendo éste un tratamiento muy suave, que no modifica la viabilidad celular, y que permite el marcaje externo solamente. Se llevó a cabo en cuatro pasos importantes :

a) Suspensiones de células en solución de fosfatos-salina (en ausencia de proteína, por razones obvias), con viabilidad superior al 95% (medida por exclusión del colorante azul tripano); en forma muy rápida; llevando a cabo el proceso en condiciones óptimas de rapidez y temperatura, para evitar la degradación de las proteínas y la obtención de artefactos.

b) Preparación de los extractos celulares : Después del marcaje las células se resuspendieron en volúmenes grandes de fosfato-salina para lavarlas por centrifugación en frío y se lisaron en solución de fosfato-salina conteniendo el 1% de Nonidato P-40 (detergente no iónico) fluoruro de fenilmetil sulfonilo - 10 mM (para inhibir la proteólisis, fundamentalmen

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te aquella provocada por la serina-esterasa) y con Iodoacetamida 100 mM (para inhibir el re-ensamble espontáneo de macromoléculas a través de los -SH). Después de dejar las muestras 5 a 10 minutos en - hielo para permitir la disolución de las membranas, se centrifugaron a 15,000 x g durante 15 minutos. - El sobrenadante se pasó por Sephadex G-25 para eliminar el material de bajo peso molecular, conservando solamente a las proteínas.

En estas condiciones esencialmente todo el material precipitable con ácido tricloroacético es solubilizado por el detergente NP40 y recuperado del Sephadex G-25, treinta minutos después del marcaje celular.

c) Aislamiento de la inmunoglobulina : La inmunoglobulina fué aislada de los lisados por inmunocoprecipitación. Se utilizó el sistema de coprecipitación indirecta, i.e. primero suero de conejo anti-inmunoglobulina de ratón y enseguida suero de cabra - anti-gama globulina de conejo, en hielo y seleccionando el suero de cabra que en estas condiciones - precipitara más rápidamente (vale la pena mencionar otra vez que se utilizaron las condiciones en que- se pudiera evitar la proteólisis y la degradación- en general de manera más eficiente). Una vez que - se hubo formado el precipitado, se centrifugó y se lavó con fosfatos-salina- 1% NP40-PMSF 10 mM en - frío, hasta que el número de cuentas por minuto del precipitado no disminuyó más del 10%. El último la

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vado fué hecho con fosfato-salina para quitar el detergente, y los precipitados fueron contados - en un contador de radiaciones $\gamma$ (LKB 2000),

d) Análisis y caracterización de la inmunoglobulina de superficie en electroforesis en Geles de poliacrilamida con dodecil sulfato de sodio (SDS). En vista de que se utilizaron antisueros isotipo - (clase)- específicos, el análisis de las especies moleculares de inmunoglobulina, se llevaron a cabo en geles disociantes de SDS, en los que la separación se debe a la diferencia de pesos moleculares y no de carga. Se puede elegir entre dos - opciones : geles reducidos y no reducidos, ya que las inmunoglobulinas son proteínas compuestas de dos tipos de cadenas : pesadas y ligeras, y pueden presentar diferentes grados de polimerización: monomérica, dimérica, trimérica y pentamérica e incluyeron un marcador interno.

1. Geles no reducidos : Los coprecipitados fueron disociados con solución de SDS-iodoacetamida-sacaros (1% - 50 mM - 7%), hirviendo a Baño María 2 - 5 minutos, con el fin de - observar la clase y grado de polimerización de las inmunoglobulinas. Los geles utilizados fueron al 4.25% de acrilamida.

2. Geles reducidos : Con el fin de analizar posibles diferencias en los tamaños de las cadenas pesadas, los coprecipitados fueron re

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ducidos con Ditio-treitol (DTT) 1 mM en fosfatos de sodio 20 mM pH 7.2 - SDS 2%, sometidos a ebullición 15 minutos. Para evitar reasociación las muestras fueron alquiladas con un exceso de iodoacetamida (10 mM) y sometidas a electroforesis en geles con marcador - interno.

Marcador interno : Los marcadores internos fueron preparados, utilizando inmunoglobulinas purificadas de mieloma (IgM de MOPC 104E e IgG de Adj PC5), marcados con $^{131}I$ por el método de Hunter y Greenwood, (37) éstas fueron :

(I) No tratadas : IgG

(II) Parcialmente reducidas : IgM, usando DTT 0.5 mM a temperatura ambiente 60 minutos, alquilando posteriormente con un exceso de iodoacetamida (50 mM). Esto permite tener las - especies moleculares $H_2L_2$ y HL.

(III) Totalmente reducidas : IgG e IgM, utilizando DTT 1-2 mM en SDS al 2%, hirviendo 15 minutos y alquilando con un exceso de iodoacetamida (50 mM).

Se hizo posteriormente una mezcla apropiada, que - incluyó :

7S IgM ($H_2L_2$), $\mu$L (HL), $\mu$ , $\gamma$ y L.

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ELECTROFORESIS EN GEL DE POLIACRILAMIDA - SDS.

Todas las muestras fueron mezcladas con marcador interno (mezcla artificial de inmunoglobulinas de mieloma de ratón en diferentes grados de reducción y marcadas con $^{131}I$) antes de ser sometidas a electroforesis, y en una proporción de 3 a 5 veces de exceso de $^{125}I$ sobre - $^{131}I$.

Todas las muestras fueron corridas en geles de - 6 cm. de longitud y 0.5 cm. de diámetro. Para geles al - 7% fueron 4 horas a 15 mA/gel y para geles al 4.2% 4 horas a 10 mA/gel. (41).

Posteriormente los geles fueron congelados, cortados en rebanadas de 1 mm. de longitud y contadas en - ambos canales ($^{125}I$ y $^{131}I$).

Las cuentas por minuto fueron corregidas por el- cruce de canales (26% de $^{131}I$ se derrama en el canal de- $^{125}I$) y graficadas.

2a. ELECTROFORESIS DE MUESTRAS EXTRAIDAS DE LOS GELES.

En los casos en que se necesitó extraer muestras de los geles, el pico fué localizado contando las rebanadas de gel, reunidas y extraidas por ebullición en fosfatos 0.02 M-SDS al 1%, Ditiotreitol 1 mM, durante quince- minutos, el sobrenadante se transfirió a otro tubo, después de haber sido bloqueado con iodoacetamida 10-50 mM, se le adicionó la sacarosa y la urea y se corrió nuevamente la muestra en electroforesis.

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PREPARACION DE MEMBRANAS DE LINFOCITOS.

Se utilizó el método de Crumpton y Snary, que consiste básicamente en hacer una suspensión de células (bazo) lavadas, romperlas y someterlas a centrifugaciones diferenciales para separar núcleos y mitocondrias de ribosomas y membranas y ésta última fracción se somete a un gradiente escalonado de sacarosa (36% y 26%). En la interfase se obtienen las membranas purificadas (42,43).

Se hicieron controles en todas las fracciones, haciendo determinaciones de proteína (la membrana constituye el 0.1 - 0.2% de la proteína total de los linfocitos), y 5' nucleotidasa (enzima característica de la membrana plasmática).

PREPARACION DE COLUMNAS DE SEFAROSA-4B CON ACTIVIDAD DE ANTICUERPOS.

1. Gamma globulina normal de conejo : se preparó usando 6 mg. de gamma globulina normal de conejo purificada y 3 ml. de sefarosa.

2. Anticuerpo de conejo anti cadena $\mu$ de ratón : se preparó con 3.6 mg. de anticuerpos y 1.8 ml. de sefarosa.

   El anticuerpo anti-cadena $\mu$ de ratón se preparó por la aplicación de suero de conejo específico anti-$\mu$ a una columna de sefarosa-IgM (MOPC 104E; $\mu$, $\lambda_1$), y eluyendo el anticuerpo con ácido acético 1 M.

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3. Anticuerpo de conejo anti-Fab (K) de ratón : Se preparó utilizando 5 mg. de anticuerpo y 2.5 ml. de sefarosa.

El anticuerpo anti Fab (K) se preparó de una manera semejante , mezclando los siguientes eluados : conejo anti-$IgG_1$ completa (K) (MPC 21) retenido por Sefarosa-$IgG_{2a}$ (K) (Adj PC5); conejo anti-$IgG_{2a}$ completa (K) retenida por sefarosa-$IgG_1$ (K) (MPC 21) y conejo anti-IgM completa (K) (TEPC 183) retenida por sefarosa-$IgG_{2a}$ (K) (Adj PC5).

PREPARACION DEL ANTISUERO ANTI-IgD.

La preparación de membranas plasmáticas de células de bazo de 1750 ratones Balb/c (160 g. de peso húmedo) rindieron 130 mg. de proteína. Las membranas fueron disueltas en 25 ml. de solución de fosfatos-salina conteniendo 1% (p/v) de nonidato P-40 y fluoruro de fenil metil sulfonido 1 mM, centrifugadas a $10^5$ x g durante tres horas, y posteriormente el sobrenadante fué pasado por tres columnas sucesivas de sefarosa-4B : (I) conjugada con gamma globulina normal de conejo; con el fin de fijar cualquier tipo de moléculas que se adhieran a estas proteínas de una manera más o menos inespecífica y pudiera competir con los anticuerpos en las columnas siguientes (p.e. receptores de Fc). (II) Anticuerpos específicos de conejo anticadena $\mu$ de ratón, con el fin de fijar a la inmunoglobulina M y (III) Anticuerpos específicos de conejo anti Fab de ratón , con el fin de fijar a la IgD.

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Los anticuerpos purificados conjugados a la sefarosa (44) dan una gran capacidad como inmunoabsorbentes.

Después de pasada la muestra, las columnas fueron lavadas con solución de fosfatos-salina con 1% de NP-40 y posteriormente solamente con fosfatos-salina. Dada la labilidad de la IgD, la sefarosa-anticuerpo-inmunoglobulina de ratón, fué emulsificada con igual volúmen de adyuvante de Freund e inyectada en los conejos (45), sin tomar el riesgo de eluirla y aprovechando nuevamente las ventajas del inmunoabsorbente que daría una vida más larga al antígeno en el conejo.

## R E S U L T A D O S  Y  D I S C U S I O N

Las suspensiones de células esplénicas con viabilidad mayor del 95% (por exclusión de azul tripano) se marcaron externamente con $^{125}I$ por el método de catálisis con lactoperoxidasa (46), la inmunoglobulina de superficie fué preparada por inmunoprecipitación con suero anti Fab-K y analizada en geles de acrilamida al 4% con dodecil sulfato de sodio al1%(SDS)(26) con la adición del marcador interno (IgM marcada con $^{131}I$ (23) parcialmente reducida a $H_2L_2$ y HL). Como ya se ha descrito antes, encontramos una proporción mayor de radiactividad de la inmunoglobulina - de la superficie celular en la parte del gel que corresponde a la subunidad $IgM_S$ ($\mu_2L_2$, monómero) (Fig.1), la cual por reducción dió cadenas pesadas y ligeras (Fig.2); sin embargo, también se observó una cantidad significativa de radiactividad, en una posición ligeramente más avanzada - (más pequeña) que la subunidad HL, que por reducción también dieron cadenas pesadas y ligeras (Fig.2) y por lo tanto - debieron ser subunidades HL.

Después de la reducción y alquilación de ambas subunidades, $H_2L_2$ y HL, en geles al 10%, mostraron dos especies diferentes de cadena pesada, una con la movilidad - (y por lo tanto peso molecular) igual al marcador interno de cadena $^{131}I\mu$, y el otro (Fig.2) con una migración mayor (por lo tanto de peso molecular menor) que $\mu$, pero menor (por lo tanto de peso molecular mayor) que la cadena $\gamma$. La proporción de radiactividad encontrada en la cadena pesada más pequeña (migración más rápida que $\mu$) fué

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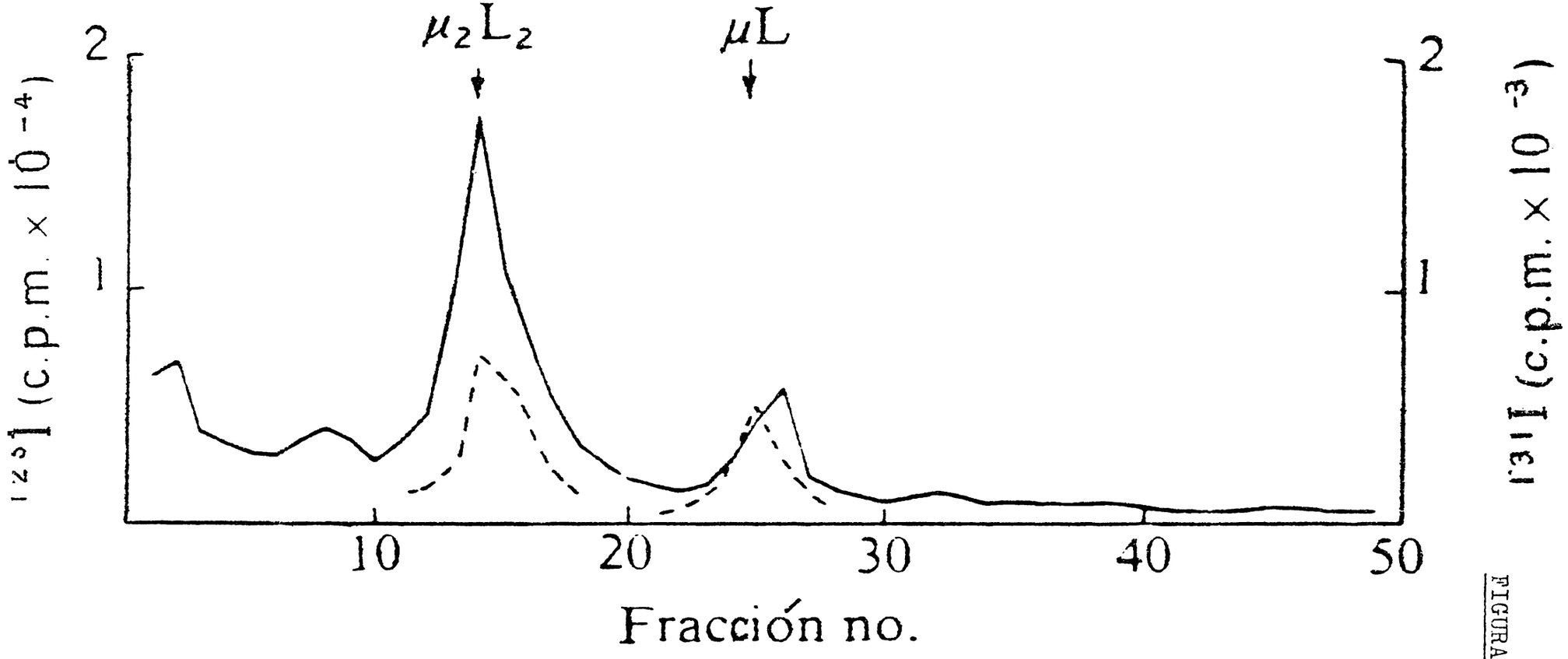
$\mu_2 L_2$
$\mu L$
2
1
10
20
30
40
50
Fracción no.
$^{125}$I (c.p.m. × 10$^{-4}$)
$^{131}$I (c.p.m. × 10$^{-3}$)

FIGURA 1

FIGURA 1 Inmunoglobulina de superficie de linfocitos de bazo de ratón.

Las suspensiones de células de bazo de ratón, - CBA, de 4 a 6 semanas de edad, fueron marcadas con $^{125}I$, por el método catalizado por lactoperoxidasa (40), lavadas con salina-fosfatos y lisadas durante 10 minutos a - 0° C en Nonidato P-40 al 1% en PBS. Los lisados centrifugados (a 4,000 x g por 10 minutos), fueron dializados - contra nonidato P-40 al 1% en PBS, en frío. La Ig de superficie fué precipitada con 10 µl de suero conejo anti-Ig de ratón (poliespecífico), seguido de 100 µl suero - de cabra anti-IgG de conejo. El precipitado lavado (NP40 al 5% en PBS : 3 veces y dos veces más en PBS, todos los lavados fueron hechos en frío), fué disuelto en dodecil - sulfato de sodio al 4%-Iodoacetamida 0.1 M-fosfatos de - sodio 0.05 M (pH 7.0), hirviendo durante 5 minutos a baño María. A la muestra así tratada, se le añadió marcador interno (ver texto), IgM de mieloma MOPC 104E marcada - con $^{131}I$ y reducida parcialmente con ditiotreitol. Para el análisis en gel, se depositó la muestra en un gel de poliacrilamida al 4.25% conteniendo dodecil sulfato de - sodio al 1%. Después de la electroforesis (4 horas a 10mA/--/gel) se rebanó el gel en segmentos de 1 mm. y se determinó la radiactividad. Los valores fueron corregidos por el cruce de canales y los datos están representados en - la gráfica, con la parte superior del gel hacia la izquierda. ———, representa la Ig de superficie marcada con $^{125}I$; - - -, representa al marcador interno ($^{131}I$).

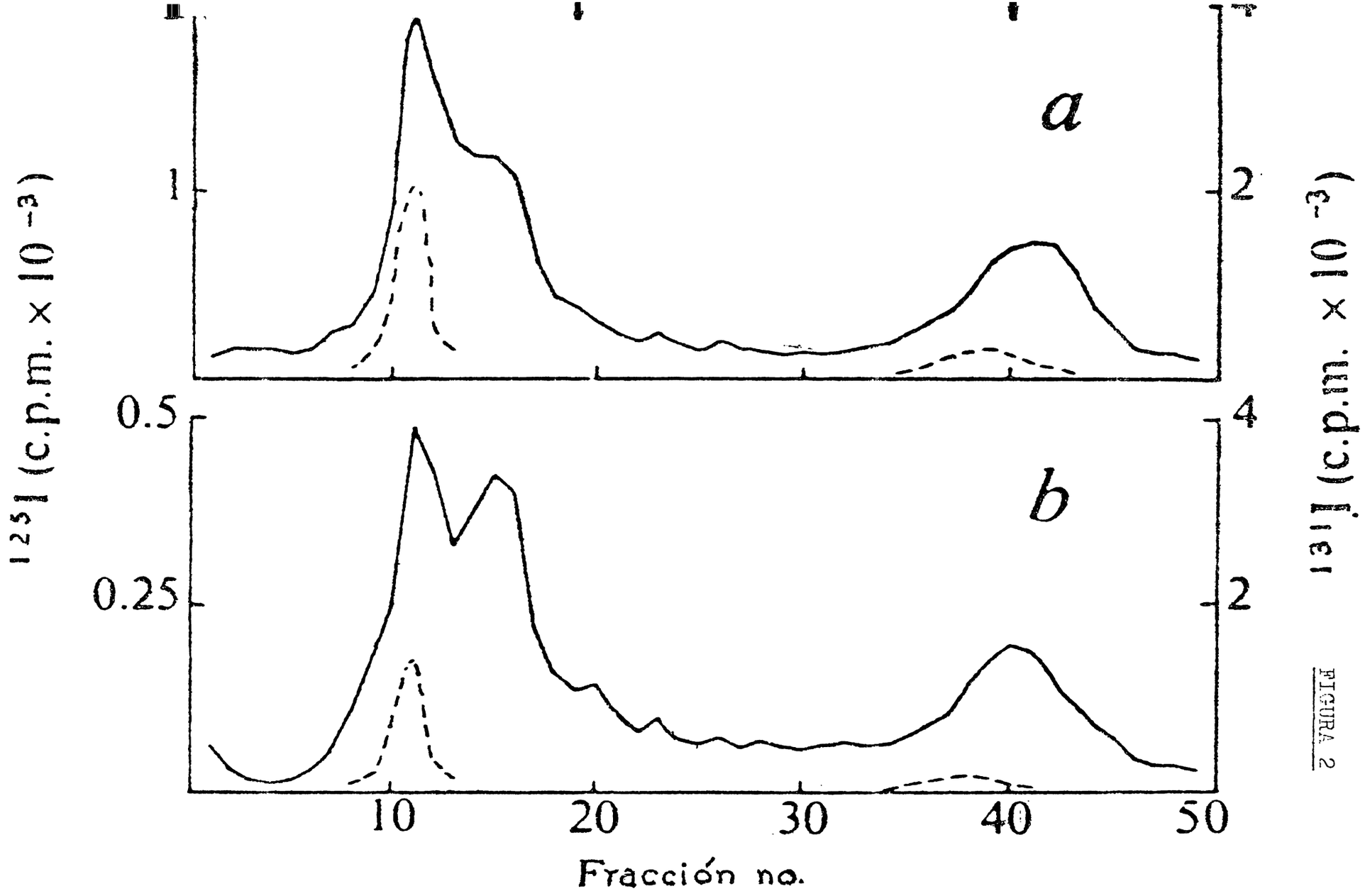
a
b
1
2
0.5
0.25
4
2
10
20
30
40
50
Fracción no.
$^{125}$I (c.p.m. × 10$^{-3}$)
$^{131}$I (c.p.m. × 10$^{-3}$)

FIGURA 2

FIGURA 2 Heterogeneidad del tamaño de la cadena pesada de la inmunoglobulina de superficie de los Linfocitos.

Las secciones del gel mostrado en la Figura 1, conteniendo los componentes $H_2L_2$ y HL fueron eluidos en condiciones reductoras (DTT 2 mM) (ver texto). El eluado fué hervido 15 minutos para asegurar la reducción de todos los puentes disulfuro, después fueron alquilados por la adición de iodoacetamida (concentración final 10 mM). El material resultante se depositó en geles de poliacrilamida al 10%, conteniendo dodecil sulfato de sodio, para resolver los componentes de la cadena pesada. Se observa que el marcador interno de $^{131}I$ proveniente del primer gel, se encuentra totalmente reducido, e indica las posiciones para la cadena $\mu$ y la cadena ligera. Los geles fueron rebanados y contados de la misma manera que en la Figura 1. La posición de la cadena $\gamma$ fué determinada corriendo un gel paralelo con una muestra de $^{131}I$-IgM (MOPC 104E) reducida y mezclada con $^{131}I$-$IgG_{2a}$ (Adj PC5) reducida, a) $H_2L_2$ de superficie, reducida, b) HL de superficie, reducida. Línea sólida $^{125}I$, línea punteada marcador interno de $^{131}I$.

menor en la muestra reducida de $H_2L_2$ (Fig.2a) que en la - de la muestra reducida de HL (Fig.2b).

Para eliminar la posibilidad de que la heterogeneidad de la inmunoglobulina pudiera deberse a la presencia de los linfocitos-T (45, 47) de las suspensiones de las - células de bazo, llevamos a cabo los mismos experimentos con células de bazo de ratones desnudos (nu/nu, atímicos congénitos) y con ratones "B" (timectomizados, irradiados, tratados con cortisona y reconstituidos con células de - hígado fetal); los resultados obtenidos fueron los mismos. Además, las células-T obtenidas por separación de las células de bazo en columnas de nylon (48), no contienen - cantidades detectables de inmunoglobulina de superficie, utilizando este tipo de metodología.

Como control de degradación de material conteniendo cadena $\mu$ durante la preparación de inmunoglobulina - marcada (Ig) se llevaron a cabo experimentos de reconstrucción. Ambas especies $^{125}I$-$H_2L_2$ y $^{125}I$-HL, preparadas por reducción parcial de IgM de mieloma MOPC 104E, se pudieron recuperar sin cambio cuando se añadieron a suspensiones de células de bazo, sin marca, y por medio de inmunoprecipitación y electroforesis en geles de SDS. Las subunidades HL encontradas en la superficie de los linfocitos esplénicos no es pues el resultado de una despolimerización reductiva de $\mu_2L_2$ durante el aislamiento. Después de reducir las $^{125}I$-$\mu_2L_2$ y $^{125}I$-$\mu L$ dieron las cadenas - pesadas con el mismo tamaño que la cadena $\mu$ no tratada;- esto es, la heterogeneidad de la Ig de superficie de la - célula no resulta de la degradación durante el proceso de aislamiento,

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Una explicación posible para la heterogeneidad de la Ig de superficie, es que pudiera existir una relación precursor-producto entre las diversas especies observadas. Dicha relación podría reflejar una vía biosintética o un recambio metabólico de la inmunoglobulina asociada a la membrana.

En el primer caso de subunidad HL podría ser el intermediario en la biosintesis de $H_2L_2$, y la cadena pesada pequeña sería $\mu$ parcialmente glucosilada, que requeriría la adición posterior de más residuos de azúcar para su terminación.

Como una situación alternativa se tendría que la dirección de la relación fuera inversa, es decir, hacia la degradación de $H_2L_2$ a HL y proteólisis de la cadena pesada.

Para definir estas posibilidades, se marcaron a las células de bazo con $^{125}I$ y se cultivaron "in vitro" a 37°, de 0 a 12 horas. Fué evidente la ausencia de cualquier forma de relación precursor-producto ya que las relaciones $H_2L_2$ a HL y de cadena pesada grande (cadena $\mu$) a pequeña determinadas a tiempo cero, permanecieron sin cambio en la Ig de superficie liberada al medio y recuperada a diferentes tiempos de incubación. Este experimento demostró también que las "vidas medias" de todas las especies de inmunoglobulina presentes en la superficie celular son similares y tienen un valor de 10 horas.

Posteriormente se exploró la posibilidad de que la cadena pesada pequeña, fuera derivada de otra clase de inmunoglobulina, diferente de la IgM, por lo que se utili

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zaron antisueros clase-específicos definidos, para el aislamiento de la inmunoglobulina de superficie.

Los anticuerpos específicos para cadenas $\gamma_1$, $\gamma_{2a}$, $\gamma_{2b}$, y $\alpha$ no fueron capaces de precipitar inmunoglobulina de los linfocitos de bazo, marcados con $^{125}I$, y solubilizados con detergente; sin embargo el antisuero específico anti-$\mu$ precipitó inmunoglobulina, que en los geles dió $H_2L_2$ y HL, las cuales por reducción demostraron tener cadenas H y L, correspondientes en tamaño a la cadena $\mu$ de secreción (Fig.3a), por lo que a este material se le identificó como cadena $\mu$.

Una vez removida la IgM de este lisado, por inmunoprecipitación específica, en el sobrenadante se llevó a cabo una segunda precipitación esta vez con el antisuero poliespecífico, es decir, con anti Fab-K; en esta ocasión, las especies $H_2L_2$ y HL estuvieron compuestas predominantemente por la cadena pesada pequeña (Fig.3b), por lo que pudimos concluir que en la superficie de los linfocitos esplénicos está presente una inmunoglobulina que tiene la cadena pesada más pequeña que $\mu$ y más grande que $\gamma$, que no precipita con ningún antisuero contra las diferentes subclases de $\gamma$, ó contra $\alpha$ ó $\mu$, pero si con el antisuero poliespecífico. En vista de que la cadena pesada pequeña no pudo ser precipitada con anti-$\mu$, la posibilidad de que correspondiese a una cadena $\mu$, glucosilada de manera incompleta, queda eliminada (49).

De esta manera la única conclusión que se puede considerar, es que esta cadena pesada, que representa el

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FIGURA 3

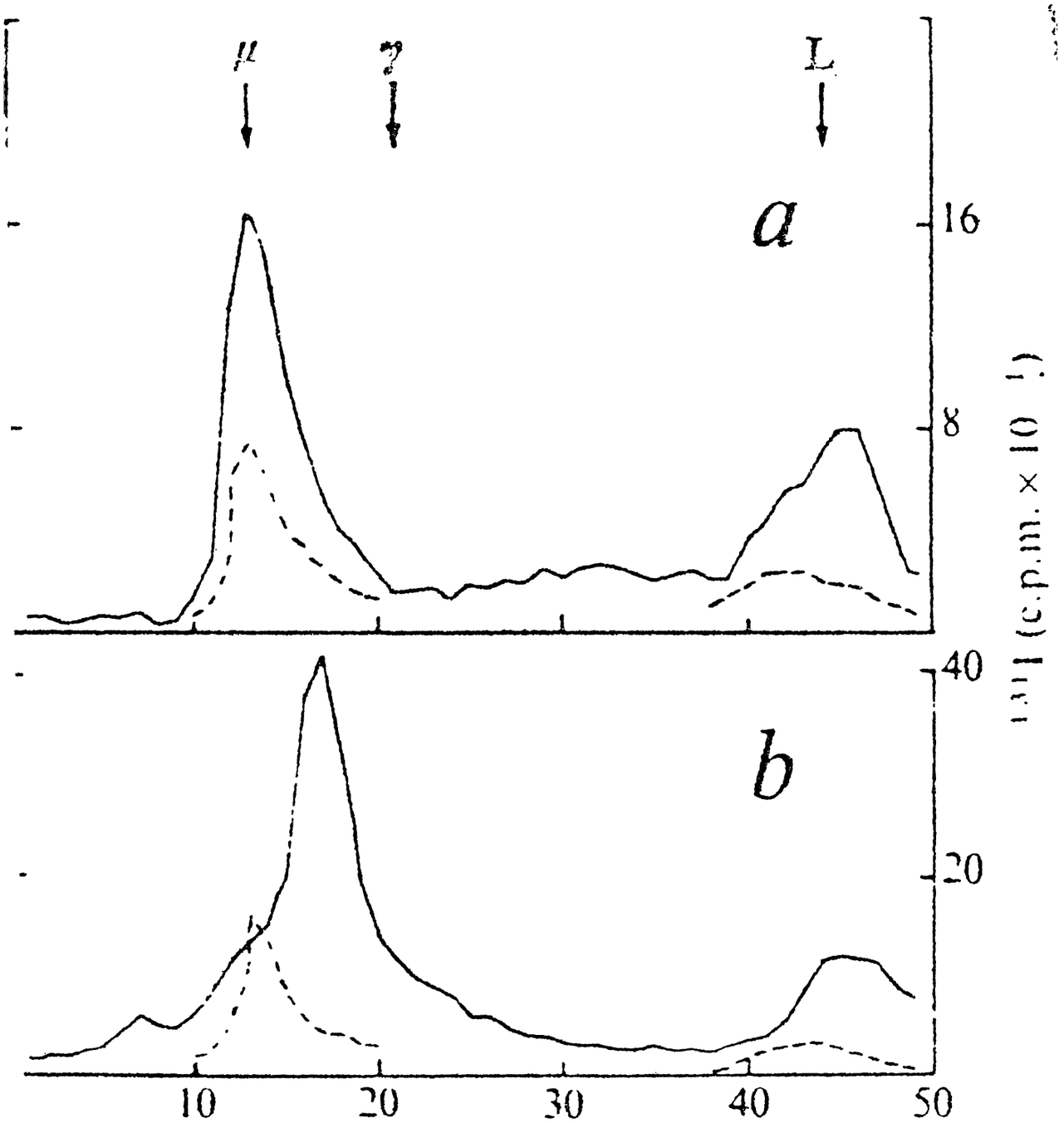

FIGURA 3 Dos clases diferentes de cadena pesada presentes en la superficie de los linfocitos de ratón.

Las células fueron marcadas, con $^{125}I$, lavadas resuspendidas en medio de Eagle- conteniendo 10% de suero de feto de res ($2 \times 10^7$ células/ml de medio), e incubadas a 37° C en atmósfera conteniendo 5% de $CO_2$ en aire, durante seis horas. Al término de la incubación, el 95% de las células estaba viable (por exclusión de azul de tripano). El medio que contenía a la inmunoglobulina de superficie, fué separado de las células por centrifugación (400 x g, - 10 minutos), y se le añadió un noveno de volúmen de NP40- al 10%. Se dializó contra PBS-NP40 al 1%. Al material no difusible se le agregaron 10 $\mu$l de suero específico de - conejo anti-cadena $\mu$ de ratón, seguido de 100 $\mu$l de suero de cabra anti $\gamma$G de conejo. Después de remover el precipitado resultante, por centrifugación, el sobrenadante fué tratado con 50 $\mu$l de suero de conejo poliespecífico anti Ig de ratón, seguido de 40 $\mu$g de Ig de ratón para asegurar la precipitación completa. Ambos precipitados fueron lavados y sometidos a electroforesis en geles de acrilamida al 4.2% (como en la Figura 1). La región 7S de ambos - geles fué eluída, reducida y sometida a electroforesis en geles al 10% de acrilamida (como en la Figura 2). Siempre con marcador interno. Línea contínua Ig de la superficie de los linfocitos marcada con $^{125}I$. Línea punteada, marcador interno con $^{131}I$. a) Precipitado con anti-$\mu$ . b) - Precipitado con anti-Ig.

40% de las cadenas pesadas aisladas de las células de bazo marcadas en la superficie, es una clase de inmunoglobulina, hasta ahora no descrita en el ratón; la conjetura más obvia, es que corresponde a la clase IgD humana.- Para apoyar esta sugerencia, hay que hacer notar, que en los geles de poliacrilamida - SDS la cadena $\delta$ humana (50) tiene una movilidad muy semejante a la cadena pesada pequeña de linfocitos de ratón. Esta inmunoglobulina de ratón, que ahora llamaremos IgD, también se parece a la IgD humana en su marcada susceptibilidad a la proteólisis.

En contra de lo esperado (52), encontramos que en el hígado fetal (fetos de 16 días), la cadena $\mu$ estaba presente, pero no la cadena $\delta$ ; la misma situación se presentó en el hígado y en el bazo del ratón neonato (menor de 16 horas). Sin embargo en el bazo del ratón de seis semanas y de seis meses, se encuentran cantidades similares de cadena $\delta$ y la relación $\mu : \delta$ es de 3 : 2. Esto se puede interpretar como que la expresión de IgM antecede a la expresión de IgD en el ratón, y de no ser éste el caso, los linfocitos que tienen IgD en la superficie deben surgir de otros órganos diferentes al bazo ó al hígado fetal.

Vale la pena mencionar que en los nódulos linfáticos del ratón, la IgD constituye a la clase más abundante en la superficie, donde la relación $\mu : \delta$ se encontró ser 1 : 3.5 - 1 : 4.0.

Es particularmente intrigante el hecho de que, las células de bazo y las de los nódulos linfáticos difieran

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en una manera tan marcada en cuanto a las cantidades relativas de cadenas $\mu$ y $\delta$ presentes en su superficie. - Respecto a este punto, hay que hacer notar que las células de bazo responden a la estimulación con lipopolisacárido de E.coli, pero las células de nódulos linfáticos - no (G. Janossy y R.M.E. Parkhouse sin publicar), ya que- mientras las células de bazo secretan fundamentalmente - IgM, las de nódulos linfáticos básicamente secretan IgG, (30).

El camino obvio a seguir en estas investigaciones fué la preparación y caracterización del antisuero específico para la inmunoglobulina-D del ratón, utilizando - un razonamiento inmunoquímico y aprovechando las ventajas que representan el uso de inmunoabsorbentes específicos, altamente purificados y de gran capacidad.

Con este fin se purificaron las membranas de los linfocitos de bazo de 1750 ratones Balb/c (130 mg de proteína), disueltas en solución de fosfatos-salina-nonidato P-40 y con fluoruro de fenilmetil sulfonilo para inhibir la acción de las proteasas dependientes de grupos-SH, y centrifugadas a alta velocidad, para eliminar cualquier material particulado.

El sobrenadante se pasó por las tres columnas de sefarosa conjugada con anticuerpos, en sucesión $\gamma$ normal de conejo, anticuerpo purificado de conejo específico - anti $\mu$ de ratón y finalmente anticuerpo purificado de - conejo, específico anti Fab-K de ratón.

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En la tercera columna se fijó la IgD y dada su labilidad, sin eluir, se usó como inmunógeno en conejos (44) emulsificando el complejo sefarosa-IgD con adyuvante completo de Freund

El suero obtenido se pasó por una columna de - $IgG_{2a}$ (K) (Adj PC5)-Sefarosa y después por otra de IgM (K) (TEPC 183)-Sefarosa. El suero absorbido se probó por radioinmunoensayo (56) y se encontró incapaz de reaccionar con :

IgM (K) (TEPC 183), IgM ($\lambda_1$) (MOPC 104E), IgA ($\lambda_2$)(MOPC315) $IgG_1$ (K) (MPC 21), $IgG_{2a}$ (K) (Adj PC5), $IgG_{2b}$ (K) (MOPC 195) $IgG_3$ (K) (FLOPC 21), cadenas $\lambda$ (RPC 20), cadena K (MOPC 41) y $\alpha_2$ macroglobulina de ratón. Todas las proteínas mencionadas fueron marcadas con $^{125}I$, y la prueba es capaz de detectar cantidades menores a 1 $\mu$g. de anticuerpo - en 1 ml. de suero.

Al utilizar el antisuero en conjunción con suero de cabra anti-conejo marcado con rodamina (38), fué capaz de teñir aproximadamente el 20% de células de bazo de ratón CBA, pero no tiñó timocitos de ratón de las cepas - CBA, C57/BL, DBA/2, $C_3H$, AKR ó Balb/c, bajo estas condiciones también fué incapaz de teñir células-T periféricas de las mismas cepas, preparadas en columnas de fibra de nylón.

Posteriormente se llevó a cabo la evaluación del antisuero, probándolo con células de bazo marcadas con -

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$^{125}I$, siguiendo el método descrito anteriormente, y se encontró que la única inmunoglobulina que precipitó fué la IgD (Fig.4a) (Ref.53, 54, 55), además se encontró el mismo perfil en los geles cuando 10 $\mu$l del antisuero fueron incubados con 100 $\mu$l de suero normal de conejo a temperatura ambiente por treinta minutos, lo que quiere decir que si hay IgD circulante en el ratón debe ser en cantidades muy pequeñas.

La recuperación de IgM fué posible, con sólo adicionar anti-$\mu$ al sobrenadante que quedó después de remover IgD (Fig.4b). En ambas figuras se observa material radiactivo que corre antes y después de las cadenas $\mu$ y $\delta$; este material está presente también cuando se lleva a cabo una precipitación no específica utilizando suero normal de conejo en lugar de cualquiera de los sueros específicos. Más aún, el material de mayor peso molecular prácticamente desaparece cuando se utilizan geles de poliacrilamida a menor concentración (4.2%) (Fig.4c y 4d), lo que permite considerar a dicho material como un artefacto debido tal vez a agregación. La desventaja que representa el uso de geles de concentración baja, es la baja-resolución, que impide la separación entre las cadenas $\mu$ y $\delta$.

Al someter a electroforesis las muestras sin reducir, en geles 4.2%, el material coincidió con la IgM monomérica (7S) unida por puentes disulfuro del marcador interno. Cuando las muestras fueron reducidas con anterioridad, indicaron la presencia de ambos tipos de cadena : pesada y ligera.

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FIGURA 4

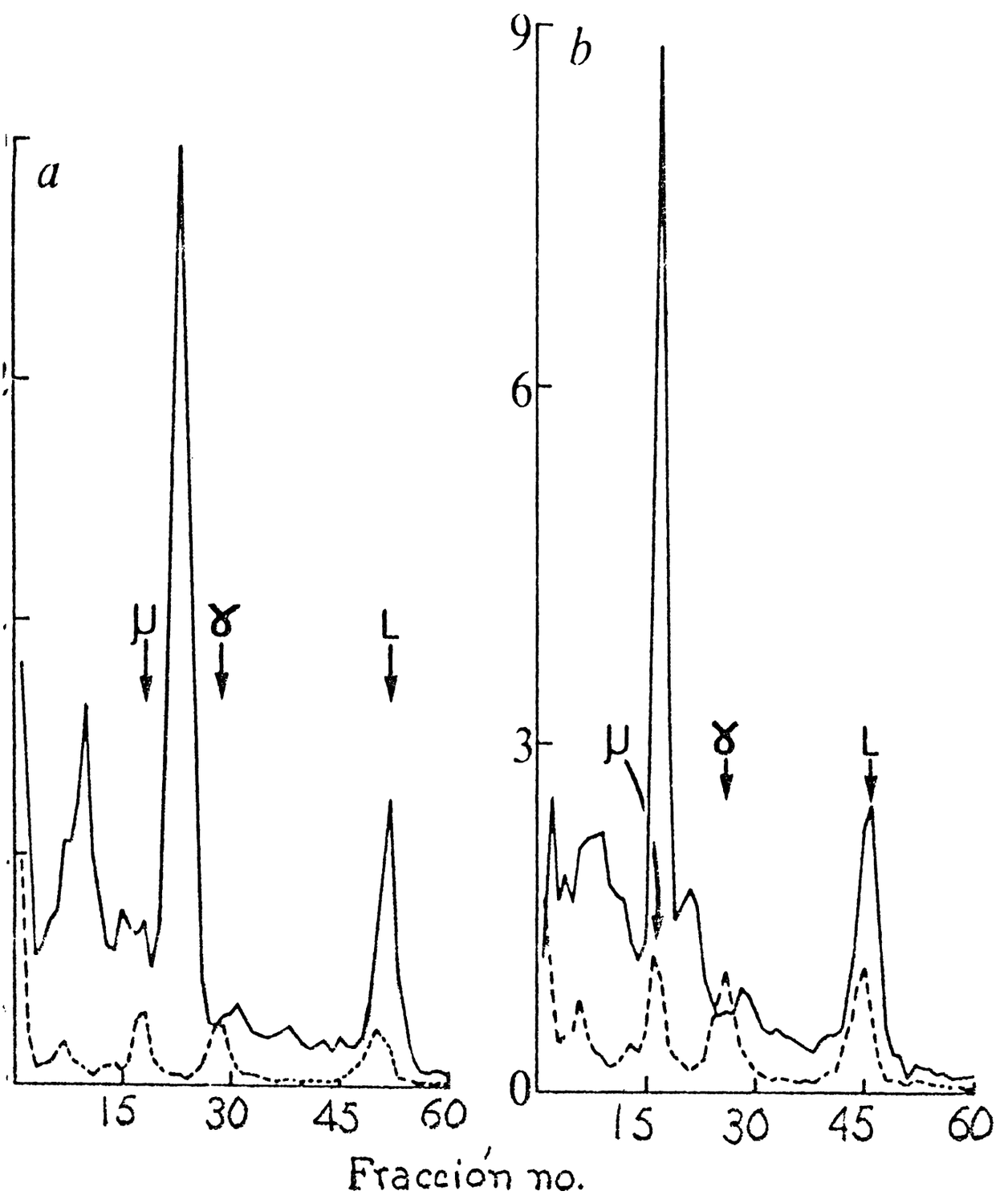

FIGURA 4

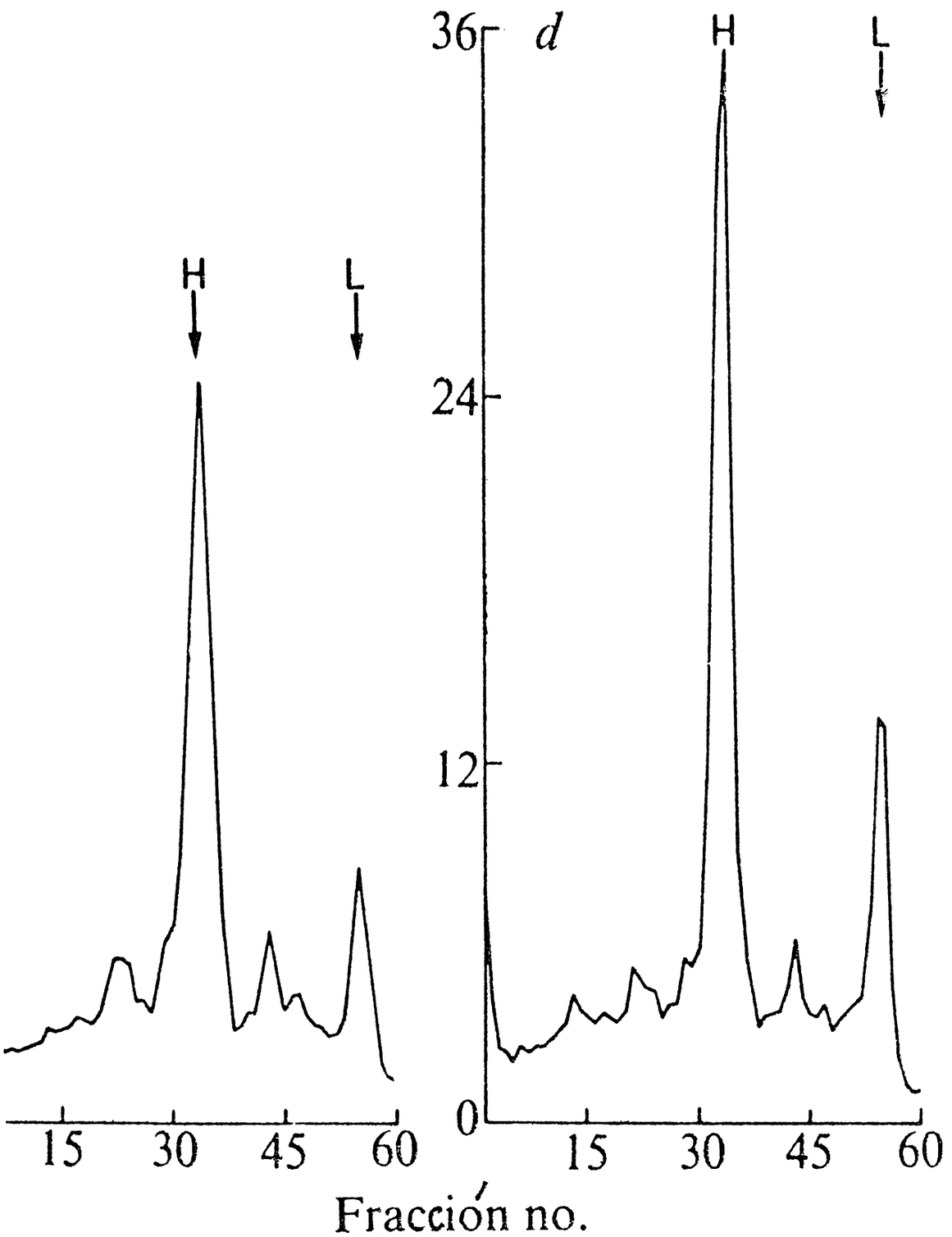

FIGURA 4 Inmunoglobulina de superficie de linfocitos de bazo de ratón.

Suspensiones de células de bazo, de ratonas de CBA (SPF, libres de patógenos específicos) de 3 a 6 meses de edad, marcadas con $^{125}I$ (ver métodos). Los lisados de las células fueron precipitados con 10 $\mu$l del suero de conejo anti-IgD, seguido de 100 $\mu$l de suero de cabra anti $\gamma$G de conejo. El precipitado fué removido por centrifugación. El sobrenadante fué sometido a una segunda precipitación con 10 $\mu$l suero de conejo anti-$\mu$ de ratón seguido de 100 $\mu$l de suero de cabra anti $\gamma$G de conejo.

Ambos precipitados fueron lavados y reducidos con ditiotreitol (ver texto) y la mitad de ambos fué sometida a electroforesis en geles al 7.5% de poliacrilamida (a y b), con marcador interno (ver leyenda de la figura 1).

La otra mitad se corrió en geles al 4.2% (c y d).

a y c corresponden al precipitado con anti IgD.

b y d corresponden al precipitado subsiguiente con anti $\mu$.

Línea contínua $^{125}I$, línea punteada, marcador interno con $^{131}I$.

En base a estos resultados químicos, tuvimos la confianza de que el antisuero es específico para la IgD de ratón, por lo que pudo ser utilizado para estudios de inmunofluorescencia, utilizando reactivos fluorescentes preparados por cromatografía en DEAE-celulosa (38), para teñir células vivas (39).

Con células de bazo de ratón CBA de una a dos semanas de edad, no se encontró un número significante de células positivas (que reaccionaran con anti-IgD), mientras que el número de células positivas con anti-$\mu$ de ratón fué de 8 - 14%. Sin embargo fué aparente la diferencia al utilizar células de bazo de ratones de seis semanas de edad, ya que el número de células positivas fué más o menos del 20% de los linfocitos totales.

La absorción 1 ml. del antisuero con $3.6 \times 10^{10}$ células de hígado fetal no removió su capacidad de reaccionar con las células de bazo o precipitar la IgD de estas células marcadas con $^{125}I$; mientras que estas propiedades se perdieron al absorber 1 ml. del antisuero con $1.9 \times 10^{10}$ células de bazo de ratón adulto. Esto confirma nuestra proposición de que la expresión de IgM antecede a la de IgD en la ontogenia.

Posteriormente determinamos la distribución de IgM e IgD en los linfocitos de diversos órganos linfoides (Tabla 1), llevando a cabo la tinción de dos maneras :

a) Tinción sencilla como método indirecto.

b) Tinción doble, como método directo, tiñendo IgD con los anticuerpos marcados con fluo-

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TABLA 1 Frecuencia de IgM e IgD.

| | Tinción sencilla | | |
|---|---|---|---|
| | Anti-Ig | Anti-$\mu$ | Anti-$\delta$ |
| Nódulos periféricos | 15.25 | 3.10 | 9.80 |
| Nódulos mesentéricos | 14.55 | 3.85 | 11.55 |
| Placas de Peyer | 23.15 | 8.95 | 24.65 |
| Bazo | 33.90 | 19.45 | 24.85 |

| | Tinción doble | | |
|---|---|---|---|
| | $\mu$ | $\mu + \delta$ | $\delta$ |
| Nódulos periféricos | 0.60 | 2.10 | 9.60 |
| Nódulos mesentéricos | 0.95 | 2.30 | 9.40 |
| Placas de Peyer | 1.20 | 8.35 | 16.65 |
| Bazo | 10.40 | 12.85 | 13.45 |

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Las suspensiones celulares fueron preparadas a partir de órganos de ratonas CBA (SPF : libres de patógenos específicos) de 7 meses de edad, en solución amortiguadora de veronal-salina conteniendo 100 mg. de albúmina de bovino (Fracción V) por cada 100 ml (VBS-BSA) y las células vivas se tiñeron de acuerdo con el método de Raff (39) (ver texto). Los nódulos periféricos colectados fueron los cervicales, axilares, branquiales e inguinales. En el caso de la doble tinción, se utilizó el suero de conejo anti IgD seguido de anticuerpo de cabra anti $\gamma$G de conejo marcado con fluoresceína, para ase

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(sigue de la TABLA 1)

gurar la formación de casquete en las células IgD positivas. Después de lavadas las células, se resuspendieron en anticuerpo de conejo anti-$\mu$ de ratón marcado con rodamina, en baño de hielo y en presencia de azida de sodio. Las células fueron lavadas nuevamente montadas en portaobjetos (ver texto) y examinadas en un microscopio Leitz Orthoplan con iluminación de Ploem. Por lo menos se contaron 500 células cada vez y en la tabla se da el resultado en porcentaje de células teñidas.

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resceína y en condiciones de formación de - casquete , seguido de anti-IgM marcada con- rodamina y en condiciones de inhibición de- la formación del casouete, es decir en pre- sencia de azida de sodio y haciendo la incu bación en frío.

Por medio de la tinción sencilla, se ve claramen te que la IgD constituye a la inmunoglobulina de super- ficie más abundante en los nódulos linfáticos y en las- placas de Peyer, como lo sugirieron las pruebas químicas (53, 54, 55, 56).

La tinción doble mostró que de las células con - inmunoglobulina positiva, una mayoría tienen solamente IgD, aunque hay algunas dobles (IgM + IgD) y menos con solamente IgM.

Sin embargo en el caso del bazo los tres tipos de células se encuentran en proporciones más semejantes.

Nuestros hallazgos en el ratón, son por lo tanto diferentes de los de otros investigadores, utilizando - células humanas (51, 57).

En el ratón hay menos células con ambas inmunoglo bulinas (IgM + IgD) con un número mayor de células que tengan solamente IgM ó IgD.

Sin embargo, estos resultados en el humano se han obtenido con linfocitos de sangre periférica por lo que la comparación puede no ser válida, ya que se está vien do a otras poblaciones celulares.

La significación biológica de estas subpoblaciones de células-B deben abrirse ahora a la investigación. - Como la IgM antecede a la IgD en la ontogenia, se puede considerar que en la maduración del linfocito-B hay diferenciación de las células que solamente expresan IgM en la superficie, a las que expresan solamente IgD (52), con un estado intermedio en que la célula exprese a ambas inmunoglobulinas.

Por lo tanto, los linfocitos-B de los nódulos linfáticos y de las placas de Peyer constituirían una población más madura y posiblemente más rica en células de memoria y precursoras de células que vayan a secretar IgG e IgA.

El hallazgo de que los precursores de células de IgA en las placas de Peyer de conejo son IgM negativas e IgA negativas, pero que tienen inmunoglobulina en la superficie (58), sugiere que estas células tengan el - equivalente de IgD en el conejo.

La expresión simultánea de dos clases de inmunoglobulina en la superficie de una sola célula tiene implicaciones de un cambio (switch) y mecanismos de - integración génica V-C; esto es, los dos genes que codifican la región constante para $\mu$ y $\delta$, se sabe que comparten la región variable (59), y ésto refleja la presencia simultánea de dos genes integrados, es decir - $V_H$-C$\mu$ y $V_H$-C$\delta$, por lo que se sugiere un mecanismo de copia y elección (copy-choice) para la integración génica (60, 61).

# C O N C L U S I O N E S

Por análisis radioquímico de la inmunoglobulina de superficie de los linfocitos de ratón, las células-B tienen :

a) Dos subunidades : $H_2L_2$ y HL : Estos resultados se obtuvieron en electroforesis en geles disociantes (con SDS), en condiciones no reductoras (ausencia de ditiotreitol) y bloqueando los sulfhidrilos libres (con iodoacetamida) para evitar reducciones durante el manejo de las muestras.

   Ambos tipos de subunidades rindieron cadenas pesadas y ligeras al ser sometidas a electroforesis en geles disociantes después de haber sido sometidos a reducción (ditiotreitol) y alquilación (iodoacetamida).

b) Dos tipos de inmunoglobulina : IgM e IgD. La identificación de la IgD se llevó a cabo por:

   1. Especificidad antigénica (no es IgM, IgG ó IgA).
   2. Tamaño de la cadena pesada (no es IgM ó IgG).
   3. Comparación con la IgD humana, es decir, tiene el tamaño predicho, se degrada muy facilmente, se encuentra presente en la superficie de los linfocitos (51).

c) La IgG se encuentra en cantidades no detectables por técnicas radioquímicas.

II. Analizando a los linfocitos de manera individual por técnicas de inmunoflucrescencia y observación al microscopio, las células B se pueden - clasificar en :

a) Las que solamente tienen IgM en la superficie.

b) Las que contienen IgM e IgD.

c) Las que presentan solamente IgD.

III. La inmunoglobulina total representa el 3 - 4% de la proteína total de la membrana linfocitaria.

IV. Tanto la expresión de la IgM de superficie - como la IgD en las células-B, es independiente de las células-T, ya que se encuentran los mismos resultados en las células de animales normales, ratones desnudos (nu/nu : atímicos congénitos) y ratones "B" (timectomizados, irradiados y reconstituídos con células de hígado fetal).

V. La expresión de IgM antecede a la de IgD en la ontogenia.

VI. En los linfocitos que tienen más de una clase de inmunoglobulina en la superficie, se presenta el mismo idiotipo (región variable) (61) esto significa que en la síntesis de inmunoglobulinas, debe existir integración génica a nivel del DNA,

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tal vez por un mecanismo de copia y elección.

VII. En lo que respecta a los timocitos y las células-T, no se les detecta inmunoglobulina en la superficie, ni por técnicas radioquímicas ni por inmunofluorescencia. La cantidad que puedan tener, será por lo menos un orden de magnitud menor que la que se encuentra en las células-B.

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*Chapter 10*

# *Biochemical Approaches to Receptors for Antigen on B and T Lymphocytes*

R. M. E. PARKHOUSE
E. R. ABNEY

1. INTRODUCTION .....
2. METHODOLOGY .....
3. ISOTYPES, ALLOTYPES AND IDIOTYPES .....
4. B LYMPHOCYTES .....
5. T LYMPHOCYTES .....
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## 1. INTRODUCTION

The classical experiments of Gowans and his colleagues[1] established that the lymphocyte was the key cell that reacts upon contact with antigen and becomes, as a result of a complex and ill-understood series of differentiative events, the effector cell of immune responses, both humoral and cellular. A feature of the immune response is its specificity and this is explained by selectivity of antigen, as predicted by proponents of selective theories of immunity.[2–4] Thus receptors for antigen on an individual lymphocyte are of one specificity only, but the specificity varies from cell to cell within the total lymphocyte population of an organism. Consequently, when an immunogenic determinant is introduced into a vertebrate animal only the very few cells with appropriate receptor structures are able to interact with the antigen, and they are therefore selected. Following interaction with the antigen those cells divide and differentiate into effector cells. During this process some cells are set aside as memory cells, thereby forming an expanded population so that subsequent exposure to the same antigen provokes the well documented secondary immune response.

Lymphocytes, however, although they may all look quite similar when viewed under the light microscope, are a heterogeneous population of cells. The simplest and broadest division is into B lymphocytes and T lymphocytes, although it is true that a small proportion of cells with the morphological characteristics of lymphocytes cannot be assigned to either category. The latter may possibly be precursors to B lymphocytes or cells which function in antibody-mediated cytotoxicity reactions. The foundation for this

division rests upon experiments with chickens.[5,6] Removal of the thymus was found to impair cellular immunity whereas removal of the bursa of Fabricius profoundly depressed development of humoral immunity. In mice similarly, removal of the thymus depressed cellular immunity.[7] To this day it has not been possible to pinpoint a mammalian equivalent of the chicken bursa, and recent evidence suggests that it is multifocal.[8] However, it was possible to show that in the mouse the precursors of antibody-secreting cells are the B lymphocytes and that their maturation frequently depends upon interaction with T lymphocytes.[9] This concept of an interaction between T and B lymphocytes originally stemmed from experiments in which mixtures of both cell types were considerably more efficient in transferring immune responses than either cell type alone.[10] Also clear was that the T lymphocyte, although it proliferates when presented with antigen, does not differentiate into an antibody-secreting cell.[11-13] The jigsaw was then completed with the advent of cell surface markers for B and T lymphocytes[14] and the demonstration that in many immune responses whilst the B lymphocyte may make the antibody, the T lymphocyte is necessary for induction ('help').[15] The most popular cell surface markers are immunoglobulin and Thy-1 for B and T lymphocytes respectively. Thus B lymphocytes carry surface immunoglobulin and differentiate into B memory and antibody-secreting cells when exposed to antigen, and usually with the collaborative influence of T lymphocytes. T lymphocytes alone are responsible for the various phenomena of cell-mediated immunity. Superimposed upon this already complex scheme are: possible roles for macrophages in immune induction and positive (help) and negative (suppression) effects of T lymphocytes on T and B lymphocyte responses (References 16 and 17, and G. E. Reelants, herein).

In this article, recent exciting and unexpected findings pertaining to receptors for antigen on lymphocytes will be considered in detail. For a thoroughly detailed review of the knowledge up to 1974 the treatise of Warner[18] is enthusiastically recommended.

## 2. METHODOLOGY

All procedures which attempt to define receptors for antigens on lymphoid cells ultimately depend on using an antiserum. For B lymphocytes antisera raised against the varied heavy and light chain isotypes are the reagents, whilst for T lymphocytes a recent development has been the use of sera with anti-idiotype specificity.

Reactivity between a given antibody and plasma membranes is usually taken as evidence for the presence of the relevant antigen on the cell surface. Whatever the system used to detect reactivity, it is obviously crucial that the antibodies used in the investigation be strictly characterized. In principle, sera should be monitored for unwanted specificities by techniques at least as sensitive as the methodology followed in the investigation. For example, absence of antibody activity to light chains as judged by Ouchterlony analysis cannot guarantee that the antibody sample will not detect light chains in more sensitive procedures, e.g. fluorescence or radio-immune precipitation.

The customary procedure for rendering an anti-immunoglobulin serum specific for one isotype is to pass it through solid phase immunoabsorbents bearing all other isotypes. After such treatment, however, it is still possible for the antiserum to contain unwanted specificities. These may be against components not normally routinely tested for, such as $\alpha_2$-macroglobulin, which is in fact found on the surface of most B lymphocytes.[19] Alternatively, an anti-immunoglobulin serum could contain antibodies directed against variable region determinants shared by some, but not all, representatives of most

immunoglobulin classes. Such specificities would frequently escape detection in many testing systems. The possibility of antibodies to immunoglobulin cross reacting with $\beta$2-microglobulin is also raised by the finding of sequence homology between these two entities.[20] Since $\beta$2-microglobulin is found in association with histocompatibility antigens on the surfaces of most cells, antibodies of this type would create obvious, but perhaps unforeseen, problems. Removal of unwanted specificities must be done with solid phase immunoabsorbents to avoid artefacts arising from the presence of antibody–antigen complexes in sera. A major problem is heterophile antibodies to cell surfaces. Whilst many of these can be removed by absorption with membranes from appropriate organs (e.g. liver and kidney for anti-immunoglobulin), on occasions it is not feasible to carry out the appropriate absorption.

The ultimate test for specificity is actually to isolate the molecule on the cell membrane that the antiserum combines with, and then to carry out biochemical characterization. This is possible if the cell surface is first labelled with radioactive iodine using the enzyme lactoperoxidase.[21] Then the cell can be dissolved in dissociating solvents[22] or detergent.[23] The total mixture of radioactive cell surface molecules is reacted with the test antibody and the resulting precipitate is characterized, usually by electrophoresis in polyacrylamide gels containing dissociating (sodium dodecyl sulphate) reagents. By doing this one is not only recording the fact that an antiserum reacts with a cell but also defining what the antiserum reacts with.

In the simplest system the antibodies are tagged with fluorescent or radioactive molecules, mixed with living lymphocytes and then visualized on the surface by microscopy. Thus the presence of immunoglobulin on the surface of B lymphocytes is readily demonstrated using fluorochrome-coupled anti-immunoglobulin.[24,25] It is important to emphasize, however, that this type of approach can only identify molecules, and identification is not rigorous proof of a functional role. When whole IgG antibodies or divalent $(Fab)_2$ fragments are used, the surface components usually aggregate and then 'cap' at one end of the cell (References 26–29, and F. Loor, herein). The process of cap formation is an energy dependent phenomenon, probably involving microfilament activity, but does depend on cross-linking of the membrane located molecules since monovalent (Fab) antibody does not result in capping. Thus capping does not occur in the cold or in the presence of such metabolic inhibitors as sodium azide. In addition, capping is selective, only molecules recognized by the antiserum or strongly associating with the target of the antiserum are capped. This finding is in agreement with the fluid mosaic model of the cell membrane, where the membrane proteins are not fixed, but are free to move within the plane of the lipid bilayer.[30,31] Of great practical importance is the fact that cells can be screened for two surface antigens by using the two appropriate antisera sequentially; the first is tagged, for example, with a green fluorochrome and allowed to cap and the second, tagged with a red fluorochrome, is reacted under conditions where capping cannot occur. The observer can then record cells with green caps, red peripheral staining, or both. Also useful is that once a membrane component is capped by an antiserum it is lost from the cell surface, either by internalization through pinocytosis or by shedding of aggregates to the exterior milieu. This process, often termed modulation, is followed by reinsertion of newly synthesized units in the membrane but does give a finite period of time when the cell surface is denuded of a given membrane component. From the rate at which a modulated molecule reappears on the cell surface, estimates of synthetic and turnover times can be gathered.

Unfortunately, although the identification of cell surface components by reacting living cells with specific antisera is undoubtedly a powerful tool, there are major pitfalls that can

be encountered, particularly with lymphocytes, even when the antisera are undoubtedly specific. The first, and most obvious, is whether identification by this type of approach necessarily means that the surface component visualized is actually an endogenous product of the cells in question. Both B and T lymphocytes can have receptors on their surfaces for the Fc portion of immunoglobulins[32–40] and it is therefore quite feasible for lymphocytes to passively absorb autologous immunoglobulin *in vitro* or the anti-immunoglobulin reagents used for studying the cells *in vitro*. These problems can be dealt with by first treating the cells in such a way that the cell surface component under study is lost. This may occur simply by incubating the cells *in vitro*, but can also be done by modulation with the antibody or by enzyme treatment. Having allowed sufficient time for resynthesis, the cells are then reacted with the chosen antibody, but in its $(Fab)_2$ or Fab form in order to avoid passive uptake mediated by Fc receptors. Ideally, the cell population used should be homogeneous. For example, in a mixture of cells it is possible that only one cell type resynthesizes a certain membrane component, but this is secreted or shed into the surrounding medium and subsequently taken up by other cell types. It is only very rarely that such stringent experimental conditions are followed and, as a result, the literature abounds with conflicting claims.

An alternative approach to identifying receptors for antigen has been to inhibit function of the lymphocytes with anti-immunoglobulin.[41] This approach has the advantage that it does have a functional correlate, but is clearly subject to all the problems discussed above. Basically, anti-immunoglobulin has been added to almost all systems possible; from injecting whole animals to attempts to inhibit the binding of antigen by lymphocytes.

The least ambiguous technique for identifying cell surface proteins with antibodies is by radiolabelling cells. In principle, the labelled cells are solubilized and cell surface components are coprecipitated by addition of appropriate antibody. The antibody will combine with whatever structures it has specificity for, and precipitation of these complexes can be effected by adding non-radioactive antigen (direct precipitation) or an antibody to the first antibody (indirect precipitation). For example, when rabbit anti-mouse immunoglobulin is used with labelled mouse B lymphocytes, precipitation can result from the addition of non-radioactive mouse immunoglobulin or goat anti-rabbit immunoglobulin. The precipitates are then washed and the amount of radioactivity they contain is expressed as a fraction of the total input radioactivity in macromolecular material (estimated by precipitation with trichloracetic acid). Since these specific immunological precipitates invariably contain non-specifically entrapped or adsorbed radioactivity, it is essential to do a non-specific control, with a parallel sample. This simply entails the formation of an immunological precipitate using an antibody lacking specificity for any of the radioactive components of the solubilized radioactive cells. For obvious reasons, the mass of precipitate formed in the specific and non-specific systems should be comparable. Although it is common practice to take the difference between the amounts of radioactivity found in the specific and non-specific precipitates, this does not necessarily give an accurate estimate of specific membrane molecules. Once again we must consider the specificity of the antisera employed. Two other important factors are: the total yield of radioactivity in the specific precipitate, and the choice of reactants for the non-specific control. Most membrane proteins individually account for a small percentage of the total membrane. Immunoglobulin, for example, constitutes about 2% of splenic lymphocyte membranes and this fraction is accordingly the maximum recovery possible in the specific precipitate. It is not uncommon to find 1% of the input radioactivity in non-specific precipitates, and so one is placed in the position of taking the difference between two numbers of fairly similar value. This is obviously a source of error. Far more serious, however, is the variable presence in

normal and immune sera of heterophile antibodies to cell surface components. Suppose an antiserum to immunoglobulin has a high titre of such antibodies and suppose it is reacted with extracts of radioactive cells which do not have surface immunoglobulin, but which do have antigens recognized by the heterophile antibodies. Now suppose that the non-specific precipitation system is one with little or no heterophile antibody content. The result will be a higher recovery of radioactivity in the specific (anti-immunoglobulin) precipitate than in the non-specific precipitate. In this case, however, the higher recovery is not due to the presence of immunoglobulin on the surfaces of the cells examined. It is in fact an artefact of the coprecipitation system. Such artefacts are more likely when direct precipitation is done, and in particular in this case when the non-specific control system is chosen because of its low yield of radioactivity. There is in fact no truly satisfactory control for direct precipitation systems that yield relatively small amounts of radioactivity, e.g. 2% total radioactivity in macromolecular material. Clearly a precipitate formed between a rabbit antibody and mouse immunoglobulin will not necessarily collect the same quantity of non-specific radioactivity (i.e. not mouse immunoglobulin) as one formed between fowl immunoglobulin and a rabbit antibody. This will depend upon the presence or absence of contaminating or unsuspected heterophile type antibodies in the reagents. In this respect, the indirect precipitation system has a major advantage, the non-specific control can be similar to the specific system simply by using normal rabbit immunoglobulin with the same second reagent, e.g. goat anti-rabbit immunoglobulin. Even this, however, is not perfect since the unsuspected specificities in the rabbit antibody and normal immunoglobulin samples can be different. Indeed the yield of radioactivity from labelled spleen cells using normal rabbit immunoglobulin and goat anti-rabbit immunoglobulin does vary from one rabbit serum to another (Abney and Parkhouse, unpublished work). The best control is to use exactly the same reagents added in the specific system, but with the addition of sufficient purified antigen to block all the combining sites of the rabbit antibody. Finally, and as a result of all the above considerations, the radioactivity precipitated by the specific system should always be characterized by analysis in dissociating gels.

There are two ways to radiolabel cells, externally or internally. In the first procedure[21-23] the external proteins of the cell membrane are labelled with $^{125}I$ using lactoperoxidase as the catalyst. A major advantage of this procedure is that the label is confined to the cell membrane. Consequently the cells can simply be solubilized and the extract analysed by coprecipitation with appropriate antibodies. The drawback is that a positive identification does not necessarily guarantee endogenous synthesis by the cell. The method is in principle subject to the same problems as immunofluorescence, but does give the molecular characterization when gel analysis is performed.

For direct proof of endogenous synthesis cells must be labelled internally *in vitro* with radioactive amino acids (or sugars). The problem here is that most of the cell is labelled and that the plasma membrane, which carries receptor for antigen, comprises a small percentage of the total cell protein, about 5% of pig lymphocytes[42,43] or mouse lymphocytes (Abney and Parkhouse, unpublished work). If we assume the receptor for antigen accounts for 5% at most of the cell membrane, then the theoretical yield from internally labelled cells must be very low. In this situation it would appear futile to solubilize labelled cells and directly look for receptors by coprecipitation. Nevertheless, this has been attempted and with some success.[44,45] In order to distinguish between that fraction of total cellular labelled immunoglobulin on the membrane as opposed to that contained within the cell, the following strategy was adopted. Labelled spleen cells were incubated with or without anti-immunoglobulin, lysed with detergent, centrifuged and then immunoglobulin was estimated by coprecipitation. The basis for this is that surface immunoglobulin will be

cross-linked on the cells treated with anti-immunoglobulin and in consequence will sediment when the lysate is centrifuged. This will not occur in the control cell sample and so in principle the difference between the two determinations yields the fraction of radioactive immunoglobulin which is surface associated. The remaining intracellular pool of immunoglobulin will be composed of material destined for the cell membrane and that which will be actively secreted—most lymphocyte suspensions contain some high-rate secreting cells. Apart from the fact that non-specific precipitation is particularly high in this system, rigorous attention to controls is essential. A particular source of error is that the anti-immunoglobulin bound to the cell surface is rarely saturated and, unless special precautions are followed, will therefore combine with intracellular immunoglobulin once the cell is lysed.[45] The failure to saturate both combining sites of anti-immunoglobulin absorbed to the cell suspension is not only possible as a result of only one of the two Fab portions interacting with surface immunoglobulin. It is also possible for added anti-immunoglobulin to interact with lymphocytes and macrophages via Fc receptors, in which case both combining sites of the antibody would be totally free to interact with intracellular immunoglobulin released by lysis. In view of these problems, the claim that mitogen-activated B lymphocytes display $10^2$–$10^3$ times more surface immunoglobulin than the unstimulated cell,[44] should be considered as an exaggerated statement. As an alternative to using whole lysates of internally labelled cells, it is possible to prepare the plasma membrane fraction from the cells and then use this as the starting material for coprecipitation.[46] The obvious disadvantage of this procedure is that it takes a long time, although its merit is that a great deal of the 'noise' is thrown away prior to the coprecipitation step.

## 3. ISOTYPES, ALLOTYPES AND IDIOTYPES

The structural basis of antibody heterogeneity is at the broadest level, the consequence of different heavy and light chain isotypes (classes and subclasses), each of which is specified by a structural gene for the constant region of the polypeptide chain. In many cases there are allelic alternatives (allotypes) at these constant gene loci but in an allotypically heterozygous animal, although both allotypes are found in the serum, individual plasma cells synthesize only one of the allelic alternatives (allelic exclusion).

Isotype and allotype heterogeneity do not, however, explain the enormous repertoire of antibody combining site specificities that can be expressed by an animal. The antibody combining site is formed by the juxtaposition of the variable regions of heavy and light chains, and therefore the uniqueness of a homogeneous antibody is the consequence of its variable region amino acid sequences. It is these unique variable region sequences that define the idiotype. The clonal theory predicts that a precursor lymphocyte will bear receptors of one idiotype, and that its clonal progeny, derived by antigenic selection, will secrete antibodies of the same idiotype. Normally, when an animal responds to an immunogen an enormous number of idiotypes with specificity to the antigen appear in the serum, i.e. many precursor lymphocytes bearing a range of idiotypes are selected by antigen. If specific antibodies could be made to each idiotypic variant, then they could be used as tools to study the genetics of the immune response. For example, what is the pattern of idiotype inheritance. It is possible to raise antibodies specific for the idiotypic determinants of a homogeneous antibody like the phosphorylcholine binding mouse myeloma protein, and in this case the anti-idiotype has proved extremely useful since the normal immune response to phosphorylcholine in Balb/c mice is remarkably homogeneous. Similarly, when immune responses are pauciclonal and the serum antibody is

restricted in its number of idiotypes, preparation of specific anti-idiotype antibodies is possible. The major example here is the anti-idiotype antibody with specificity for the antibody formed by mice injected with streptococcal Group A carbohydrate. Very exciting results have come from this system which, with others, will be discussed in detail in later sections.

## 4. B LYMPHOCYTES

The characteristic property of B lymphocytes in all species examined is their possession of easily detectable surface immunoglobulin. Each lymphocyte presents about $10^5$ immunoglobulin molecules on its surface.[18] Questions of interest, therefore, are: what is the evidence that the surface immunoglobulin serves as a receptor for antigen and which classes of immunoglobulin are expressed on B lymphocytes?

That the immunoglobulin on the surface of B lymphocytes acts as receptor for antigen, there is absolutely no doubt. The most direct evidence comes from studies on antigen binding cells (reviewed in Reference 18). In most of these experiments an antigen is usually labelled with $^{125}I$, mixed with lymphocytes and following a period of incubation to allow interaction between antigen and cell, the cells are washed and then examined for labelled cells by autoradiography. With certain exceptions, in unimmunized animals the number of antigen-binding cells range about a mean of about 0·05% of cells examined. Very few lymphocytes will bind two unrelated antigens, as would be expected from the clonal selection theory, and the frequency of antigen binding cells increases when animals are immunized. The frequency of 0·05% may seem rather high for one antigen, but this does not represent the number of cells specific for one antigenic determinant, nor the number of cells bearing exactly the same variable region pair for creation of a unique combining site (idiotype). Apart from the fact that most of the antigens used in antigen binding studies have multiple antigenic sites (e.g. bovine serum albumin), the repertoire of antibodies produced in response to a simple hapten is vast. By transferring limiting numbers of hapten (NIP)-primed cells from primed mice and then analysing the isoelectric heterogeneity of antibodies formed in recipients, it is possible to estimate that CBA strain mice can synthesize 3000–16 000 different antibodies to the one hapten.[47] The frequency of cells with one defined idiotypic specificity will therefore fall by 2–3 orders of magnitude, becoming somewhere between 50 and 500 cells per mouse spleen. In fact, the frequency of mouse lymphocytes binding phosphorylcholine, an antigen which elicits an immune response of restricted clonality, is in this range, being about 1 in 100 000.[48] The potential of the system is further expanded by the possibility of one antibody combining site having specificity for more than one antigen.[49] This consideration derives from the description of clonal antibodies which can bind two unrelated determinants (e.g. dinitrophenol and menadione), and there is evidence for the occurrence of similar double specificities in the course of a normal immune response.

Having shown that there are small numbers of cells which will bind antigens in a specific way two questions arise. What structure on the cell membranes is binding the antigen, and are the antigen-binding cells precursors of those cells which eventually synthesize and secrete the relevant antibody?

The suspicion that the receptor was immunoglobulin was clearly demonstrated by the failure to find antigen-binding cells when the lymphocytes were pretreated with anti-immunoglobulin.[50] The most elegant experiments of this type are with the phosphorylcholine system.[48] Here it was possible to examine individual antigen-binding cells and to

show that they all possessed surface immunoglobulin, IgM in fact. In addition, the immunoglobulin receptors for phosphorylcholine on B lymphocytes of Balb/c mice possessed the same idiotype as a phosphorylcholine-binding myeloma protein, thereby demonstrating identical specificity of surface and secreted immunoglobulin as well as the restricted nature of this response in Balb/c mice. Further, when an anti-idiotype (anti-(anti-phosphorylcholine)) was used in conjunction with externally radiolabelled lymphocytes it was found to be reacting with IgM by the stringent criteria of isolation and gel analysis.[51] One note of caution relates to the last experiment, and that is the high yield of immunoglobulin precipitated by the anti-idiotype (about 10% of total).

Additional evidence for identification of the receptor as immunoglobulin came from experiments based on the antibody induced capping of surface immunoglobulin.[26–29] Using red blood cells it was shown that caps of red blood cells and surface immunoglobulin were contiguous.[52] Along similar lines, but with flagellin as the antigen, it was shown that at least 95% of the surface immunoglobulin of antigen-binding cells is drawn into a cap when antigen is bound.[53] To some extent the latter experiment shows that all of the immunoglobulin receptor molecules on the surface of a lymphocyte have the same specificity for antigen. A similar conclusion has been reached more recently using anti-idiotype antisera and chronic lymphatic leukaemia (CLL) cells.[54,55] Here, anti-idiotype raised against a monoclonal IgM from the patient's serum was found to cap all of the immunoglobulin on the CLL cell surface.

Having demonstrated that B lymphocytes can bind antigen via cell surface immunoglobulin, we must now ask whether the antigen-binding cells demonstrated *in vitro* are functional. In other words, are they the precursors of high-rate antibody secreting cells to the antigen they bind? One immediate problem is that the frequency of antigen-binding cells to a given antigen is higher than the frequency of precursor cells, as determined by measuring the number of antibody-secreting clones developed upon exposure of a mouse spleen to antigen.[56] Of course, it is possible that the difference is entirely the result of methodological limitations, but since there is a difference it is comforting to find that there is evidence to suggest that some, at least, of the antigen-binding cells are in fact precursors. The earliest experiments which bear on this issue have been termed 'suicide' experiments,[57,58] although Michael Sela is terminologically correct to say that they should more properly be described as 'murder' experiments. In these experiments the antigen-binding cells are formed using a $^{125}$I-labelled antigen of very high specific activity. The cells are washed, transferred into an X-irradiated host, challenged with antigens and an antibody response to the relevant and to control, irrelevant, antigens is measured. If the specific activity of the original radiolabelled antigen is sufficiently high, then the antibody response is specifically abolished, the antigen-binding cell having received a lethal radiation dose from the bound radioactive antigen. Further evidence for the immunocompetence of antigen-binding cells comes from selective depletion or enrichment of precursor cells by passing lymphocytes through columns of antigen bound to inert supports,[59–61] or by separation in a fluorescence activated cell sorter following treatment of the cells with fluorescent labelled antigen.[62]

Some beautifully conceived work with anti-idiotype antibodies is apposite at this point. Since anti-idiotypes specifically react with the variable region of antibodies, frequently at or close to the combining site, could they not mimic the action of antigen? The answer is that they do. What is more, depending on the experimental system they can either inhibit induction of an immune response ((?)tolerance),[63–66] or stimulate production of the appropriate idiotypic antibody.[67,68] Using the streptococcal Group A carbohydrate system, anti-idiotypic antisera were raised (in guinea pigs) with specificity for the anti-Group

A carbohydrate antibody synthesized by A/J mice.[66,67] The guinea pig anti-idiotype was separated into the two major subclasses, $IgG_1$ and $IgG_2$, found in guinea pig serum. Whereas the $IgG_2$ fraction inhibited induction of the idiotype by the antigen,[66] the $IgG_1$ fraction was able to prime B lymphocytes so that they would respond vigorously to the streptococcal Group A carbohydrate when tested in a cell transfer system.[67] In A/J mice responding to the Group A carbohydrate antigen about 25% of the antibodies react with the anti-idiotype, i.e. the response is highly restricted. Nevertheless, upon priming with the anti-idiotype essentially all of the antibody subsequently synthesized is of the idiotype in question. There is therefore a selective expression of the one idiotype when stimulation is provoked by the relevant anti-idiotype. Even more impressive was when the same experiment was done with C57L mice, a strain in which this particular idiotype is undetectable. Upon priming with the anti-idiotype, however, there was production of a cross-reacting idiotype, now in easily detectable quantities. Eichmann and Rajewsky[67] draw three important conclusions from this work. That their and similar data[62] 'leave hardly any doubt that the precursors of idiotype-secreting B lymphocytes express the idiotype as functional receptor molecules. This is close to formally proving the basic assumption of selective theories of antibody formation'. The next point comes from the anti-idiotype provoked expansion of a previously undetectable clone in C57L mice. They point out that the idiotype produced here is cross-reactive with the A/J mouse product (i.e. it is not exactly the same) and that similar experiments with, for example, CBA mice failed to cause the expression of either a completely or incompletely cross-reacting idiotype. These observations 'support the view that the potential receptor *répertoire* of the immune system is strictly germ line controlled'. The inclusion of the word 'potential' in the sentence offers two alternative interpretations: that somatic diversification is preprogrammed or that antibody diversity is carried in the germ line. Many proponents of the germ line theory will almost certainly take satisfaction from the second interpretation and pay scant attention to the first. Meanwhile those favouring the somatic generation of antibody diversity will point out that there is no final conclusion possible. Nevertheless, what is obvious is that further experimentation of this nature will certainly contribute evidence, perhaps decisive evidence, to allow a final decision on whether antibody diversity is encoded in the germ line or somatically generated. The third conclusion is a practical one: 'in certain instances anti-idiotypic antibody may thus serve as a new type of vaccine'.

At this point it might seem churlish to ask the question whether immunoglobulin is actually synthesized by the B lymphocytes. It is, nevertheless, a question which has been posed and which has been answered in the affirmative. The simplest method has been to remove immunoglobulin by modulation with anti-immunoglobulin and then to record its reappearance after tissue culture (e.g. References 69 and 70). Additional evidence has also been presented which is based on internally labelling lymphocytes *in vitro* with radioactive protein and carbohydrate precursors. Surface immunoglobulin is then either defined by treatment of the cells with anti-immunoglobulin,[44,45] or by isolation from a purified plasma membrane fraction of the labelled cells.[46] The underlying methodology was described in an earlier section. The finding of radiolabelled immunoglobulin in these experiments constitutes unequivocal proof of synthesis by the cells under investigation. These and other studies[71–73] using cells externally labelled with $^{125}I$ also provide information on the turnover of membrane associated immunoglobulin. Most immunoglobulin released from the surface is found in the medium in association with fragments of plasma membrane, which is why the process has been termed shedding rather than secretion.[72] The analysis of surface immunoglobulin turnover is complicated by heterogeneity of surface immunoglobulin classes, for example the simultaneous presence of IgM and IgD or the same

cell.[69,70] In addition there is a heterogeneity of B lymphocytes. Thus one subpopulation consisting of relatively large cells, releases cell surface immunoglobulin with a half-life of 1–3 hours. Another subpopulation, consisting of smaller cells, releases immunoglobulin at an appreciably slower rate, the half-life being 20–28 hours.[73]

The lymphocyte, unlike the plasma cell, does not contain the well developed membranous elements that characterize secreting cells. In plasma cells it is now clear that immunoglobulin destined for secretion is translated on membrane bound polyribosomes.[74] The nascent polypeptide chains are vectorially released from the polyribosomes into the cysternal spaces of the rough endoplasmic reticulum. From there, after their assembly into disulphide linked $H_2L_2$ structures, they pass to the exterior milieu via the Golgi apparatus. Some biosynthesis data obtained with B lymphocytes[45] are consistent with a similar transport route for membrane immunoglobulin, but the evidence is by no means conclusive. In the same series of experiments it was also possible to biosynthetically radiolabel cell surface immunoglobulin with labelled fucose and galactose, in contradiction to some earlier work which indicated that surface immunoglobulin was devoid of these sugars.[44]

There is therefore no doubt that the receptor for antigen on immunocompetent B lymphocytes is endogenous immunoglobulin, and that a given lymphocyte bears immunoglobulin of only one idiotype. Upon stimulation its clonal product then secretes antibodies of the same idiotype. Before passing to the vexed question of constant region expression on lymphocytes, it is worth mentioning the current argument over whether the receptor immunoglobulin acts as a signal to the cell when it binds antigen. At first sight this might appear to be a very silly question indeed. On the basis of experiments with the polyclonal B lymphocyte activator, lipopolysaccharide, Coutinho and Möller[75] have argued that the signalling site, is not immunoglobulin. The immunoglobulin merely serves to focus the antigen on the cell surface where it then interacts with another membrane protein, which is postulated and for which no concrete evidence exists. This membrane protein then provides the signal which directs the cell into a state of tolerance or immune induction, depending on the number of interacting sites: just right for induction and too much for tolerance. The major arguments against this 'one-signal' model are presented by Cohn and Blomberg in an article which also very fairly summarizes the Coutinho–Möller point of view.[76] The major point is that the one-signal model was designed to account for observations with lipopolysaccharide which is a very specialized system in that it deals with a polyclonal B cell activator, unlike most situations of B lymphocyte induction. There are therefore a number of observations which cannot be accounted for by the Coutinho–Möller model, e.g. maintenance of B cell paralysis to self, breaking of tolerance with material that cross-reacts with the tolerogen. Challenging and thought-provoking though the one-signal hypothesis may be, it more underlines our ignorance of basic mechanisms of immune induction than it explains them. The real truth is that we really do not understand the dual mystery of antigen recognition, the rendering of the target cell to a non-responsive or differentiative event.

Having said that, it is time to consider the expression of constant regions on immunocompetent cells. From a number of observations, and bearing in mind some apparent exceptions,[18] it is now clear that allelic exclusion occurs. The most conclusive experiments which demonstrate this are those of Jones, Cebra and Herzenberg.[77] These experiments are particularly instructive since they follow what is certain to be a model protocol which sidesteps problems due to cytophilic antibody. They took lymphocytes from allotypically heterozygous rabbits, stripped off endogenous and non-endogenous immunoglobulin by treatment with proteolytic enzymes *in vitro* and then placed the cells in

tissue culture in order to allow for resynthesis of endogenous membrane proteins. Then, with the aid of fluorochrome-coupled specific anti-allotype antibodies they were able to sort the cells, on the basis of their surface immunoglobulin allotype, by using the fluorescence activated cell sorter. The results were quite compelling; lymphocytes of a given allotype went on to make that, and not the alternative allotype. This evidence taken together with that reviewed by Warner,[18] makes a persuasive case for allotypic exclusion at the level of constant genes on the lymphocyte surface.

In considering the constant regions expressed on B lymphocytes it would be as well to first define the questions that will be discussed. For example, does a given lymphocyte and its clonal progeny always synthesize one and the same constant region or can there be a switch from one constant region to another, either in ontogeny or upon an immunogenic challenge? In the first case, stem cells would give rise directly to lymphocytes bearing surface immunoglobulin of only one of the known classes, either IgM or IgG or IgA and so on. These, upon immunogenic induction, would eventually give rise to cells secreting exactly the same isotype represented on the original precursor cell surface. Thus, IgM- and IgG-secreting cells would derive from lymphocytes bearing IgM and IgG respectively. For this type of situation only one V–C gene integration event would be necessary. However, it is now almost certain that a model of this nature is untenable. Somewhere in the development of many individual B lymphocytes a change of $C_H$ gene expression occurs. The major arguments therefore concern the sequence of heavy chain expression in lymphocytes and their progeny, and whether this 'switching' of $C_H$ genes is antigen driven or not. Further, if exposure to antigen is responsible for switching of $C_H$ genes, then at what level of differentiation does it occur? In an antigen independent model,[78] stem cells first develop into lymphocytes bearing surface IgM. Some of these cells are postulated to give rise to lymphocytes bearing IgA via a population with IgG on their membranes. This developmental progression, it is argued, occurs without the necessity for antigenic intervention, and involves two $C_H$ gene switching steps, i.e. from $C\mu$ to $C\gamma$ and from $C\gamma$ to $C\alpha$. When antigen does interact with the cells, they differentiate into high-rate antibody secreting cells expressing the same isotype as the original precursor lymphocyte, e.g. cells with IgG receptors develop into IgG-secreting cells. The model was originally proposed by Cooper, Kincade and Lawton to explain a series of pioneering experiments with chickens.[78] By judicious timing of bursectomy and administration of anti-$\mu$ chain serum *in vivo* they were able to render the animals completely agammaglobulinaemic. A suitable delay in these manoeuvres could either cause appearance of IgM, but not IgG or IgA (bursectomy at 16–17 days), or selective suppression of IgA only (neonatal bursectomy). An important point is that once cells are seeded from the bursa to the periphery suppression of IgG and IgA requires a very prolonged regimen of anti-$\mu$ chain injections. It is therefore easier to inhibit the development of IgG expression with anti-$\mu$ when bursal B lymphocytes, rather than peripheral cells, are the target of the anti-$\mu$ chain reagent. Nonetheless, chronic administration of anti-$\mu$ chain *in vivo* can prevent the appearance of all antibody, both in mice and chickens,[78,79] and is strong evidence for the occurrence of $C_H$ gene switching in lymphocyte differentiation. Other frequently quoted evidence for switching is the presence within one individual of myeloma protein, which is heterogeneous with respect to isotype, but homogeneous for the variable regions.[80,81] For example, in one case the myeloma protein was shown to consist of IgM, IgG and IgA, but all three isotypes shared the same idiotypic determinants.[81]

Taking the Cooper–Kincade–Lawton model as the starting point for discussion, we shall now pose some questions. In doing this, it should be pointed out that their model, first published in 1971,[82] has probably stimulated more alternative forms than any other

attempted description of B cell development (cf. Reference 18). There are no serious objections to the idea of a switch in $C_H$ expression during differentiation and development of lymphocytes. The major alternatives centre on whether the $C_H$ switch is antigen independent or not, and whether the sequence of IgM → IgG → IgA occurs entirely on the lymphocyte surface so that the receptor and eventual secreted product are of the same isotype.

The original postulate of antigen independent switching was based on the bursa-dependence of the switch in chicken, and also the fact that deliberate administration of antigens to chicken embryos failed to influence the development of IgM- and IgG-containing cells in the bursa. On the other hand, unprimed mouse spleen cells, which respond to sheep red blood cells *in vivo* by synthesizing IgM, IgG and IgA antibody, fail to do so if treated with anti-$\mu$.[83–85] Furthermore, exposure of normal spleen cells to anti-$\mu$ chain caused a marked suppression of IgG production by the cells when tested in an *in vivo* transfer system.[86] If the appropriate precursor cells were generated in an antigen independent way it is difficult to see how responses to IgG and IgA could be inhibited by anti-$\mu$. An alternative explanation, and one that will be discussed below, is that the precursors of the IgG and IgA secreting cells rarely have IgG and IgA on their surfaces.

Having said that, this is as good as any time to say that the situation regarding $C_H$ representation on the surface of B lymphocytes is thoroughly confused, as perusal of the review by Warner[18] will indicate. There are three very clear reasons for the confusion. First, much of the data comes from immunofluorescent staining and, as pointed out in an earlier section, there are many problems associated with material binding through Fc receptors, or perhaps even being non-specifically absorbed. What is clear, however, is that the number of B lymphocytes recorded positive for IgG and IgA steadily decrease to a very low number (1%–3%) as the experimenter takes more care to avoid these problems.[87–90] Second, and in particular in the mouse, when surface immunoglobulin is characterized by external labelling and immunochemical procedures there is little, if any, IgG or IgA detected.[22,23,91] Third, is the problem of distinguishing between a precursor lymphocyte that has not been in contact with antigen and one that has, and, as a result, has embarked upon an irreversible differentiation pathway leading to the high-rate antibody secreting cell. To pose an example: suppose a lymphocyte with surface IgM interacts with antigen and its clonal progeny secrete IgG, as could be inferred from data discussed above.[78,79,82–85] At some stage of the differentiation process the cells will begin to express IgG, possibly on their surfaces. If so, are these surface IgG molecules to be regarded as receptors for antigen? For the purposes of this article, the answer will be in the negative. Nonetheless, this type of cell would be positively recognized in an immunofluorescence procedure. It is also possible that a cell at this stage of differentiation could be stimulated into an abnormally extensive clonal expansion under certain experimental conditions, for example, transfer into an irradiated host together with antigen, and this would create obvious problems in the design and interpretation of experiments. In this context it is also important to point out that many IgG-secreting mouse plasmacytoma cells can be shown to have surface immunoglobulin,[92] but here the distinction is clear. The surface associated immunoglobulin molecules are not functional receptors for antigenic stimulation, but once again an immunofluorescence assay would record a positive result. Not all the B cells stimulated by antigen go on to become high-rate antibody-secreting cells. Some will be set aside as memory cells and the nature of their receptor immunoglobulin is more a matter for conjecture than informed discussion. There is one beautiful series of experiments which does suggest that the isotype of memory cell surface immunoglobulin corresponds to the eventual secreted product.[93] Memory cells and high-rate antibody-secreting cells from

mice primed 1–6 months prior to sacrifice were retained on antigen-coated columns. Assay for memory was done by transfer of the cells to lethally irradiated recipient mice. Pretreatment of the cells with either anti-$IgG_1$ or anti-$IgG_2$ before passage down the insoluble antigen column caused a selective 'sneaking through' of presumed precursor cells for $IgG_2$ and $IgG_1$ synthesis respectively. Impressive as the experiments are, they were done well in advance of our current appreciation of the problems arising from cytophilic antibody and Fc receptors. They cannot therefore be regarded as in any way conclusive.

At present we are therefore faced with the embarrassment of a total lack of correlation between isotypes expressed systemically and on lymphocytes. Since the number of IgG and IgA bearing lymphocytes is low, it is reasonable to ask whether this minor population of cells has biological significance. It is also a matter of urgency to define the immunoglobulin on the vast majority of B lymphocytes.

In answer to the first question we can say that it is entirely possible that a small population of, for example, IgG bearing cells could be rapidly expanded by cell division in such a way as to account for all of the serum IgG. Indeed, and as mentioned above, there are experiments suggesting that IgG is present on the surface of some, but not necessarily all, precursors of IgG secreting cells.[93,94] With the prevailing paucity of information, however, it is fair to ask if this is the exception rather than the rule. The situation with IgA secreting cell precursors is more confused. These are reported to have or not to have surface IgA, depending on the antiserum used to make the identification.[95,96] A positive identification was made with an anti-IgA allotype antiserum (directed towards the Fd part of the heavy chain), whereas IgA could not be detected when a conventional anti-isotype antiserum was used. For reasons given below, we find the results with the anti-isotype antiserum more convincing, and therefore predict that IgA will not be on the surface of precursor lymphocytes destined to secrete IgA.

If most immunoglobulin on B lymphocytes is not absorbed, then what is it? For some time IgM has been recognized as a major component of B lymphocyte membranes. External radiolabelling procedures showed that surface IgM differs from the secreted variety by being the monomeric, $H_2L_2$, subunit rather than the fully assembled $(H_2L_2)5$ pentamer.[22,23] In these and other[91] similar studies, neither IgG nor IgA were detected (lower limit of detection about 5% of total cell surface immunoglobulin). A major breakthrough came with the discovery that IgD was, in addition, present on many human peripheral B lymphocytes.[97,98] This was quickly followed by the demonstration of its endogenous synthesis[69,70] and occurrence on many human chronic lymphatic leukaemia cells. Although some cells expressed either IgM or IgD, on many both isotypes were simultaneously present.[99,100] Using chronic lymphatic leukaemia cells and an anti-idiotype raised against their IgM product it was possible to show that where the two isotypes co-exist on the same cell, they share the same idiotype,[54,55] and hence presumably V regions. The simultaneous expression by one cell of two isotypes sharing the same V region has implications for the mechanism of V–C gene integration. Because of the very long life of chronic lymphatic leukaemia cells, it seems probable that in those cells expressing both IgM and IgD the genome contains integrated genes for both heavy chains. To explain this, reiteration of the V gene or a copy-choice[101] mechanism for V–C gene integration is required. These speculations are based on the long life of chronic lymphatic leukaemia cells, and conclusive evidence (e.g. simultaneous transcriptions of mRNA for $\delta$ and $\mu$ chains) is lacking. Having raised the possible existence of two integrated heavy chain genes in the chronic lymphatic leukaemia cell, however, it does suggest the following hypothetical scheme. At some time in the development of a B-lymphocyte there is integration of a

unique V gene, with the constant region genes of all heavy chain isotypes. Provision of multiple copies of the same V gene could be germ line determined or somatically generated by making a series of copies (i.e. copy-choice) at the time of integration. The advantage of simultaneous integration of all $C_H$ genes early in the ontogeny of B lymphocytes is that questions relating to isotype expression would then revolve entirely around differential gene activation and repression; the requirement to account for V–C gene integration events at later stages of differentiation would no longer exist.

The paucity of serum IgD contrasts with its high frequency on B lymphocytes and if a special role for this class of membrane immunoglobulin is to be entertained, then the importance of establishing the existence of a similar molecule in an animal species is obvious. To date, there have been claims for a similar immunoglobulin in the mouse[91,102,103] and suspicions of one in the rabbit.[104]

In the mouse, the candidate for IgD is a disulphide-linked $H_2L_2$ molecule which can be precipitated from lysates of externally labelled nude or normal mouse B lymphocytes with anti-light chains, but not antisera to any of the known mouse heavy chain isotypes. It is therefore clearly an isotype hitherto undescribed in the mouse. This candidate for IgD resembles the human counterpart in heavy chain size, marked susceptibility to proteolysis, and in its occurrence on lymphocyte surfaces. In the absence of sequence data this new immunoglobulin class in the mouse cannot be formally identified as IgD. However, for ease in presentation, and because of the similarities noted above, we shall henceforward refer to this molecule as IgD. In addition, the probability that sequence studies will confirm this assignment appears to be very high.

In both humans[105] and mice[91,103] IgM precedes IgD in ontogeny, IgD appearing about two weeks after birth in mice and at some time between 3 and 4 months of gestation in the human. Earlier assumptions that IgD precedes IgM in the human were based on comparisons between cord and adult blood[69,70,98] and appear to be incorrect. Interestingly, what little we know of the amino acid sequence of human IgD would suggest that it evolved some time after the gene for $\mu$-chains.[106] Thus IgD appears subsequent to IgM in both evolution and ontogeny.

There is a marked difference between the relative amounts of IgM and IgD in murine spleen lymph nodes and Peyer's patches.[91,102,103,107] By external labelling techniques IgM and IgD were found in approximately equal amounts in the spleen; in lymph nodes and Peyer's patches, IgD accounted for 70% and 90%, respectively, of the total immunoglobulin. It is important to emphasize that in these studies IgM and IgD are the *only* immunoglobulins recovered.

The external labelling procedure can only give the total yield of IgM and IgD, and not their distribution on individual cells. Based on the rarity of immunoglobulins other than IgM and IgD on the surface of mouse B lymphocytes, fluorescent staining was used to indicate that in the mouse, as in the human, there are splenocytes bearing IgM or IgD, or both IgM and IgD.[108] In those experiments IgM was first capped with rhodamine-labelled anti-$\mu$ chain, and then subsequent ring staining with fluorescein-labelled anti-Fab in the presence of sodium azide was taken as evidence for the presence of IgD. Recently, an antiserum specific for mouse IgD has been developed,[109] and the above result has been confirmed. Using this antiserum it could be shown that the majority of B lymphocytes in lymph nodes and Peyer's patches were expressing only IgD, although some doubles (IgM and IgD) and an occasional IgM-bearing cell were seen. In the spleen, however, all three categories of cells were present in similar proportions. Inspection of the cells doubly stained for IgM and IgD suggested a considerable variation in the relative intensities of the two fluorescent markers, suggesting a variation in the IgM and IgD ratio from cell to cell

This, together with the observation of the three cell types (i.e. IgM, IgD and IgM–IgD) and the fact that IgM precedes IgD in ontogeny, suggests a developmental sequence of immunoglobulin expression on B lymphocytes from IgM to IgD via an intermediate cell type with both isotypes. Thus the B lymphocytes of lymph nodes and Peyer's patches would in the main constitute [illegible] cells. [illegible] this [illegible] the finding that large, immature splenocytes from mice are enriched for IgM whereas the smaller splenic lymphocytes, thought to be more mature, are enriched for IgD.[110]

It is an extraordinary fact that IgD was not detected on B lymphocytes earlier. However, it is present in very low amounts in normal serum,[111] and thus its very extensive representation on B cells must imply a specialized role as receptor for antigen. This is easy to say but is not particularly illuminating. At present, we can only guess at possible roles for IgD. As discussed above, there may be a developmental sequence. According to this, immature lymphocytes bearing IgM will develop into cells with surface IgD via an intermediate cell type with both isotypes expressed. Do each of these three cell types exercise different immunological functions? Do all B lymphocytes pass through these three distinguishable stages, or are some arrested or diverted in other directions (e.g. IgG) at any stage of the differentiation pathway? Time, and experiments with the fluorescence activated cell sorter will almost certainly provide an answer. A most intriguing question is whether IgM and IgD give different signals when simultaneously present on one lymphocyte. In many ways this would seem unlikely since they both share the same variable region. However, a key question could be the exact mode of insertion of these two immunoglobulins in the membrane, e.g. whether they are attached to and/or associated with the same or different cell surface components. An important question is exactly when IgD arose in evolution. If this isotype is not found in, for example elasmobranch fishes, then some clues as to the function of IgD might be suggested. The marked susceptibility of IgD to proteolysis[111] could conceivably be a clue. In fact, a model for B lymphocyte stimulation which incorporates proteolytic cleavage of IgD as a crucial event has been suggested.[112]

An interesting observation has recently been made with a line of CBA mice (CBA/N) which have an X-linked defect and associated failure to respond to thymus-independent antigens. It appears that these mice have reduced but detectable levels of IgD,[113] and consequently it is possible that IgD plays a critical role as a receptor for thymus-independent responses. In the experiments reported the immune responses measured were of the IgM variety, and so there is some conceptual difficulty in interpretation. In addition, there are reduced numbers of B lymphocytes in CBA/N mice and so the failure to respond to thymus-independent antigens could be the result of defects other than at the IgD level.

Experiments in the rabbit provide circumstantial evidence for IgD bearing lymphocytes as the precursors of IgA secreting cells. Peyer's patch cells were stripped with a proteolytic enzyme and then cell surface immunoglobulin was allowed to regenerate *in vitro*. The cells were then sorted in the fluorescence cell sorter using fluorescent-labelled anti-$\mu$ chain to give $\mu$-negative and $\mu$-positive subpopulations.[95] Upon transfer or stimulation with pokeweed mitogen *in vitro*, the $\mu$-positive cells developed primarily into an IgM secreting population. The $\mu$-negative cells, which contained lymphocytes stained with anti-light chain but not heterologous anti-$\alpha$ chain, contained precursors of IgA secreting cells; it is reasonable to assume that the precursors were the light chain bearing cells. In another publication,[96] it was claimed that surface IgA regenerated upon culture of the cells *in vitro* after enzymic stripping. For identification of IgA an anti-allotype antiserum thought to be specific for the IgA class was used. No functional studies were done with the IgA-positive

population. Nevertheless, the authors concluded that precursors of IgA secreting cells have IgA on their surfaces. However, when untreated rabbit Peyer's patch cells were externally labelled with $^{125}I$, the immunoglobulin recovered resolved into three components after reduction and alkylation.[104] The major components corresponded to $\mu$ and $\delta$ chain, and in addition there was a minor fraction which appeared to be $\alpha$ chain. Unfortunately, the presumptive $\delta$ chain was not taken into consideration in these experiments. Furthermore, the radiolabelling was conducted on untreated Peyer's patch cells and these are known to bear cytophilic IgA. Two controls are suggested. First the specificity of the anti-allotype antiserum should be checked by the stringent criteria of external labelling and subsequent immunochemical analysis; the cells for labelling should of course be first stripped with enzyme and then allowed to regenerate cell-surface components *in vitro*. Second, the same population of cells should be fractionated with appropriate anti-immunoglobulin reagents and the subpopulations submitted to functional testing. We predict that when this is done the majority of IgA precursor cells will be found to express IgD on their surfaces.

Finally, any model of B lymphocyte differentiation must be superimposed upon the major two surface immunoglobulins, IgM and IgD. Whether or not IgG secreting cells derive from a small population of IgG bearing lymphocytes, their origin is almost certainly a cell with IgM, IgD or both. Furthermore, expression of surface IgD is independent of thymic[91,103] or antigenic[103] influence.

Is there anything that distinguishes membrane-bound from secreted immunoglobulin? At first sight there are two clear differences. First, surface IgM is assembled as far as the $H_2L_2$ monomer, whereas secreted IgM is polymeric, consisting of five disulphide linked monomer subunits. Second, a large proportion of lymphocyte associated immunoglobulin is IgD, an isotype which is a very minor component of serum. These differences do not, however, immediately afford an understanding of the mode of membrane insertion. Thus monomeric IgM can be secreted in certain situations[114,115] and so this particular subunit structure does not necessarily guarantee insertion into the membrane. In addition, and although the comparison cannot be made for IgD, the known sequence of IgM[116] does not suggest a C-terminal region of marked hydrophobicity. As noted above, there is confusion over whether surface-associated and secreted IgM differ in their relative amounts of carbohydrate.[44,45] However, while small carbohydrate differences cannot be ruled out, it does seem that surface immunoglobulin does possess some, if not all, of the fucose and galactose incorporated into secreted IgM. Similarly, other small structural differences between surface-associated and secreted immunoglobulin cannot be dismissed. One possible line of enquiry would be to ask whether membrane bound immunoglobulin heavy chains do or do not have the extra N-terminal sequence of about twenty amino acids found in the intracellular precursors of secreted immunoglobulins.[117] It is not inconceivable that this sequence, normally cleaved from immunoglobulin polypeptide chains prior to secretion, could be a feature of surface immunoglobulins. The actual integration of immunoglobulins into the membrane structure is another intriguing unknown, the solution of which could well have implications for the mechanism of antigenic stimulation.

## 5. T LYMPHOCYTES

In one year's time the controversy over the molecular nature of the T cell receptor for antigen will almost certainly be solved by extension of recent studies using anti-idiotype antisera. There is therefore no useful purpose in a detailed review of past errors and

successes in this extraordinarily murky area. Instead, this section will be brief and, as a result, incomplete. For a voluminous account of the many approaches by which attempts have been made—some apparently successful and others contradictory—to demonstrate immunoglobulin on T cells, the reader is referred to Warner's excellent review.[18] At the outset we should like to draw attention to the fact that for years Simonsen has maintained consistent scepticism towards the idea that conventional immunoglobulin is the T-cell antigen receptor.[118,119] There is now increasing evidence to justify his scepticism.

To start with the least contentious points: it can be asserted that T lymphocytes do not generally express high density immunoglobulin determinants on their membranes: they do not stain when treated with fluorochrome coupled anti-immunoglobulin. Furthermore, in most systems where immunoglobulin has been demonstrated on the surface of T cells, when appropriate experiments have been carried out the immunoglobulin can be shown to be exogenously derived. For example, chickens deprived of B lymphocytes by bursectomy and anti-$\mu$ chain treatment do not form rosettes with sheep red blood cells unless previously injected with an anti-sheep red blood cell serum obtained from a normal, immunized bird.[120] Similarly, the low density labelling of chicken T cells observed using radiolabelled anti-chicken light chain disappears when the source of serum antibody, the B cells, is removed by bursectomy.[121] Therefore in chickens there is excellent evidence for an absence of endogenous immunoglobulin on the surface of T lymphocytes. In mammals too there is good evidence for T cells with exogenously derived immunoglobulin. Thus using allotypic markers it has been convincingly demonstrated that T cells in the thoracic duct of mice[122] and rats[123] passively acquire immunoglobulin. From these experiments it appears that activated T cells acquire more surface immunoglobulin than resting T cells, although in both situations it seems likely that the uptake is due to Fc receptors.[35-40]

Of the many reports that anti-immunoglobulin can inhibit various manifestations of cell-mediated immunity, there are certainly an equivalent number of contradictory findings.[18,118] We have discussed at length in an earlier section the sorts of unwanted specificities that can occur in poorly characterized anti-immunoglobulin antisera and agree with an explanation offered by Simonsen:[118] namely that positive findings are the result of contaminating antibodies to cell surface structures other than immunoglobulin constant regions.

An enormous amount of work has been devoted to antigen-binding T lymphocytes,[18] and in many cases it has been inferred (through the use of anti-immunoglobulin) that antigen is bound to T lymphocytes via immunoglobulin. However, as Simonsen points out,[118] control absorptions of the test antiserum with T lymphocytes are never done. More recent studies using the synthetic copolymer (T, G)-A-L indicate major differences in the specificity and nature of antigen-binding by T and B lymphocytes.[124-126] The differences are in temperature requirements, metabolic state, susceptibility to anti-histocompatibility antisera and specificity. Antigen-binding to T cells occurs more efficiently at 37 °C than at 4 °C, so that more antigen-binding cells are observed at the higher temperature. Consequently more or less antigen-binding T cells can be obtained simply by shifting the temperature up or down. The reversible nature of this phenomenon could be inhibited by addition of a metabolic inhibitor (sodium azide). Thus if T cells were incubated at 37 °C and then shifted to 4 °C in the presence of azide, the frequency of antigen-binding cells was the same as that observed at 37°C. With B cells, on the other hand, equal numbers of antigen-binding cells are found at either temperature. Another difference was that pretreatment with antisera against the K or D locus of H-2b abolished the formation of T, but not B, antigen-binding cells. Finally, the specificity of antigen-binding was quite different for T and B cells. Whereas binding of (T, G)-A–L to B cells was inhibited by an

excess of (H, G)-A-L or (Phe, G)-A-L, this was not the case for T cells. The simplest conclusion from this series of experiments demonstrating marked differences in the characteristics of antigen binding by B and T lymphocytes is that the two cell lines bear different structures for antigen recognition. This is the same as saying that immunoglobulin is not the antigen receptor on T cells. Those who wish to quarrel with the conclusion can assert that there is immunoglobulin, and argue that the observed differences in antigen-binding characteristics are a consequence of differences in membrane organization between B and T cells.

The most elegant experiments designed to demonstrate the immunoglobulin nature of the receptor on antigen-binding T cells are those of Roelants and his colleagues.[127,128] Basically they showed that pretreatment of T lymphocytes with anti-immunoglobulin caused capping of the radiolabelled antigen subsequently bound to T cells. To be sure that they were not dealing with an artefact resulting from cytophilic antibody they used unimmunized mice, and incubated the cells *in vitro* after the treatment with anti-immunoglobulin. Under these conditions the receptor for antigen would be lost by internalization and, or, shedding as a result of the anti-immunoglobulin-induced capping. After a suitable time *in vitro* endogenous receptors would be synthesized and reinserted into the cell membrane. The prediction then is that at short times (1 hour) after treatment with anti-immunoglobulin there would be no antigen-binding T cells, and at long times (6–18 hours) there would be. In fact, the observations were in agreement with the prediction but the experiments are nonetheless subject to the criticisms raised above concerning the specificity and use of antisera. It is exactly this type of situation that demands biochemical characterization of the material on the cell surface reacting with the antiserum. Furthermore, the experiments were with unfractionated populations of lymphocytes (i.e. containing B cells) and so an artefact due to the contaminating B lymphocytes cannot be strictly ruled out.

By far and away the most compelling evidence for immunoglobulins on T cells appears to come from external labelling studies. Using the lactoperoxidase-catalysed procedure for radiolabelling cell surfaces, immunoglobulin has been recovered in similar[22] or even greater[129] yields from thymocytes as B lymphocytes. The former group[22] has extended their observation to other sources of T cells[130–132] with similar findings, but others have failed to confirm their observation with thymocyte or peripheral T lymphocytes.[133–141] We shall not discuss here the presence or absence of immunoglobulin on T cell lymphomas[132,142] since this type of cell is malignant and therefore not necessarily a precise model for an immunocompetent T lymphocyte.

This situation is disturbing and almost without precedent. Here we are dealing with precise biochemical techniques including gel analysis of the labelled cell surface material reacting with anti-immunoglobulin. On the one hand, it is claimed that thymocytes and T cells have immunoglobulin which is a disulphide-linked 7S 'IgM-like' molecule. What is more, in these studies the yield of 7S 'IgM-like' molecule is comparable when either normal thymocytes or splenocytes from nude mice are labelled.[22] On the other hand, however, using similar techniques this observation cannot be reproduced by a number of different investigators. The only possible conclusion that can be drawn is that one of the two groups of protagonists in the controversy is wrong. We take the view that there is not a disulphide-linked 7S 'IgM-like molecule' on thymocytes and T lymphocytes. This point of view has been arrived at for a number of reasons, other than the irreproducibility of the work.

In the first place, in none of the experiments where 7S 'IgM-like' immunoglobulin has been isolated from radiolabelled T lymphocytes have three crucial controls been done.

The first is to see if the anti-immunoglobulin does or does not precipitate the putative T cell receptor when cold immunoglobulin is added to block the combining sites of the first antibody used in an indirect coprecipitation test. As discussed in detail in an earlier section, this is the most exact control for non-specific precipitation. The second is to absorb the anti-immunoglobulin with thymocytes prior to the coprecipitation step. The third important control is to analyse the radioactive immune coprecipitates on dissociating gels without a prior chemical reduction. Any radioactive material then appearing in the 7S region of the gel should then be eluted, reduced and submitted for a further gel analysis to show that the radioactivity now migrates in the predicted positions of heavy and light chains. We predict that the 7S 'IgM-like molecule' on T lymphocytes will not stand up to examination by these controls. Of particular relevance here, is the finding that anti-mouse immunoglobulins antisera can contain antibodies reactive with a component of the thymocyte surface which co-electrophoreses with 7S IgM in gel electrophoresis.[143] This high molecular weight material, however, is certainly not immunoglobulin since it does not dissociate into heavy and light chains upon chemical reduction. Furthermore, it is not recognized by specific anti-immunoglobulin reagents.

Another reason for doubting that immunoglobulin is the receptor for antigen on T lymphocyte comes from recent work on T cell helper[144] and suppressor[145] factors. Both types of molecules have been shown to be biologically active and antigen specific. They react with anti-Ia antisera but not with anti-immunoglobulin reagents.

The final pertinent point is that the molecules on T lymphocytes recognized by anti-idiotypic reagents (see below) have been characterized and are clearly not 7S 'IgM-like'.[146] Instead, and without chemical reduction, the major material appears to have a molecular weight of about 35 000, although a 50 000 molecular weight component was also present.

Now we come to the recent excitement generated by the use of anti-idiotype antisera for the characterization of antigen receptors. The conclusion that will be drawn is that T and B lymphocytes with specificity towards the same antigen have recognition structures that share the same idiotypic determinants. However, such indications as there are, suggest that on T cells these idiotypic determinants are not associated with conventional immunoglobulin molecules. These conclusions come from two independent lines of study. One using classical anti-idiotype antisera in the mouse[67] and the other with an anti-idiotype system provided by the rat.[146]

The simplest system to describe is in the mouse, and this has been considered earlier in relation to stimulation of B lymphocytes with anti-idiotypic antibodies. In these experiments the crucial reagent was the $IgG_1$ fraction of a guinea pig anti-idiotype directed towards a monoclonal anti-streptococcal Group A carbohydrate antibody formed in the A/J strain of mice. The critical observation was that mice injected with small amounts of this reagent were subsequently found to have generated 'helper' T cells with specificity for the Group A carbohydrate. Since B cells were also sensitized to Group A carbohydrate with the anti-idiotype, the obvious conclusion is that in this restricted system both B and T lymphocytes recognize the streptococcal antigen via structures displaying the antigenic determinants defined by the anti-idiotype.

In the rat system the anti-idiotypic antibodies were raised against antibodies to the major histocompatibility Ag-B determinants. The principle was based on earlier observations in mice[147] and rats,[148] and this will be presented in a general, rather than a specific, way. Assume an $F_1$ hybrid is derived from the two parental strains A and B. Within the $F_1$ hybrid there will be mutual tolerance to histocompatibility antigens of both parental strains. Or, to say it the other way round, cells of either parental type with immunological

reactivity towards the other half represented in the $F_1$ hybrid will be deleted. Thus, for example, A strain cells capable of recognizing B strain cells will be absent. If we now make the reasonable assumption that recognition takes place through surface located receptors, then receptor molecules of this specificity must also be absent. Therefore, should such receptors be introduced into the $F_1$ hybrid, they should be immediately recognized as foreign and, accordingly, an immune response towards them should result. However, the foreign antigenic determinants in this situation can only be related to that part of the molecule which combines with the antigen (i.e. the idiotypic determinants), and thus the humoral immune response must be the production of anti-idiotype antibody. To pose a simple example: the general structure of B lymphocyte antigen receptors is the same, it is immunoglobulin. Thus the major structural disparity between the various receptor immunoglobulins of different clones is in the idiotypic determinants. In principle, therefore, it should be possible to use $F_1$ hybrid animals to make anti-idiotype reagents with specificity for recognition structures expressed in either parental strain, but not the $F_1$ hybrid, i.e. anti-(receptor of those strain•A lymphocytes which recognize B strain lymphocytes); anti-(receptor of those strain B lymphocytes which recognize strain A lymphocytes). It should also be possible to design the immunization schedules so that the anti-idiotypes are directed either towards the immunoglobulin receptor of the appropriate B lymphocytes or the recognition unit expressed by T lymphocytes of the chosen specificity. Having achieved this, then the anti-idiotypic reagents can be used as probes for antigen receptors of B and T lymphocytes.

Anti-idiotype directed towards the T cell receptor was made by injecting parental peripheral T lymphocytes (A) into $F_1$ hybrid animals (A.B). The resulting antiserum (which should be anti-(A anti-B)) bound to the surface of T lymphocytes of the appropriate parental strain (A), but not to cells from the other half of the $F_1$ (B) or appropriately chosen third party controls. Moreover, the anti-idiotype would also specifically bind to immunoglobulin carrying the relevant idiotype: made, for example, by injecting B strain cells into A strain animals and purifying the alloantibody, i.e. A anti-B. Thus receptors towards the same antigenic determinants, whether expressed by T or B cells, share the same idiotypes. Using this type of anti-idiotype it was possible to inhibit graft versus host reactions or mixed lymphocyte responses, in an appropriately specific way, and thereby to deduce a functional receptor role for the idiotype-bearing T lymphocytes. It was also possible to directly visualize those T and B lymphocytes bearing the idiotypic determinants by immunofluorescence. In all of the studies appropriate specificity controls were included. When used in conjunction with radiolabelled T lymphocytes the anti-idiotype appeared to recognize a single chain molecule with a molecular weight of 35 000; some material with a molecular weight of 50 000 was also recovered. With radioactive B lymphocytes, the indications were that immunoglobulin was selected by the anti-idiotype.

There are therefore two different lines of evidence to suggest that both B and T lymphocytes share the same idiotypic determinants when they are directed towards the same antigen. In one case the anti-idiotype was prepared against serum antibody, and in the other it was raised against the T cell receptor for antigen. Preliminary characterization of the material on T lymphocytes suggests that it is not conventional immunoglobulin. A common interpretation of the data is that the T cell antigen receptor may consist of an immunoglobulin variable region gene integrated with a polypeptide coded for by a gene (or genes) in the major histocompatibility locus. The problem here is that there is no known linkage between genes specifying immunoglobulin and the histocompatibility locus. This could mean a subset of V genes exclusively for T cells. However, any speculation on this point is of very little value since it is quite clear that most of these

problems will shortly be better understood by experimentation in the types of systems discussed above.

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# SURFACE IMMUNOGLOBULIN ON MURINE LYMPHOCYTES

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## INTRODUCTION

A key question in biology is regulation of gene expression following interaction of cells with hormones, drugs, antigens and other cells. In most cases the critical event is combination between the biologically active effectors and specific receptor molecules in the cell membrane. It is therefore of utmost importance that such receptor molecules be isolated and identified, the long term aim being to explain in molecular terms the alteration of cell phenotype following receptor-ligand interactions. Although the studies outlined below are confined to cells of the lymphoid series, the methodology is of general application.

The division of lymphocytes into thymus-derived (T) cells and bursa-equivalent-derived (B) cells is well established. In the mouse, the major Ig present on B-lymphocytes has been thought to be IgM (Lisowska-Bernstein et al, 1973; Marchalonis & Cone 1973; Vitetta & Uhr 1973) but, as will be demonstrated below, this is not entirely correct. The presence or absence of Ig on thymocytes is controversial. Using the lactoperoxidase-catalysed procedure for radio-labelling cell surfaces, some workers have prepared comparable amounts of radioactive Ig from thymocytes and B-lymphocytes, (Marchelonis and Cone, 1973). Others, (Grey et al, 1972; Lisowska-Bernstein et 1973; Vitetta and Uhr, 1973), as well as ourselves, have failed to repeat this work. In view of the role of T-lymphocytes in cellular immunity and immune surveillance, it is crucially important that this controversy be resolved.

## METHODS

Spleen and thymus cell suspension were labelled internally by culturing in the presence of 3H-leucine (Parkhouse and Askonas, 1969), or externally with 125I using lactoperoxidase (Marchalonis et al, 1971). From the leucine-labelle cells a plasma membrane fraction was isolated (Crumpton and Snary, 1974), which was solubilized in 1% (w/v) sodium deoxycholate. 125I-labelled cells were directly solubilized by a variety of procedures. In all cases, Ig was purified from the soluble extract by precipitation with rabbit anti-(mouse Ig) (Parkhouse and Askonas, 1969), and then characterized by electrophoresis in sodium dodecyl sulphate-polyacrylamide gels (SDS gels) (Summers et al, 1965) with addition of an internal 131I-labelled Ig marker.

Purified mouse IgM (myeloma MOPC 104E) and IgG (myeloma Adj PC5) were labelled with 131I (Hunter and Greenwood, 1962) and totally reduced to heavy and light chains (2mM dithiothreitol -2% (w/v) SDS-0.05M sodium phosphate, pH 7.0; 15min, 100°). The IgM was partially reduced to contain disulphide-linked $\mu 2L2$ and $\mu L$ subunits (1mM dithiothreitol -0.05M sodium phosphate, pH 7.0; 1 hour at room temp.). Reduced samples were alkylated by the

addition of a three fold molar excess of iodoacetamide. In all of the SDS gel profiles presented, the top of the gel is to the left, the profile of 125I-cell surface material is given by the solid line, and the 131I internal marker is given by the dotted line.

## RESULTS

T-lymphocytes. We have been unable to demonstrate Ig on the surface of thymocytes or peripheral T-lymphocytes (Julius et al, 1973) when the cells were labelled externally with 125I. The cells were extracted with 1% (w/v) Nonidet P40, 1% (w/v) sodium deoxycholate or 9M urea -1.5M acetic acid. In all cases, essentially all of the radioactivity incorporated into the cells was recovered in soluble form after adding the extractant. Removal of low molecular weight material by dialysis resulted in excellent recovery of TCA-precipitable material in detergent lysates, but aggregation caused highly variable yields (10-80%)when acid-urea lysates were dialysed. Addition of of a variety of anti-(mouse Ig) reagents (with specificities to all known classes of heavy and light chains) to dialysed lysates failed to precipitate detectable amounts of mouse Ig. Ig was also absent when inhibitors of proteolysis (Trasylol, phenylmethylsulphonyl fluoride, iodacetamide and ε-aminocaproic acid) were added at the moment of cell lysis and during the isolation procedures. These results contrast with those we obtained with splenic lymphocytes, where Ig was readily detectable; Nonidet P40 gave best results, sodium deoxycholate was satisfactory and acid-urea was erratic. Because Marchalonis and Cone have emphasised that thymocyte Ig is not solubilised by Nonidet P40, the pellet obtained after centrifuging Nonidet P40 extracts of labelled thymocytes was extracted with acid-urea, but results were again negative.

The controversy of whether thymocytes do, or do not, possess surface Ig has resolved around methodological issues, in particular, choice of extracting solvent, choice of antibody used for purification of surface Ig and the possibility of proteolysis of surface Ig. Bearing in mind that T cell surface Ig has been characterized as a disulphide-linked $\mu 2L2$ molecule (Marchalonis and Cone, 1973), we designed an experiment where these methodological variables could be ignored. Cells labelled with 125I were extracted for 3 min at 100° with 4% (w/v) SDS -0.1M iodoacetamide - 0.05M sodium phosphate, pH 7.0. The extract was dialysed for two hr (2% (w/v) SDS - 50mM iodoacetamide - 0.05M sodium phosphate, pH 7), mixed with some partially reduced 131I-IgM and then directly applied to an SDS gel. Three cell populations were examined: normal spleen cells (Fig.1a), "B spleen" cells (thymectomized at 2 weeks of age, lethally irradiated at 2 months of age (850r) and reconstituted with foetal liver cells) (Fig.1b) and thymocytes (Fig.1c). With the 131I-internal marker as a reference, the $\mu 2L2$ region of each gel was eluted with 2% (w/v) SDS -2mM dithiothreitol -0.05M sodium phosphate, pH 7.0. The eluate was heated (15 min, 100°) to ensure complete reduction, alkylated (10mM iodoacetamide) and electrophoresed once more. It was therefore possible to determine whether 125I-labelled cell surface material with the expected mobility of $\mu 2L2$ could be resolved into heavy and light chains.

When the second gels were analysed, it was clear that the internal marker (131I-$\mu 2L2$) had been reduced to $\mu$ and L chains. A considerable portion of the 125I cell surface material from normal and "B spleen" cells co-migrating with 131I-$\mu 2L2$ was also sensitive to reduction, yielding a distinct peak corresponding to $\mu$ chains (Fig.2a, 2b). The comparable fraction obtained from thymocytes was not sensitive to reduction (Fig.2c). Thymocytes therefore do not possess surface Ig, similar to disulphide-linked $\mu 2L2$, which can be revealed by external labelling. The material present of the surface of mouse thymocytes which co-migrates with $\mu 2L2$, and which is preset in

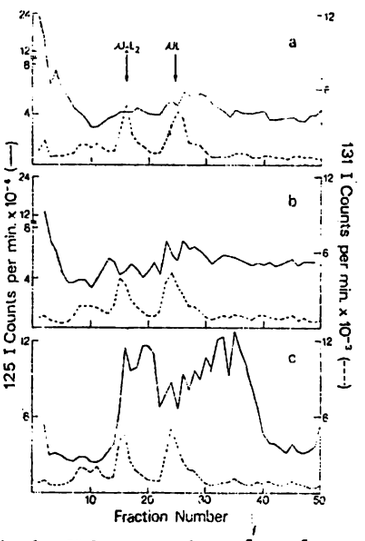



Fig.1. SDS extraction of surface labelled cells. Normal spleen cells (a), "B spleen cells (b), and thymocytes (c) were labelled with 125I and run on 4.2% gels with an internal 131I marker.

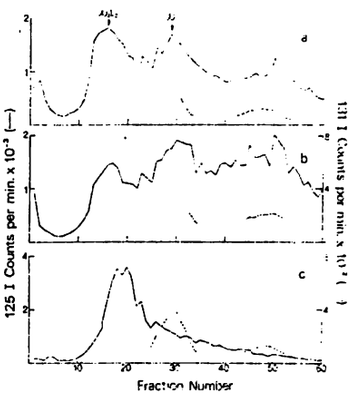



Fig.2. Reduction of labelled 7S material from normal spleen cells (a), "B spleen" cells (b), and thymocytes (c). The portion of the gels shown in Fig. 1 containing the 131I-μ2L2 internal marker was eluted, reduced and alkylated and applied to 4.2% gels.

relatively high amounts (Fig.1c), does not have a counterpart in spleen cells and may therefore be a surface protein unique to thymocytes

If Ig was present on thymocytes, but inaccessible to iodination, then this should be revealed by internal labelling. Accordingly, thymocytes were labelled in vitro with 3H-leucine for 4-24 hr and their plasma membranes were prepared. However, we were unable to detect Ig in these plasma membrane samples (detection limit, 0.1% of total radioactivity), although similarly prepared plasma membranes from spleen cells contained easily demonstrable Ig (3% of total radioactivity). When internally labelled spleen cells were added either to a gross excess of unlabelled spleen cells or thymocytes, the plasma membranes prepared in both cases contained similar amounts of Ig. We are therefore confident that our negative data for thymocytes is not due to proteolysis.

The experiments described above argue against the presence of Ig on the surface of T-lymphocytes, and we suggest that an explanation for previous positive findings be sought in 1) Selective purification and, or, labelling of contaminating B-lymphocytes in thymocyte suspensions. 2) The presence of material in thymocyte membranes with mobility similar to μ chain on polyacrylamide gels. Such material, although not Ig, would be fortuitously precipitated by the antibody used for purification. 3) The Ig on T-lymphocytes is hitherto undescribed class composed of unique heavy and light chains and which is not covalently assembled to the H2L2 form.

Should T-lymphocytes be conclusively demonstrated to lack any form of Ig subunit, polypeptide chain or fragment, then a new system of receptor molecules must be present in order to explain the specificity of T-cells. One possibility is the Ir gene product (McDevitt, 1972).

B-lymphocytes. Although the original discovery of human IgD is almost ten years old (Rowe and Fahey, 1965), it is only recently that a possible function

for this class has been suggested. Present in normal human serum in very small amounts (Spiegelberg, 1972), IgD is found on the surface of relatively large numbers of human peripheral lymphocytes, in particular those obtained from cord blood and cases of chronic lymphatic leukaemia (Van Boxel et al, 1972; Rowe et al, 1973a and 1973b; Fu et al, 1974; Kubo et al, 1974). Interestingly, IgD is frequently associated with IgM on the same cell (Rowe et al, 1973b; Fu et al, 1974; Kubo et al, 1974). These findings suggest a fundamental role for IgD, perhaps as a primitive recognition unit or for regulation of the immune response. Were this to be so then IgD would be expected in species other than Man. We now present identification of a molecule similar to IgD on the surface of mouse B-lymphocytes.

Radioactive surface Ig prepared from Nonidet P40 extracts of externally labelled spleen cells was analysed on 4.2% SDS gels. As has been previously described (Marchalonis and Cone, 1973; Vitetta and Uhr, 1973), we found a major portion of the radioactivity in that part of the gel corresponding to the $\mu 2L2$ subunit of IgM (Fig.3). In addition, however, there was a significant component running very slightly in advance of the $\mu L$ subunit, but which contained heavy and light chains (see Fig.4b) and must therefore be an HL subunit. Following reduction and alkylation, both the H2L2 and HL subunits were shown to contain two distinct species of heavy chain. Appropriate sections of the gel shown in Fig. 3 were eluted and reduced and alkylated as described above, and then applied to 10% gels in order to resolve the heavy chain components. Under these conditions the 131I markers in the first gel are also extracted and reduced, thereby providing internal markers for $\mu$ and L chains in the second electrophoretic separation. Upon fractionation of the 10% gels it became obvious that the heavy chain was heterogeneous, there being one component with the mobility (and therefore size) of the internal 131I-$\mu$ chain marker, and another which migrated faster than $\mu$ chain, but slower than $\gamma$ chain. The proportion of radioactivity found in the smaller (faster migrating) heavy chain was lower in H2L2 (Fig. 4a) than HL (Fig. 4b). Surface Ig of murine splenic lymphocytes is therefore not homogeneous but consists of two species of heavy chains, one the size of $\mu$ chain and the other significantly smaller. We discount the possibility that the heterogeneity results from the presence of T-lymphocytes since similar results were obtained when spleen cells from nude mice were analysed by the same techniques. In addition peripheral T-lymphocytes purified by passage of spleen cells through nylon wool columns (Julius et al, 1973) did not contain detectable amounts of surface immunoglobulin using the methods described above.

The heterogeneity of surface Ig does not result from precursor-product relationships, degradation of $\mu$ chain-containing material or variable glycosylation of $\mu$ chains. Instead, the small heavy chain appears to be a new

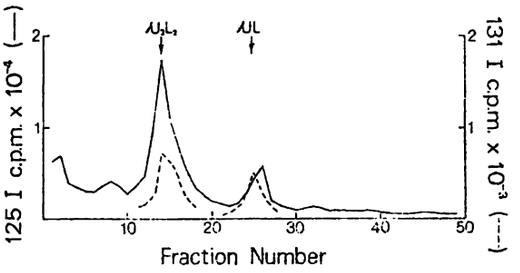
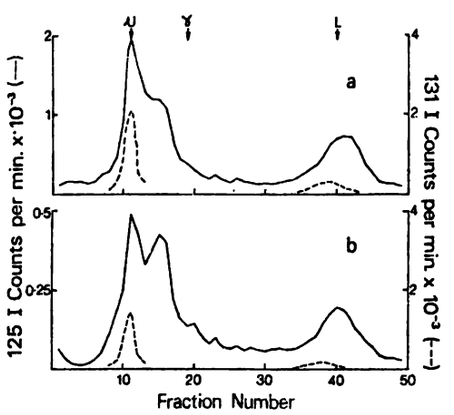



Fig.3. Radioactive surface Ig from externally labelled normal spleen cells analysed on 4.2% SDS gels.




.4. Heavy chain heterogeneity of surface Ig. Segments of the gel shown ig. 3 containing the μ2L2 and μL components were extracted, reduced, lated and analysed on 10% SDS gels. (a) Reduced μ2L2. (b) Reduced μL.

y chain class, since it is not precipitated by anti-μ, anti-γ or anti-α ents, but is precipitated by polyspecific anti-Ig.
The most obvious surmise is that it corresponds to the human IgD class. upport of this suggestion we may note the similar mobility on sodium cyl sulphate-polyacrylamide gels of human δ chain (Spiegelberg, 1972), the novel heavy chain of mouse lymphocytes described above. This newly ribed mouse immunoglobulin, which we shall now refer to as IgD, is also lar to human IgD in its marked susceptibility to proteolytic degradation .A. and R.M.E.P., unpublished observations). A similar conclusion has been drawn by others (Vitetta and Uhr, personal communication).
Contrary to our espectation, however, was the finding of μ chain, but not in, in foetal liver (16 day embryos) and neonatal spleen and liver. In ion, splenic lymphocytes of 6 week and 6 month old mice contained a ar amount of δ chain, the ratio of μ:δ being about 3:2. This could mean the expression of IgD in the mouse is subsequent to the appearance of If this is not the case, then IgD-bearing lymphocytes must arise in s other than spleen or foetal liver. Perhaps relevant to this point is inding that IgD constitutes the major immunoglobulin class present on e lymph node cells, where the μ:δ ratio was found to be 1:3.5-4.
t is certainly intriguing that spleen and lymph node cells differ mark- in terms of the relative amounts of μ and δ chains present on their es. In this respect we may note that spleen cells, but not lymph node , respond to lipopolysaccharide (Janossy and Parkhouse, unpublished work) at spleen cells secrete largely IgM, whereas the major product of lymph ells is IgG (Parkhouse, 1973). However, the presence or absence of a onal relationship between these separate observations remains to be ined. Whether the molecular heterogeneity of total cell surface Ig is ted in a similar pattern on individual cells is not known. Nonetheless, tionship between this observed heterogeneity and regulation of the hocyte response to antigen is suggested.

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# THE DETECTION OF IgD AND IgM ON MURINE B-LYMPHOCYTES IN CONDITIONS WHERE NO Ig CAN BE FOUND ON T-LYMPHOCYTES

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The crucial event in immune responses is recognition of antigen by lymphocytes. A massive body of evidence has established that the molecule responsible for antigen binding by B-lymphocytes is immunoglobulin. Curiously, there is little, if any, IgG on the surface of B lymphocytes. Instead, the major classes of Ig found are IgM and an Ig thought to be IgD (1,2,3). The presence of Ig on T-lymphocytes is not conclusively proved. Using the lactoperoxidase catalysed procedure for radiolabelling cell surfaces, some workers have prepared similar (4) or even greater (5), amounts of radioactive Ig from thymocytes as B-lymphocytes. Others (6,7,8) as well as ourselves (9), have failed to repeat this work. We can, however, demonstrate a component of thymocyte membranes which behaves like μ-chain on electrophoresis, but which is not immunoglobulin in nature.

## METHODS

Spleen and thymus cell suspensions were labelled internally in the presence of $^{3}$H-leucine (10), or externally with $^{125}$I using lactoperoxidase (11). From the leucine-labelled cells, a plasma membrane fraction was isolated (12), which was solubilised in 1% (w/v) sodium deoxycholate. Cells labelled with $^{125}$I were solubilised by a variety of procedures. In all cases Ig was purified from the soluble extract by precipitation with rabbit anti-(mouse Ig) and goat anti-(rabbit IgG) (10), and then characterised by electrophoresis in SDS-gels (13) with the addition of an internal $^{131}$I-labelled marker consisting of Ig and Ig subunits (1).

Rabbits were immunised with purified MOPC 104E IgM ($\lambda_1$) or the Fab portion of Adj PC5 ($\gamma_{2a}$K) (14). After passage through

normal mouse Ig coupled to Sepharose 4B (15), the anti-IgM precipitated $\mu\lambda_1$ (MOPC 104E) and $\mu K$ (TEPC 183), but was negative towards $\gamma_1 K$ (MOPC 21), $\gamma_{2a} K$ (Adj PC5), $\gamma_{2b} K$ (MOPC 195), $\gamma_3 K$ (FLOPC 21) and $\alpha\lambda_2$ (MOPC 315) in a sensitive radio-immuno-assay ($^{125}$I-myeloma proteins (5-10 ng) mixed with test serum (10 µl) and then precipitation of the rabbit Ig with goat anti-(rabbit Ig). The anti-Fab reagent precipitated all classes of immunoglobulin but no other serum protein. The IgG fractions of both antisera were prepared, coupled to fluorescein isothiocyanate, and conjugates with fluorescein-protein ratios of 2-3:1 were selected by chromatography on Whatman DE 52 (16). A goat antiserum specific for rabbit IgG was similarly treated to yield a rhodamine conjugate. Rabbit antisera specific for other mouse heavy chain classes were also prepared as indicated above.

## RESULTS

B-lymphocytes. Spleen cells labelled with $^{125}$I were lysed in 1% (w/v) Nonidet P40-0.1M iodoacetamide-1mM KI-1mM phenylmethylsulphonyl fluoride-PBS, centrifuged and the supernatant, containing at least 95% of the acid-precipitable material, was passed over Sephadex G:25 equilibrated with the solution used for cell lysis. All operations, including precipitation of Ig, were carried out in the cold in order to minimise proteolysis. When radioactive surface Ig precipitated with polyspecific anti-(mouse Ig) was analysed on SDS gels, the predominant component observed was a disulphide-linked $H_2L_2$ structure, similar in size to monomeric IgM. Some Hl subunits were also present but neither IgG nor 19S IgM were observed. Furthermore, on using antisera specific for mouse heavy chain classes, we were unable to detect IgG or IgA. With anti-µ chain, however, some, but not all, of the surface Ig was precipitated, and on reduction yielded a heavy chain the same size as authentic (secreted) µ chain. The Ig remaining after precipitation with anti-µ chain was isolated by addition of anti-K chain, but in this case reduction revealed a heavy chain of size intermediate between µ and $\gamma$. Thus in addition to monomeric IgM, there exists on the surface of mouse B-lymphocytes an Ig class which does not react with anti-µ, anti-$\alpha$ or anti-$\gamma$ chain sera. Because of similarities in heavy chain size, susceptibility to proteolysis, and location (i.e. found on lymphocyte surfaces), we have concluded that this Ig class is the murine homologue of IgD. The size of the heavy chain excludes the possibility of it being IgE.

This candidate for IgD is a major cell surface component, comprising 40% of surface Ig from splenic lymphocytes and 70% of surface Ig from lymph node cells. Remaining Ig is entirely accounted for as IgM. The difference between spleen and lymph nodes may reflect the presence of a more mature population of B-lymphocytes in the latter location. Interestingly, the ratio of IgM:IgD was the same

in spleen cells from normal, 6 week old, CBA mice and nude mice. Expression of IgD on the cell surface is therefore independent of T-lymphocytes. Furthermore, using the same methodology we never recovered either IgM or IgD from thymocytes or peripheral T-lymphocytes. In foetal liver or neonatal spleen and liver, however, only IgM was found. Thus IgM precedes IgD in embryological development in the mouse, as would be expected if, as seems the case, IgD appears subsequent to IgM in evolution (17). Earlier assumptions that IgD precedes IgM in the human were based on comparisons between cord and adult blood (18,19), and appear to be incorrect (20).

The distribution of IgM and IgD on individual cells was studied by staining live cells with fluorescent antibodies in an experimental design based on the radiolabelling experiments described above. The crucial assumption, which is supported by the labelling data (1,2) is that IgM and IgD are the major Ig classes present of lymphocyte surfaces. The possibility of very low numbers of IgG-bearing cells or the presence of very small amounts of surface IgG will not influence the experimental approach. Since IgM and IgD cap independently on human lymphocytes (18,19), our protocol consisted of first capping IgM using rabbit anti-(mouse μ chain) and rhodamine-labelled goat anti-(rabbit IgG). The cells were then reacted with a polyspecific fluorescein-coupled rabbit anti-(mouse $\gamma_{2a}$K Fab) under non-capping conditions (0.03M sodium azide, 0°). For reasons given above, the green rings visualised by the second reagent must represent IgD. Using this double staining procedure, splenic lymphocytes from 6 week old, SPF, female CBA mice could be classified into three groups. A large number (31-41% of total Ig-bearing cells) which were capped with anti-μ subsequently stained peripherally with anti-Fab (i.e. surface IgM and IgD simultaneously present). The remaining stained cells were divided between those showing only caps (i.e.surface IgM only) (17-29% of Ig-bearing cells) and those showing only green rings (i.e. surface IgD only)(37-45% of total Ig-bearing cells). In the doubly stained cell population there was considerable variation in the relative intensities of the two fluorochromes, suggesting a variation in the IgM to IgD ratio from cell to cell. Complete capping of IgM occurred in the first stage, since fluorescein-labelled anti-μ failed to reveal ring staining of cells previously capped in the first staining step.

The biological significance of these subpopulations of B-lymphocytes remains to be established. However, the fact that IgM precedes IgD in ontogeny does suggest a developmental sequence. Thus the B-lymphocytes, originally expressing only IgM, matures to a cell expressing only IgD via an intermediate cell type with both classes of Ig on its surface.

The simultaneous expression of two Ig classes on the cell surface has implications for "switch" and V - C gene integration mech-

anisms. While it is quite possible that an explanation be sought in the half-life of mRNA for μ and δ chains, it is intriguing to consider that there is simultaneous transcription of the genes for the two heavy chains. Were this to be so, then the possibility of simultaneous integration of all $C_H$ genes with $V_H$ genes is raised. Based on the probability that a given lymphocyte expresses only one $V_H$ gene (21), then simultaneous integration of all $C_H$ genes would require a copy-choice mechanism (22) for V - C gene integration. The advantage of simultaneous integration of all $C_H$ genes early in the ontogeny of a B-lymphocyte is that questions relating to Ig class expression would then revolve entirely around differential gene activation and repression; the requirement to account for a V - C gene integration event at this stage of differentiation would no longer exist.

T-lymphocytes. We have been unable to demonstrate Ig on the surface of thymocytes or peripheral T-lymphocytes (23) when the cells were labelled externally with $^{125}I$. The cells were extracted with 1% (w/v) Nonidet P40, 1% (w/v) sodium deoxycholate or 9M urea - 1.5M acetic acid. In all cases, essentially all of the radioactivity incorporated into the cells was recovered in soluble form after adding the extractant. Removal of low molecular weight material by dialysis resulted in excellent recovery of TCA-precipitable material in detergent lysates; but aggregation caused highly variable yields (10-80%)when acid-urea lysates were dialysed. Addition of a variety of anti-(mouse Ig) reagents (with specificities to all known classes of heavy and light chains) to dialysed lysates failed to precipitate detectable amounts of mouse Ig. Ig was also absent when inhibitors of proteolysis (Trasylol, phenylmethylsulphonyl fluoride, iodoacetamide and ε-aminocaproic acid) were added at the moment of cell lysis and during the isolation procedures. These results contrast with those we obtained with splenic lymphocytes, where Ig was readily detectable; Nonidet P40 gave best results, sodium deoxycholate was satisfactory and acid-urea was erratic. Because Marchalonis and Cone have emphasised that thymocyte Ig is not solubilised by Nonidet P40, the pellet obtained after centrifuging Nonidet P40 extracts of labelled thymocytes was extracted with acid-urea, but results were again negative.

If Ig was present on thymocytes, but inaccessible to iodination, then this should be revealed by internal labelling. Accordingly, thymocytes were labelled in vitro with $^3H$-leucine for 4-24 hr and their plasma membranes were prepared. However, we were unable to detect Ig in these plasma membrane samples (detection limit: 0.1% of total radioactivity), although similarly prepared plasma membranes from spleen cells contained easily demonstrable Ig (3% of total radioactivity): When internally labelled spleen cells were added either to a gross excess of unlabelled spleen cells or thymocytes, the plasma membranes prepared in both cases contained

similar amounts of Ig. We are therefore confident that our negative data for thymocytes is not due to proteolysis.

The experiments above argue against the presence of Ig on the surface of thymocytes. In order to complete our study, we turned to the system of "metabolic release" (24).

Here, $^{125}$I-labelled thymocytes are incubated in vitro for 2-4 hours and the medium is used as a source of cell surface proteins. Using this system it has been claimed (24) that Ig is released from thymocytes and is cytophilic for macrophages. The Ig was characterised as a polypeptide with the mobility of a μ-chain on SDS-gels. In our experiments, surface labelled thymocytes were cultured in vitro for 4 hr. The culture medium was dialysed against cold PBS and divided into three portions: (a) Control, no treatment, (b) Nonspecific precipitation, normal rabbit IgG plus goat anti-rabbit IgG (c) Anti-Ig precipitation, rabbit anti-(mouse IgM ( $\lambda_1$), IgA ($\lambda_2$), IgG$_1$ (K), IgG$_{2a}$(K), and free K chains) plus goat anti-(rabbit IgG). The precipitates were removed and the supernatants were incubated with macrophages (peritoneal exudate cells from mice

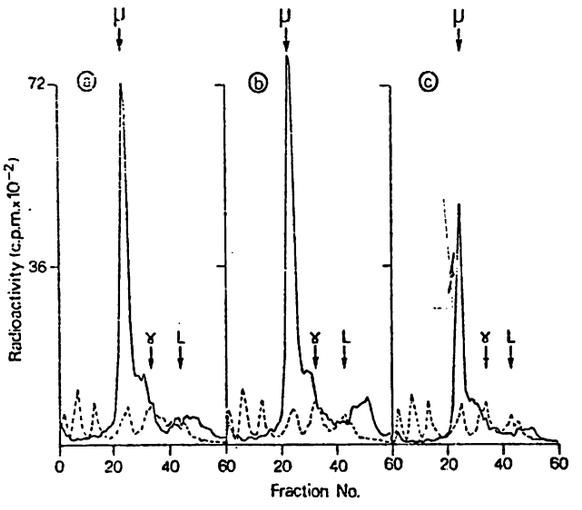


Fig.1. Absorption of material released from labelled thymocytes in vitro to macrophages. Analysis on 4.2% (w/v) SDS gels of reduced samples. Macrophages incubated in (a) Control medium (b) Medium after removal of non-specific Ag-Ag precipitate (c) Medium after removal of anti-(mouse Ig) precipitate. $^{125}$I-surface labelled material is represented by the solid line and $^{131}$I-labelled internal markers by the dotted line.

given 1 ml 2% protease peptone intraperitoneally 4 days previous to sacrifice) for 90 min at 0°. The incubation conditions were such that $2.5 \times 10^5$ macrophages were mixed with released material from $2 \times 10^6$ thymocytes. The cells were pelleted, washed in PBS and applied to SDS gels with or without reduction in dithiothreitol.

Comparable amounts of radioactivity (about 4% total input) was absorbed to macrophages when all three samples were tested. On gel analysis of reduced samples, most of the absorbed material was found to migrate in the same position as μ-chain (Fig.1). However this component was found when the cells were incubated with all three sources of cell surface material. Since one of the samples was absorbed with polyspecific anti-(mouse Ig) (Fig.1c), the radioactive material in the μ chain position cannot be immunoglobulin. Furthermore, when the samples were not reduced prior to gel analysis, the major peak in the μ-chain position persisted in the absence of a radioactive component in the position of monomeric IgM. It is possible, but not conclusively proved, that this molecule has previously been mistaken for immunoglobulin.

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# Location of Nucleotide Pyrophosphatase and Alkaline Phosphodiesterase Activities on the Lymphocyte Surface Membrane

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1. Isolated mouse spleen lymphocytes hydrolysed UDP-galactose added to the medium. Nucleotide pyrophosphatase activity that accounted for this hydrolysis was enriched to a similar extent as alkaline phosphodiesterase and 5′-nucleotidase in a lymphocyte plasma-membrane fraction. 2. The cell surfaces of mouse spleen and thymus lymphocytes were iodinated with $^{125}I$ by using the lactoperoxidase-catalysis method. Detergent extracts of the cells were mixed with a purified anti-(mouse liver plasma-membrane nucleotide pyrophosphatase) antiserum and the immunoprecipitates analysed by polyacrylamide-gel electrophoresis. Only one major radioactive component, similar in size (apparent mol.wt. 110000–130000) to the liver enzyme, was observed. 3. Electrophoresis of an iodinated spleen plasma-membrane fraction indicated peaks of radioactivity, including one of apparent mol.wt. 110000–130000. 4. When detergent extracts of spleen lymphocytes were passed through a Sepharose-bead column containing covalently attached anti-(nucleotide pyrophosphatase) antiserum, the nucleotide pyrophosphatase activity was retained by the beads, whereas protein and leucine naphthylamidase activity were eluted. 5. The results indicate that nucleotide pyrophosphatase and alkaline phosphodiesterase activities are due to the location of the same or similar enzymes at the outer aspect of the lymphocyte plasma membrane. Some possible functions of enzymes at this location are discussed.

?lasma-membrane enzymes whose active sites -e the external medium rather than the cytoplasm /e been designated 'ectoenzymes' (De Pierre & rnovsky, 1974*a*). It now appears that a number of mmalian plasma-membrane marker enzymes can classified as ectoenzymes (De Pierre & Karnovsky, '4*b*; Trams & Lauter, 1974) and that these enzymes, -en located in different tissues and organs may ;sess similar properties (Riemer & Widnell, 1975). cleotide pyrophosphatase was shown to be an oenzyme in hepatocytes (Evans, 1974; Bischoff *al.*, 1976), and in various cultured cell lines (Sela *al.*, 1972; Deppert *et al.*, 1974). The present studies ow that the enzyme activity present in lympho--e plasma membranes occupies a similar position.

## perimental

### imals

ɔpecific pathogen-free mice 8–12 weeks old of the ıred strain CBA/Ca (equivalent to CBA/H) were d as a source of normal spleen and thymus ıphocytes. Mice were deprived of thymus-derived ıphocytes (T-lymphocytes) by being thymecto-ed as young adults and subsequently irradiated l reconstituted with syngeneic foetal liver (Miller & Mitchell, 1969). The lymph nodes and spleens of these mice [(CBA×C57) $F_1$ hybrids] had 70 and 80% immunoglobulin-bearing cells (B-lymphocytes) respectively. Also, spleen lymphocytes from these mice contained less than 5% of cells with the antigenic determinant Thy-1 on their surface; this antigen has been shown to be a marker for T-lymphocytes (Reif & Allen, 1964). A lymphocyte plasma-membrane fraction was prepared from male mice, 6 months old, of the CBA/Ca strain.

### *Preparation of antiserum*

Nucleotide pyrophosphatase was purified from a sarcosyl extract of mouse liver plasma membranes by rate-zonal centrifugation in sucrose/sarcosyl gradients, followed by gel filtration (Evans *et al.*, 1973). Polyacrylamide-gel electrophoresis and chemical analyses indicated that the enzyme was a sialoglycoprotein of apparent mol.wt. 120000–130000. Antiserum, raised in rabbits against the purified enzyme suspended in Freund's complete adjuvant (Difco Labs., Detroit, MI, U.S.A.), was purified as a γ-globulin fraction by $(NH_4)_2SO_4$ precipitation and gel filtration on Sephadex G-200 columns (120cm×2.5cm) (Gurd *et al.*, 1972). Antiserum was concentrated by Diaflo filtration on UM 50 filters (Amicon, High Wycombe, Bucks., U.K.) to 8mg/ml and immunoelectrophoretic analyses

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indicated one component. The antiserum was covalently coupled to CNBr-activated Sepharose 4B as recommended by the manufacturers (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

*Preparation of lymphocyte cell suspensions*

Lymphocytes were teased from minced mouse spleens into a phosphate-buffered saline medium (124mM-NaCl / 4mM-KCl / 2mM-$KH_2PO_4$ / 8mM-$Na_2HPO_4$), pH7.4, and were separated from erythrocytes and damaged lymphocytes by layering on to a cushion of Isopaque/Ficoll solution [14% (w/v) Ficoll (Pharmacia)/32.8% (w/v) metrizoate (Isopaque) solution (Nycgaard and Co. A.S., Oslo, Norway) (12:5, v/v)] and centrifuging with rapid acceleration in plastic tubes at 20°C for 15min at 2000*g* (Davidson & Parish, 1975). The lymphocytes present at the interface were collected, and resuspended in phosphate-buffered saline, pH7.4, containing 10mM-glucose. They were then divided into two equal portions, one of which was homogenized in a small tight-fitting homogenizer, and the breakdown of UDP-galactose determined immediately as described below.

*Preparation of plasma membranes*

A plasma-membrane fraction was prepared from washed spleen lymphocytes of 70 mice. Spleen cell suspensions were prepared by mincing the organs in a Moulinex parsley mincer with Hanks gelatin (Dresser & Greaves, 1973), that was adjusted to pH7.4 by addition of $NaHCO_3$. Cells were washed twice in Hanks gelatin and once in 10mM-Tris/0.14M-NaCl, adjusted to pH7.4 by addition of HCl. Cells (approx. $7.4\times10^9$) were ruptured by a single passage through a Stansted disruption press (Stansted Fluid Power, Bentfield Road, Stansted, Essex, U.K.) operating at a disrupting valve pressure of 138kPa (Crumpton & Snary, 1974). A low-speed pellet was collected by centrifuging the cell homogenate at 5000*g* for 15min. An intermediate-speed pellet was collected by centrifuging the supernatant at 31000*g* for 30min. This pellet was resuspended in 10mM-Tris/HCl, pH7.4, and re-centrifuged at 31000*g* for 30min before resuspension in 10ml of 37% (w/v) sucrose/10mM-Tris/HCl, pH7.4, and overlayed with 25% (w/v) sucrose/10mM-Tris/HCl, pH7.4. After centrifuging overnight (18h) at 97000*g* (Beckman SW27 rotor) a plasma-membrane fraction was collected at the 25–37% sucrose interface; the pellet at the bottom consisted mainly of endoplasmic reticular membranes (Crumpton & Snary, 1974).

*$^{125}I$ labelling of membrane proteins*

Lymphocyte cell suspensions were labelled with $^{125}I$ by using lactoperoxidase (Marchalonis *et al.*, 1971), washed once in ice-cold phosphate-buffered saline (130mM-NaCl/4mM-KCl/10mM-$Na_2HPO_4$, pH7.4) and then lysed for 10min at 4°C in 1% Nonidet P40 (Shell Chemicals, London W.C.2, U.K.) in phosphate-buffered saline containing 1mM-phenylmethylsulphonyl fluoride and 10mM-iodoacetamide (recrystallized; added to limit proteolysis). The lysate, after centrifuging at 30000*g* for 15min, was passed over Sephadex G-25 equilibrated in the solution used for lysis of the cells. Uptake of $^{125}I$ by the cells was 30–40% of added radioactivity, and at least 95% of the trichloroacetic acid-precipitable radioactivity was recovered in the supernatant after centrifugation of the cell lysate. Radioactivity determined in the excluded peak from the G-25 column was 70–90% trichloroacetic acid-precipitable and accounted for 10–15% of the total radioactivity applied. A 'non-specific' immunological precipitate was first formed in the radioactive sample by addition of normal rabbit serum (10$\mu$l) and a goat anti-(rabbit immunoglobulin G) serum (100$\mu$l). The resulting precipitate was removed by centrifugation, and then further precipitation was effected by addition of rabbit anti-(mouse plasma-membrane nucleotide pyrophosphatase) antiserum (120$\mu$g) and, after 30min, goat anti-(rabbit immunoglobulin G) serum (100$\mu$l). The immunological precipitations were performed at 4°C. Specific precipitates were washed three times with ice-cold 0.5% Nonidet P40 in phosphate-buffered saline (see above for composition), once with 50mM-sodium phosphate, pH7, and then dissolved by heating at 100°C for 10min in 50mM-$Na_2HPO_4$/$NaH_2PO_4$, pH7.0, containing 2% (w/v) sodium dodecyl sulphate. When the samples were reduced, the solvent also included 1mM-dithiothreitol and iodoacetamide was added (final concn. 100mM) after the heating step; for non-reduced samples iodoacetamide was present at 100mM. Internal $^{131}I$-labelled markers (Abney & Parkhouse, 1974) were added to the samples, which were then resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Summers *et al.*, 1965). After electrophoresis the gels were sliced into 1mm segments and radioactivity was determined by using a gamma counter (Packard model 5230).

Lymphocyte plasma membranes washed in 0.15M-NaCl/5mM-Tris/HCl buffer, pH7.4, were iodinated by using lactoperoxidase, and a glucose/glucose oxidase enzymic system was used to generate $H_2O_2$ (Hubbard & Cohn, 1973; Evans, 1974). Approx. 1mg of lymphocyte plasma membranes was iodinated with 0.5mCi of $^{125}I$ in 0.5ml of phosphate-buffered saline/10mM-glucose, containing 65$\mu$g of lactoperoxidase (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) and 100$\mu$l of glucose oxidase (from *Aspergillus niger*; approx. 100 units; Sigma). The membranes were washed several times in phosphate-buffered saline before dissolution

in 1% sodium dodecyl sulphate/5mM-mercaptoethanol/10mM-Tris/HCl, pH7.4, by heating at 90°C for 5min for polyacrylamide-gel electrophoresis (Evans, 1974).

*Enzymic determinations*

5'-Nucleotidase, alkaline phosphodiesterase, leucine naphthylamidase activities and protein were determined as previously described (Evans *et al.*, 1973).

Nucleotide pyrophosphate was assayed with UDP-galactose as substrate as described by Sela *et al.* (1972) and Mookerjea & Yung (1975). Enzyme activities of disrupted lymphocytes and plasma membranes are determined at pH8.6, since maximum hydrolysis of sugar nucleotides occurred at alkaline pH (Sela *et al.*, 1972; Evans *et al.*, 1973). However, comparison of the enzyme activities of intact and disrupted lymphocytes was made at pH7.6 when cells remain viable and about 50% of the activity is measured (Sela *et al.*, 1972). The complete incubation mixture contained in 100$\mu$l: 10–80$\mu$g of protein; 3$\mu$mol of cacodylate buffer, pH8.6, or 5$\mu$mol of $KH_2PO_4/Na_2HPO_4$, pH7.6; 2nmol of UDP-galactose ($10^5$c.p.m.); 0.5$\mu$mol of $MnCl_2$; 5$\mu$l of a 10% solution of Triton X-100 (used in pH8.6 incubations only). Enzymic activity was measured at 37°C for 15min and stopped by adding 100$\mu$l of ethanol and 50$\mu$l of acetic acid. Particulate material was removed by centrifuging the reaction mixture at 3000rev./min for 5min, and applying a portion of the supernatant to Whatman 3MM paper. Reaction products were separated by descending chromatography for 18h in a solvent system containing ethanol/1M-sodium acetate buffer, pH3.8 (15:6, v/v), and monitored by scanning in a Packard radiochromatogram scanner model 7201 before cutting out strips corresponding to the peak areas for determination of radioactivity by scintillation counting in a toluene-based scintillant [4g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene dissolved in 1 litre of toluene]. By using the appropriate standards, it was found the UDP-galactose was hydrolysed initially to galactose 1-phosphate and then to galactose, the latter reaction occurring especially with isolated plasma membranes.

## Results

Although 5'-nucleotidase is a well established lymphocyte plasma-membrane marker (Crumpton & Snary, 1974), there is little information available on the subcellular location of nucleotide pyrophosphatase and alkaline phosphodiesterase activities. Table 1 shows that all enzymes were enriched in a lymphocyte plasma-membrane fraction, relative to the cell homogenate. The hydrolysis of UDP-galactose to UMP and galactose 1-phosphate by nucleotide pyrophosphatase is the first of a two-step reaction that proceeds further, catalysed by a monoester phosphohydrolysis activity, to yield free sugar and nucleosides. This two-stage enzymic hydrolysis of sugar nucleotides has been examined in detail by Sela *et al.* (1972) and Deppert *et al.* (1974) in cultured cell lines. Further details of the range of substrates hydrolysed by the nucleotide pyrophosphatase of lymphocyte plasma membranes have yet to be established, but a similar general specificity towards the pyrophosphate bands of NAD, ATP etc. as in hepatocytes (Evans *et al.*, 1973) is to be expected.

The most direct method of demonstrating a location on the external face of the plasma membrane of an enzymic activity is to show that substrates impermeable to cells are rapidly hydrolysed when

Table 1. *Distribution of lymphocyte-membrane enzymes*

The enzymic activities of spleen lymphocytes, cell homogenates and subcellular fractions were determined. The preparation details and the incubation conditions are described in the Experimental section. Values in parentheses indicate specific activity relative to that of cell homogenate.

| Fraction | Protein (mg) | 5'-Nucleotidase ($\mu$mol/h per mg of protein) | Alkaline phosphodiesterase ($\mu$mol/h per mg of protein) | UDP-galactose pyrophosphatase: Galactose 1-phosphate and galactose produced (c.p.m./h per mg of protein) | UDP-galactose pyrophosphatase: Galactose 1-phosphate and galactose produced (c.p.m./unit cell number) |
|---|---|---|---|---|---|
| Intact cells | — | — | — | — | 23170 |
| Cell homogenate | 240 | 0.90 (1) | 0.35 (1) | 6800 (1) | 32658 |
| 75000*g*-min pellet | 200 | — | 0.31 (0.97) | — | — |
| 900000*g*-min pellet | 5 | — | 3.4 (9.9) | — | — |
| 900000*g*-min supernatant | 11 | — | 0 9 | — | — |
| Plasma membranes | 2 | 16.3 (18.1) | 7.3 (20.7) | 102700 (15.1) | — |
| Endoplasmic-reticulum membranes | 3 | — | 1.6 (4.6) | — | — |

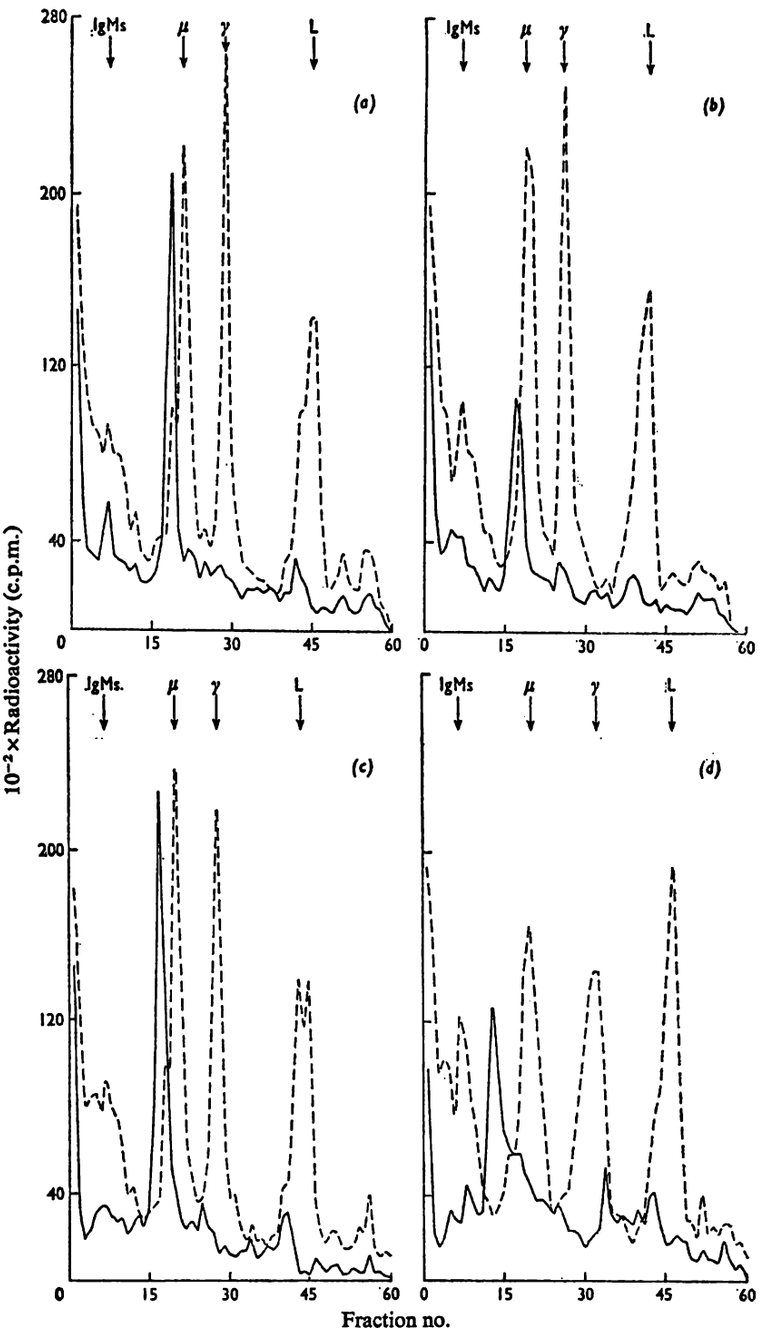


Fig. 1. *Characterization of lymphocyte surface proteins immunoprecipitated after addition of a anti-(mouse liver plasma-membrane nucleotide pyrophosphatase) antiserum*

(*a*) Normal spleen lymphocytes, (*b*) lymphocytes from spleens from eight mice, (*c*) and (*d*) thymus cells. Lymphocytes were isolated and labelled with $^{125}I$ as described in the Experimental section, and specific immunoprecipitates were either unreduced (*a–c*) or reduced and alkylated (*d*), and then resolved in 7% (w/v) polyacrylamide gels (——) that also contained the following reduced and alkylated internal $^{131}I$-labelled markers (----): immunoglobulin Ms (IgMs) ($\mu_2L_2$), $\mu$-chains ($\mu$), $\gamma$-chains ($\gamma$) and light chains (L). The anti-(mouse liver plasma-membrane nucleotide pyrophosphatase) antiserum immunoprecipitated 0.32, 0.26 and 0.52% of the total $^{125}I$ radioactivity precipitated by addition of trichloroacetic acid to lysates of labelled normal spleen lymphocyte, spleen lymphocytes from B mice and thymocytes respectively.

added to the medium. Table 1 indicates that washed spleen lymphocytes and disrupted lymphocytes hydrolysed UDP-galactose. However, De Pierre & Karnovsky (1974*a*) and Trams & Lauter (1974) have shown that this approach to demonstrate ectoenzymic activity on cell surfaces is fraught with technical difficulties and can yield equivocal results. In the present case, the difference between a pH optimum of 8.5–9.0 of nucleotide pyrophosphatase/alkaline phosphodiesterase activities (Evans *et al.*, 1973; Sela *et al.*, 1972) and a pH of 7.4–7.6 necessary to maintain lymphocyte viability and hence the permeability of the cell membrane towards added substrates, compounds these difficulties. To circumvent such problems, advantage was taken of combining techniques for enzymically iodinating cell-surface components with the availability of an antiserum raised against the purified mouse hepatocyte enzyme to investigate whether the enzyme, if iodinated on the lymphocyte surface membrane, could be immunoprecipitated. In addition, this technique yields information on the molecular size of the enzyme.

Lactoperoxidase-catalysed iodination of lymphocytes (Vitetta & Uhr, 1973) and erythrocytes (Hubbard & Cohn, 1973) results mainly in the labelling of tyrosine residues exposed to the medium. The nature of the labelled molecules on the lymphocyte surface membrane was studied directly by extraction of $^{125}$I-labelled cells with the detergent Nonidet P40, which, in the present experiments,

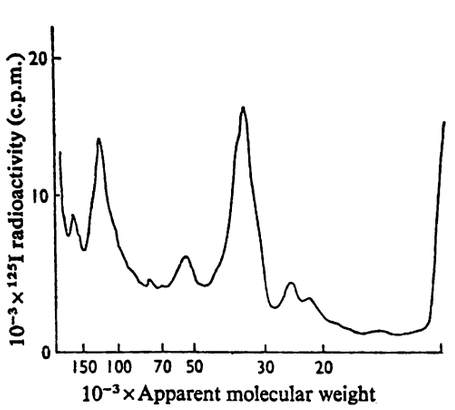


Fig. 2. *Polyacrylamide-gel electrophoresis $^{125}$I-radioactivity profile of enzymically iodinated lymphocyte plasma membranes*

The plasma-membrane fraction was prepared and labelled with $^{125}$I by using the lactoperoxidase/glucose oxidase catalysis method described in the Experimental section. The apparent-molecular-weight scale was obtained by using a series of reoviral protein markers of known molecular weight.

Table 2. *Retention of alkaline phosphodiesterase activity on a Sepharose 4B column containing covalently bound liver anti-(plasma-membrane nucleotide pyrophosphatase) antiserum*

Approx. 2mg of anti-(mouse liver plasma-membrane nucleotide pyrophosphatase) was attached to 2g of CNBr-activated Sepharose 4B. Washed spleen lymphocytes ($2.6\times10^8$ cells) were extracted with 2ml of 0.5% Nonidet P40 in phosphate-buffered saline and added to the column, followed by 10ml of a phosphate-buffered saline, pH7.4. Approx. ten 1ml fractions were collected. Protein and leucine aminopeptidase activity were present in fractions 4–6. For other details, see the Experimental section.

| | Protein (mg) | Phospho-diesterase activity (μmol/h) | Leucine naphthyl-amidase activity (μmol/h) |
|---|---|---|---|
| Addition to column | 5.1 | 2.28 | 0.72 |
| Recovery from column | 5.3 | 0.336 | 0.503 |
| Recovery (%) | 104 | 14.7 | 70 |

solubilized >90% of the radioactivity present in washed lymphocytes. Addition of a mouse anti-(liver plasma-membrane nucleotide pyrophosphatase) antiserum to detergent extracts of lymphocytes resulted in an immunoprecipitate, that, when analysed by polyacrylamide-gel electrophoresis, contained only one major peak of radioactivity of apparent mol.wt. 110000–115000 in non-reducing conditions (Figs. 1*a–c*) and 130000 under reducing conditions (Fig. 1*d*). The iodinated protein was present on both B and T classes of lymphocytes (see the Experimental section). The extremely close correspondence of the molecular weight of the iodinated immunoprecipitated product to that of the mouse liver plasma-membrane enzyme strongly suggested that nucleotide pyrophosphatase was iodinated, and thus located at the outer aspect of the lymphocyte plasma membrane.

Iodination of isolated spleen lymphocyte plasma membranes showed that one of the peaks of radioactivity present was of apparent mol.wt. 130000 (Fig. 2), in agreement with other reports (Ladoulis *et al.*, 1974; Juliano & Behar-Bannelier, 1975; Anderson *et al.*, 1976). A component of similar apparent molecular weight to the immunoprecipitated peak is thus available for iodination in isolated plasma membranes. Since isolated lymphocyte plasma membranes consist mainly of closed vesicles of a right-side out configuration (Allan & Crumpton, 1970; Misra *et al.*, 1974), this result can also be interpreted to suggest a location for nucleotide pyrophosphatase on the outer aspect of the plasma membrane.

To demonstrate a direct combination of the mouse liver plasma-membrane nucleotide pyrophosphatase antiserum with the enzyme in lymphocytes attempts were made to demonstrate antigen–antibody interaction. Passage of Nonidet P40 extracts of spleen lymphocytes through a Sepharose column containing covalently attached antiserum against mouse liver plasma-membrane nucleotide pyrophosphatase indicated retention of enzyme activity, whereas protein and leucine naphthylamidase activity were recovered (Table 2). This result suggested a direct combination between the liver anti-(nucleotide pyrophosphatase) antiserum and the enzyme of lymphocytes.

## Discussion

Enzymically catalysed iodination of perfused liver and isolated hepatocytes showed that nucleotide pyrophosphatase is a sialoglycoprotein of apparent mol.wt. 130000 located at the outer aspect of the rat and mouse hepatocyte plasma membrane (Evans *et al.*, 1973; Evans, 1974; Bischoff *et al.*, 1975, 1976). Previous observations (e.g. Bischoff *et al.*, 1970) showing that various nucleotides were rapidly hydrolysed when added to the isolated haemoglobin-free perfused liver were thus explained. The purified nucleotide pyrophosphatase of mouse liver (Evans *et al.*, 1973) and rat liver (Bischoff *et al.*, 1974) were shown to hydrolyse a range of substrates containing a nucleotide pyrophosphate bond, and also to behave as an alkaline phosphodiesterase, hydrolysing the artificial substrate thymidine 5′-monophosphate *p*-nitrophenyl ester. The predictions of Touster *et al.* (1970), based on similarities in the kinetics of hydrolysis of the physiological and artificial substrates by the liver plasma-membrane enzyme, were thus confirmed. However, although more direct evidence for activity towards nucleotide pyrophosphate and phosphodiester bonds in the case of lymphocytes must await the purification of the enzyme, the results strongly argue that the present enzyme has a similar location and substrate specificity in lymphocytes. Isolated lymphocytes hydrolysed UDP-galactose, and this enzyme was found to be increased in specific activity (relative to the homogenate) in a plasma-membrane fraction to an extent similar to that shown by alkaline phosphodiesterase and 5′-nucleotidase activities. An antiserum raised against the purified mouse hepatocyte plasma-membrane enzyme immunoprecipitated a single iodinated component that was of similar molecular size in extracts of lymphocytes that were previously surface-labelled with $^{125}I$. By studying the inhibitory properties of an antiserum raised against mouse liver plasma membranes towards the 5′-nucleotidase activity of mouse and rat liver and pig lymphocyte plasma membranes, Gurd & Evans (1974) showed that there are strong antigenic similarities between plasma-membrane ectoenzymes that cross not only tissue barriers, but also species barriers. Riemer & Widnell (1975) showed that a 5′-nucleotidase partially purified from a number of rat tissues showed similar properties. The present results then add to the picture that there are classes of glycoproteins with enzymic activity on mammalian cell surfaces that show common antigenic and molecular properties. Indeed, studies of cells of disparate function, e.g. lymphocytes and platelets (Tanner *et al.*, 1974), seem also to indicate that polypeptides of similar molecular-weight classes are iodinated by the lactoperoxidase procedure.

The present demonstration of nucleotide pyrophosphatase/alkaline phosphodiesterase activity on the outer aspect of the lymphocyte plasma membrane adds to the list of cells in which this activity has been similarly located, e.g. hepatocytes (Bischoff *et al.*, 1970) and a variety of cultured cell lines (Deppert *et al.*, 1974; Sela *et al.*, 1972). Monneron (1974) also showed histochemically that UDP-galactose was hydrolysed by thymocyte plasma membranes. Since glycosyltransferases are also claimed to be present on the lymphocyte cell surface (Cacan *et al.*, 1976), the presence of two categories of ectoenzymes that may compete for the same substrate should be borne in mind in devising models [see Shur & Roth (1975) for a review] invoking cell-surface glycosyltransferases to explain cell recognition and adhesion. Ectoenzymes hydrolysing ATP were shown to be present on a variety of eukaryotic cell surfaces by Trams & Lauter (1974) and De Pierre & Karnovsky (1974*a*,*b*), and nucleotide pyrophosphatase activity may account for at least part of this hydrolytic activity.

The exact function of a nucleotide pyrophosphatase residing on lymphocyte and other mammalian cell surfaces is not fully understood. The enzyme may function to exclude the entry of nucleotides into cells e.g. RNA-degradation products, or may serve to conserve cellular nucleotides, by ensuring, in conjunction with nucleotide phosphohydrolases (e.g. 5′-nucleotidase), their hydrolysis in the plasma membrane to nucleosides. Specific mechanisms exist for the transport of nucleosides into cells (Berlin & Oliver, 1975). Liberation of adenosine into serum by lymphocytes could cause vasodilation (Dobson *et al.*, 1971), thus ensuring movement of these cells through narrow blood vessels. Clearly, knowledge of the topography of plasma-membrane enzymes is a first step towards determining their possible implication in the transport and metabolism of nucleotides by mammalian cells.

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# Identification of a high molecular weight protein on the surface of murine thymus and thymus-dependent cells

Rabbit anti-mouse Ig reacted with mouse thymocytes resulting in the formation of caps which were shed into the medium and subsequently injected into rabbits. The antiserum from these animals (AMTP) reacted strongly with thymocytes and peripheral T cells and weakly with B cells. The antiserum did not react via the Thy-1 antigen and could be made specific for T lymphocytes by absorption with B lymphocytes. By surface labeling of lymphocytes with $^{125}I$, it could be shown that the major T lymphocyte antigen recognized by AMTP was one, or possibly two, large, single chain molecules with a molecular weight of approximately 200 000. This molecule was not Ig and, furthermore, the AMTP did not react with cell surface Ig of B lymphocytes. The implications of this finding for previous reports on the existence of immunoglobulin on T lymphocytes are discussed.

[I 1311]

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viations: AMBA-Θ: Anti-mouse brain-associated theta antigen : Anti-mouse thymus protein BM: Bone marrow FCA: d's complete adjuvant GAM-TRITC: Rhodamine-coupled goat ouse Ig GAR-FITC: Fluorescein-coupled goat anti-rabbit Ig nmunoelectrophoresis IF: Immunofluorescence RAMIg: t anti-mouse Ig TCA: Trichloroacetic acid

## 1. Introduction

During a previous investigation into the possible presence of an immunoglobulin-like protein on the surface of murine thymus cells the observation was made that certain polyvalent rabbit anti-mouse immunoglobulin (RAMIg) sera, exhaustively absorbed and diluted, retained the ability to stain living thymocytes by indirect immunofluorescence (IF). After incubation

ls for 10 min at 37 °C, the stained molecules moved
ne of the membranes and formed small caps, thus
g that they might be homogeneous in structure. Enzy-
atment of normal thymocytes prior to staining re-
ese molecules, which reappeared after 20 h of culture
1].

sent paper, further studies on the nature of the anti-
ecules stained by an RAMIg serum on the surface
s cells are described. It was observed that after in-
stained thymocytes for 2 h at 37 °C, these cells were
fluorescent, presumably because the caps had been
the medium. Therefore, cell supernatants containing
as protein complexed to antibodies in the RAMIg
re used in the production of a specific antiserum in
he activity of this antiserum against lymphoid cells
ous organs in mice, was then studied by indirect IF.
n, the nature of the protein molecules specifically
ed by this antiserum was investigated by lactoperoxi-
yzed radioiodination of the lymphoid cell mem-

## ls and methods

nice, 4–6 weeks old, were used in most experiments.
l mice (B mice) for absorptions were (CBA x C57)$F_1$
hese mice had been thymectomized, irradiated, re-
d with fetal liver, and their lymph nodes and spleens
d 80 % B cells, respectively.

### era

*t anti-mouse Ig (RAMIg) serum* was raised in rabbits
njections, given at weekly intervals, of 1 mg of par-
fied whole mouse Ig. This Ig was prepared as follows:
ouse serum was adjusted to contain 30 % saturated
n sulfate, and the precipitate was then dissolved in
buffered saline and reprecipitated a further four
he first injection the antigen was emulsified in
omplete adjuvant (FCA) and given intramuscularly;
subsequent injections, the antigen was diluted in
injected intramuscularly and intravenously, respec-
animals were bled 7 days after the last injection.
ving for clot retraction, the serum was separated
l at 57 °C for 30 min. Absorptions to avoid non-
ining were carried out as described previously [1].
*e μ-chain* was raised in rabbits as previously de-

*e thymus protein (AMTP) serum* was raised in rab-
nmunogen was prepared as follows: 12 x $10^7$ thy-
reviously treated with the absorbed and appro-
luted RAMIg serum for 30 min at room tempera-
thoroughly washed with TC 199 medium until no
otein was found as estimated by absorbance at
the washing fluid. The cells were then incubated
for 2 h at 37 °C. After centrifugation, the super-
found to contain protein at a concentration which
the different batches of cells between 0.14–0.2
(1 cm, 280 nm) units. The supernatants were
ophilized and stored at −30 °C until used. They
sed as an immunogen. Rabbits were immunized according to the following protocol: they received four monthly intramuscular injections of the immunogen, each containing 0.2 absorbance units in 1 ml. In the first injection, the immunogen was emulsified in Freund's incomplete adjuvant (FIA); in the others, the immunogen was in saline. Saline alone or supernatants from untreated thymocytes cultured at 37 °C in medium alone for the same length of time (2 h) were injected into control animals. One week after the last injection the animals were bled and their sera were heated at 56 °C for 30 min. Both the anti-thymus protein serum (AMTP) and the controls were examined by double diffusion in agar gel and by immunoelectrophoresis (IEP) against normal mouse serum. No precipitation lines were seen.

*Anti-mouse brain-associated theta serum (AMBA-Θ)* was produced in rabbits using the technique described by Golub [3].

### 2.3. Conjugates

Goat anti-rabbit Ig coupled to fluorescein or to rhodamine (GAR-FITC or GAR-TRITC) were produced as previously described [4]. Goat anti-mouse Ig coupled to fluorescein (GAM-FITC) was batch no. 21–272, purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands); it did not stain thymocytes or T lymphocytes.

### 2.4. Cell suspensions, indirect IF tests and microscope

These were as previously described [1]. The tissues examined were thymus, lymph nodes, spleen and bone marrow. Bone marrow cells were obtained from the femurs by flushing the marrow cavities with cold buffer. Peripheral T lymphocytes prepared by the nylon wool procedure [5] contained less than 5 % of surface Ig positive lymphocytes.

### 2.5. Double-labeling immunofluorescent tests

Lymphoid cells from the thymus, lymph nodes or spleen were first treated with the AMTP serum for 30 min, washed three times and treated with the conjugate GAR-TRITC for a further 30 min. The cells were again thoroughly washed and counterstained with GAMIg-FITC, to identify B cells. Finally, the cells were washed twice and prepared for examination.

### 2.6. Radioiodination of membrane proteins

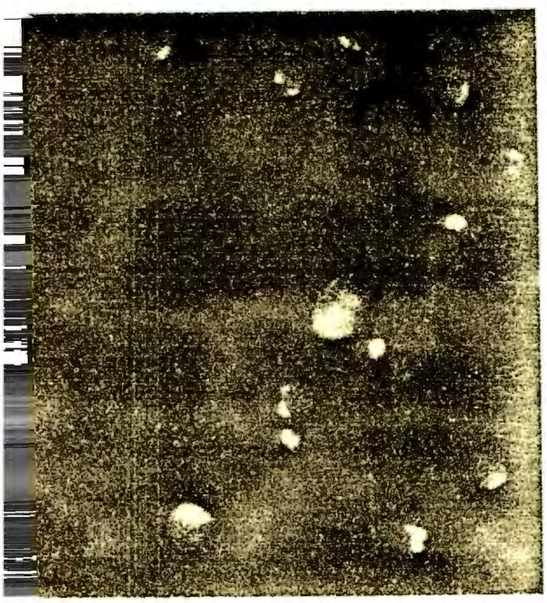
Cell suspensions were labeled with $^{125}I$ using lactoperoxidase [6], washed once in ice-cold phosphate buffered saline (PBS) and then lysed for 10 min at 0 °C in 1 % (w/v) Nonidet-P40 in PBS containing 1 mM phenylmethylsulphonyl fluoride and 100 mM recrystallized iodoacetamide. The centrifuged lysate (30 000 x g, 15 min) was passed over Sephadex G-25 equilibrated with the solution used for lysis of the cells. Uptake of $^{125}I$ by the cells was 30–40 % of input, and at least 95 % of the trichloroacetic acid (TCA)-precipitable radioactivity was recovered in the supernatant after centrifugation of the cell lysate. Radioactivity in the exclusion peak from G-25 was 70–90 % TCA-precipitable and accounted for 10–15 % of the total radioactivity applied. A "nonspecific" immunological precipitate was first formed in the radioactive sample by addition of normal rabbit serum (10 μl) and a goat

bbit IgG serum (100 μl). The resulting precipitate was
:d by centrifugation, and then further precipitation
'ected by addition of various rabbit antisera (10 μl)
'ter 30 min, goat anti-rabbit IgG serum (100 μl). The
ıological precipitations were performed at 0 °C. Spe-
·ecipitates were washed three times with ice-cold 0.5 %
Nonidet-P40 in PBS, once with 50 mM sodium phos-
pH 7, and then dissolved by heating at 100 °C for
in 50 mM sodium phosphate, pH 7.0, containing 2 %
odium dodecyl sulfate. When the samples were reduced
vent also included 1 mM dithiothreitol, and iodoaceta-
·as added to 100 mM after the heating step; for non-
d samples iodoacetamide was present at 100 mM. In-
$^{131}$I-labeled markers [7] were added to the samples,
were then resolved by sodium dodecyl sulfate-poly-
ıide gel electrophoresis [8]. Following electrophoresis
ı were sliced into 1 mm segments, and radioactivity
.ermined. Values were corrected for cross-channel
d plotted with the top of the gel to the left-hand side
'igure.

## lts

### action of polyvalent RAMIg serum with thymocytes

·eatment with the polyvalent RAMIg serum, over
` thymocytes stained by indirect IF and formed very
ıps. The fate of these caps was investigated by incu-
ıtained cells in TC 199 medium at 37 °C and then
ıamples for examination every 30 min. It was ob-
hat the small caps were progressively projected at
of a pseudopodium-like structure provided by the
ıembrane of the living thymocytes until their final
ɔn into the medium (Fig. 1). By the end of 2 h in-
n, virtually no cell remained fluorescent. This finding
· suggests that thymocytes dispose of this aggregated
on their membranes by shedding rather than by pino-



Thymocytes stained with RAMIg and GAR-FITC. Details
ı 2.5. and in the text.

### 3.2. Reaction of the AMTP serum with lymphocytes

Thymocytes indirectly stained with AMTP serum and GAR FITC displayed strong fluorescence which was visible in a cap pattern if the cells were incubated at 37 °C for 10 min (Fig. 2). The majority of peripheral lymphoid cells in the lymph nodes and spleen showed some degree of positivity which varied from faint to strong fluorescence. The ratio of cells staining strongly (like thymocytes) to those staining weakly was 2:1 and 1:1 in lymph nodes and spleen, respectively. Thymocytes were not stained with sera from rabbits that had received saline or supernatants from thymocytes not pretreated with the RAMIg serum. Bone marrow (BM) cells were negative.

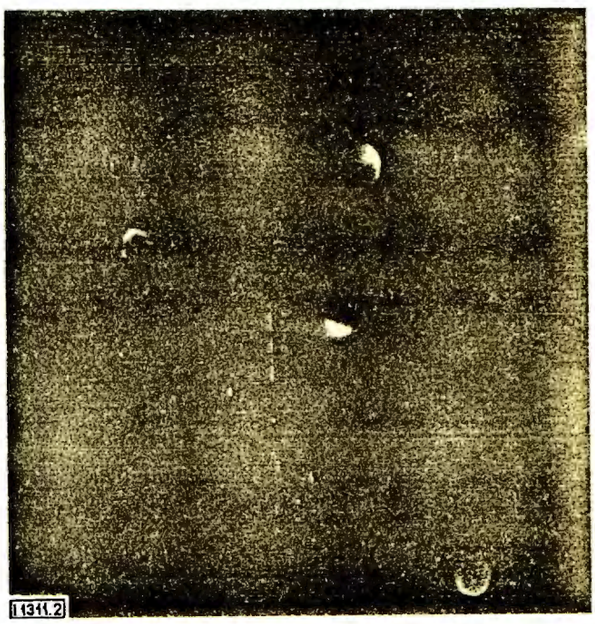


*Figure 2.* Thymocytes stained with AMTP and GAR-FITC. Details in Section 2.5. and in the text.

### 3.3. Specificity of the AMTP serum

Since the animals used for immunization with the thymus protein/RAMIg complexes were rabbits, the antiserum produced (AMTP) should contain antibodies directed mainly to the mouse thymus protein(s). The complete absence of staining of BM cells indicated that nonspecific antibodies, if present, were in a low proportion. However, to ensure specificity, the antiserum was absorbed on different sets of cells, and its activity after the various absorptions controlled by indirect IF. As shown in Table 1, absorptions on BM cells and brain tissue did not alter the staining of unabsorbed serum. Absorptions on spleen cells from T-deprived mice (B cells) abolished the weak staining found on part of the peripheral lymphoid cells. Absorptions on thymocytes completely abolished the staining of all cells.

Double-labeling experiments were then carried out to investigate whethe- Ig-bearing cells also had thymus antigen(s) on their membranes. The AMTP reagent was absorbed on B cells prior to use. Peripheral lymphoid cells from lymph node and spleen were first labeled with AMTP/GAR-TRITC and immediately counterstained with GAM-FITC. The great ma-

Immunofluorescent staining of lymphoid cells of BALB/c
:h an anti-mouse thymus protein serum[a)]

| ıed with | Thymus | Tissue Lymph node | Spleen |
|---|---|---|---|
| ·g | 100 | 93 | 87 |
| ıarrow | 100 | 92 | 88 |
| | 100 | 93 | 88 |
| | 100 | 63 | 42 |
| s | 0 | 0 | 0 |

-uspensions were prepared and stained by indirect IF with un-
d and absorbed AMTP, as described in Section 2.5. and in the
At least 200 cells were examined in each group, and the figures
ıted represent the percentage of cells which were positive. B
/ere spleen cell suspensions from T-deprived mice.

' cells which were stained by the first antiserum were
ıed by the second, *i.e.*, cells which fluoresced strong-
:he AMTP serum had no "easily detectable" surface
ever, a small proportion of lymphoid cells in the spleen
;) was found to be strongly double-labeled. The signi-
ɔf this observation will remain obscure until appro-
•ntrols to evaluate the role of cytophilic antibody and,
:ceptors are carried out.

ɔf some similarities in the staining pattern of the
•nd the AMBA-Θ sera, identical absorptions were also
ıut on the latter for comparison. After absorptions on
ɜue, a striking difference became apparent: while the
ɔf the AMBA-Θ serum had completely disappeared,
•he AMTP remained unaltered. In addition, unabsorbed
-) serum gives a substantial degree of nonspecific stain-
ʍ cells.

### -ıunochemical characterization of the thymus cell -ace protein

:rial on the cell surface reacting with the AMTP was
ized using lysates of $^{125}$I-surface-labeled cells. Rab-
:ra were added to the radioactive samples, and sol-
plexes of antibody and radioactive antigen were
-ted by the addition of goat anti-rabbit IgG, counted
characterized by electrophoresis in polyacrylamide

2 is presented the recovery of radioactivity using a
ʿic control (normal rabbit serum), anti-mouse μ-
-l AMTP. It is clear that radioactivity precipitated
ti-mouse μ-chain from thymocytes and peripheral
ɔcytes is at the level of the nonspecific control pre-
. With spleen cells, on the other hand, there is a
-al difference, which is accounted for by the known
of IgM on the surface of B lymphocytes. With these
s, therefore, there is no evidence for IgM on thymo-
ıeripheral T lymphocytes. The AMTP precipitated
tly more radioactivity than normal rabbit serum
nocytes, but not from peripheral T lymphocytes
cells. However, in the case of peripheral T lympho-
re was a significant difference when the radioacti-
ıpitated by anti-mouse μ-chain and AMTP was com-
is simply indicates that there is variability in non-
recipitating systems, some rabbit sera giving high

Table 2. Coprecipitation of radioactive cell surface molecules[a)]

| Cells | Antiserum | Total radioactivity x $10^{-8}$ (cpm) | Radioactivity in precipitate (%) |
|---|---|---|---|
| Thymocytes | Normal rabbit serum | 1.70 | 0.16 |
| Thymocytes | Anti-mouse μ-chain | 1.70 | 0.12 |
| Thymocytes | AMTP | 1.70 | 0.50 |
| Periph.T. | Normal rabbit serum | 1.87 | 0.17 |
| Periph.T. | Anti-mouse μ-chain | 1.87 | 0.08 |
| Periph.T. | AMTP | 1.87 | 0.23 |
| Spleen | Normal rabbit serum | 1.41 | 0.29 |
| Spleen | Anti-mouse μ-chain | 1.41 | 1.55 |
| Spleen | AMTP | 1.41 | 0.36 |

a) Lymphocytes were labeled externally with $^{125}$I, lysed, and precipitation with various rabbit sera was performed as described in Section 2.6. Total radioactivity in macromolecular material was given by precipitation with 10 % (w/v) TCA, and the radioactivity recovered in the washed antibody-antigen precipitates is expressed as percentage of the total radioactivity of the original sample.

values and others, low. It is consequently always essential to define the molecular composition of these coprecipitates by gel analysis.

Results obtained by gel analysis with the three different cell populations are presented in Fig. 3. It is important to emphasize that these gel profiles, obtained from samples reduced with dithiothreitol, were unchanged when the samples were not reduced prior to electrophoresis. Thus, for example, the high molecular weight component present in thymocytes (Fig. 3 A), which co-electrophoreses with the internal marker of IgMs (7 S IgM), is a single chain molecule. It is certainly not a disulfide-linked multichain molecule similar in structure to immunoglobulin, although it could be mistakenly identified as such if the sample were analyzed without reduction.

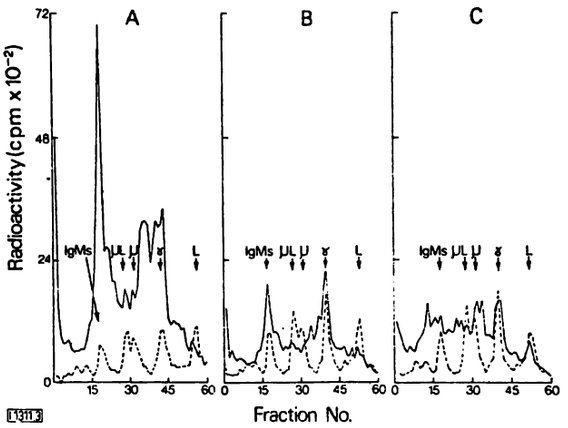



*Figure 3.* Characterization of surface proteins reacting with AMTP. Thymocytes (A), peripheral T lymphocytes (B) and normal spleen cells (C) were labeled externally with $^{125}$I and precipitations with AMTP were performed as described in Section 2.6. The precipitates were reduced, alkylated and applied to 4.2 % (w/v) polyacrylamide gels with the addition of an internal marker of $^{131}$I-labeled proteins; (——) $^{125}$I cell surface material, (- - -) $^{131}$I internal marker. The components of the $^{131}$I internal marker are indicated by the arrows on each gel profile.

re are two immediate conclusions from the data presented. t, in no sample is there radioactivity corresponding to a ain component, and thus the AMTP is without anti-IgM vity. Second, in all samples there is a radioactive peak in position of γ-chains. However, since the component in γ-chain position is also precipitated by control (*e.g.* nor- rabbit IgG and goat anti-rabbit IgG) systems, it is not ·ifically recognized by AMTP and will be discussed no ·her.

s the major components of the thymocyte surface re- ized by AMTP (Fig. 3 A) are single chains with apparent ecular weights of about 200 000 and 60 000. These mole- r weight assignments are approximate, and based on the that the larger molecule co-electrophoreses with IgMs the smaller is intermediate in size between μ and γ-chains. the larger of these two is found on the surface of peri- al T lymphocytes (Fig. 3 B) and in lower yield (40 % of recovered from thymocytes). The profile of radioactivity the spleen cell sample (Fig. 3 C) was similar to that given he nonspecific, normal rabbit serum, control. Thus the nce of the 200 000 molecular weight component, known e present on splenic T lymphocytes (Fig. 3 B), is obscured ackground "noise", and we may conclude that this, as as the 60 000 molecular weight molecule, must be absent resent in very low amounts) from B lymphocytes. This lusion was confirmed by direct analysis of spleen cells a nude mouse. Finally, careful inspection of the 200 000 cular weight peak (Fig. 3 A) suggests the presence of at two components.

## iscussion

a earlier report we described how, using an indirect IF nique, we found that not only pure anti-light chain and IgM antisera stained thymocytes, but also that some po- ent RAMIg sera had the same effect. The molecules ed by the latter were removed with pronase and were shown to be resynthesized by the cells in culture.

major observation reported here is that the reaction be- n a rabbit anti-mouse Ig and murine thymocyte surfaces rred via surface material which was not Ig in nature. This lusion is based on the fact that following interaction be- n the rabbit anti-mouse Ig and thymocytes, complexes ed on the cell surface were shed into the medium and then immunogenic in rabbits to give an anti-mouse thy- protein (AMTP) reagent, which did not react with mouse was not possible to raise sera with similar properties by tion of supernatant from thymocytes which had been d with normal rabbit serum instead of RAMIg. The pro- on of the specific antiserum AMTP could be related to act that thymocytes, unlike B lymphocytes, largely form over the pole of the cell distal to the Golgi region [9]. It ssible that shedding, rather than pinocytosis, is a conse- ce of this type of capping pattern.

AMTP reacted with all thymocytes when tested by fluor- ce. However, the basis of this reaction was not Ig on ocytes. Thus lysates of surface-labeled thymocytes did ontain IgM (or IgD; Abney and Parkhouse, manuscript itted) under conditions where Ig was readily detectable leen cells (see Table 2). Furthermore, when these radio- active lysates were reacted with the AMTP reagent, there was no evidence for μ or L-chains (see Fig. 3). Instead, the major component reacting with the antiserum was a single chain molecule with a mobility very similar to IgMs (7 S IgM) and thus having a molecular weight of about 200 000. There was, in addition, a component with a molecular weight of 60 000.

These findings raise two interesting questions: the first is to explain the activity present in the AMTP, and the second has implications for the sometimes claimed presence of Ig on T lymphocytes. The simplest explanation to the first question is that the original anti-MIg contained antibodies to the thymocyte surface proteins that we have characterized. Treatment of thymocytes with the anti-MIg then caused shedding of these thymocyte surface proteins which, on subsequent injection into rabbits to raise the AMTP, elicited the formation of relevant antibodies. Presumably, therefore, these surface protein(s) are shed *in vivo* and are consequently present in the blood. We may then raise two points in relation to the second question. These are: is it possible that anti-MIg reagents used by other workers positively to identify Ig on T lymphocytes similarly contained antibodies to non-Ig molecules, and could this high molecular weight protein have been mistakenly identified as 7 S IgM? Unfortunately, it is impossible to answer these questions, but we raise them because they do create doubt.

There is now little doubt that Ig can be found on the membranes of some T lymphocytes [10–13], but the critical question is whether they are in fact synthesized by these cells. Apart from two laboratories [14–18], who have consistently maintained that Ig exists endogenously on thymocytes and T lymphocytes, there are several others who have convincingly failed to confirm this point of view using similar techniques [19–28]. In an elegantly designed series of experiments which sought to prove synthesis of Ig by antigen-binding T lymphocytes [29, 30], the criticism still exists that there was no biochemical characterization of the T cell receptor. Instead, the effect of anti-mouse Ig on subsequent binding of antigen was studied. Moreover, the experiments were conducted in the presence of B lymphocytes, and thus cytophilic antibody cannot strictly be ruled out. Further, the finding *in vitro* that thymocytes and T cells bear receptors for IgG [31, 32] and also IgM (Santana, V., manuscript submitted for publication) adds to the difficulties.

The high molecular weight (200 000) antigen(s) defined on thymocyte membranes was present on peripheral T lymphocytes, but absent from B lymphocytes. Absorbtion with brain tissue clearly indicates that this molecule does not correspond to the Thy-1 antigen. However, it does seem quite possible that the material we have described is similar, if not identical, to the 200 000 molecular weight protein(s) noted as a characteristic component of T, but not B, lymphocyte membranes [33]. The 60 000 molecular weight component, present on thymocytes and absent from peripheral T lymphocytes, could conceivably be a thymus-specific antigen, but that remains to be demonstrated.

Finally, it is worth drawing attention to the fact that the weakly staining cells of spleen and lymph nodes disappear when the AMTP is absorbed by B lymphocytes (Table 1). Whatever the molecular basis of this weak staining, it is not

/hen the results of surface labeling are examined.
)le explanation is therefore traces of antibodies to
: components, perhaps shared by B and T lympho-
ıese circumstances the cell surface labeling tech-
ld not be expected to detect the antigens involved.

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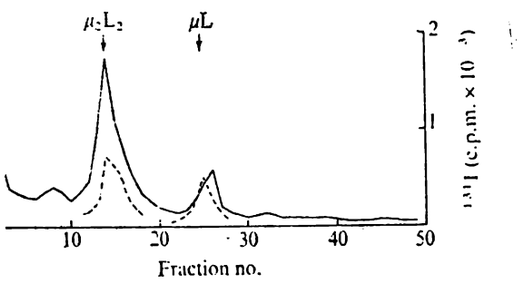
# late for immunoglobulin D t on murine B lymphocytes

ɜH the original discovery of human IgD is almost ten ¹, it is only very recently that a possible function for ; of immunoglobulin has been suggested. Present in uman serum in very small amounts[2], IgD is found on ce membrane of relatively large numbers of human i lymphocytes, in particular those obtained from cord d the circulation of patients with chronic lymphatic a[3-7]. IgD is frequently associated with IgM on the -7 and this, together with the fact that the frequency aring lymphocytes is higher in cord blood than adult suggests a fundamental role for IgD, either as a recognition unit or for regulation of the immune Were this to be so, then clearly IgD would be expected in species other than man. Here we present the ion of an immunoglobulin present on the surface of enic lymphocytes which is not IgM, but which has the characteristics of IgD.




urface immunoglobulin of murine splenic lymphocytes. ell suspensions of 4–6-week-old female CBA mice were with $^{125}$I by the lactoperoxidase catalysed procedure[8], nce in phosphate-buffered saline (PBS) and then lysed n at 0° C in 1% (w/v) Nonidet P40 in PBS. The centri- sate (4,000$g$ × 10 min), containing 95% of the total cipitable radioactivity, was dialysed against 1% (w/v) P40-PBS in the cold. Surface-labelled immunoglobulin ipitated by the addition of a polyspecific rabbit anti- serum (10 μl) followed by goat anti-rabbit IgG (100 μl). ipitate was washed three times with ice cold 0.5% (w/v) P40 in PBS, twice with ice cold PBS, and then dissolved v/v) sodium dodecyl sulphate (0.1 M iodoacetamide) um phosphate, $p$H 7.0, by heating at 100° C for 5 min. e of mouse myeloma MOPC 104E IgM was labelled ref. 16), partially reduced to $\mu_2L_2$ and μL by treatment nM dithiothreitol for 60 min at room tempera- ylated with 10 mM iodoacetamide, and then added lated $^{125}$I-cell surface Ig to serve as an internal marker el analysis (4.25% (w/v) polyacrylamide gel containing v) sodium dodecyl sulphate[9]. Following electrophoresis, was sliced into 1 mm segments and radioactivity ed. Values were corrected for cross channel spill, and ith the top of the gel on the left hand side of the figure. incorporated into cell surface Ig; – – –, $^{131}$I-labelled internal marker.

ell suspensions containing more than 95% viable an blue exclusion) were labelled with $^{125}$I by the idase-catalysed procedure[8], and the surface immuno- as prepared and analysed on sodium dodecyl sulphate [9] with addition of an internal marker ($^{131}$I-labelled ally reduced to $\mu_2L_2$ and μL). As has been previously [12], we found a major portion of radioactive cell nunoglobulin in that part of the gel corresponding As ($\mu_2L_2$) subunit of IgM (Fig. 1). In addition, ere was a significant radioactive peak running very advance of the μL subunit, but which contained light chains on reduction (see Fig. 2) and must e an HL subunit.

Following reduction and alkylation both the $H_2L_2$ and HL subunits were shown to contain two distinct species of heavy chain, there being one component with the mobility (and therefore size) of the internal $^{131}$I-μ chain marker, and another which migrated faster than μ chain, but slower than γ chain (Fig. 2). The proportion of radioactivity found in the smaller (faster migrating) heavy chain was lower in the sample of reduced $^{125}$I-surface $H_2L_2$ (Fig. 2*a*) than the reduced $^{125}$I-surface HL (Fig. 2*b*).

We discount the possibility that the heterogeneity of surface

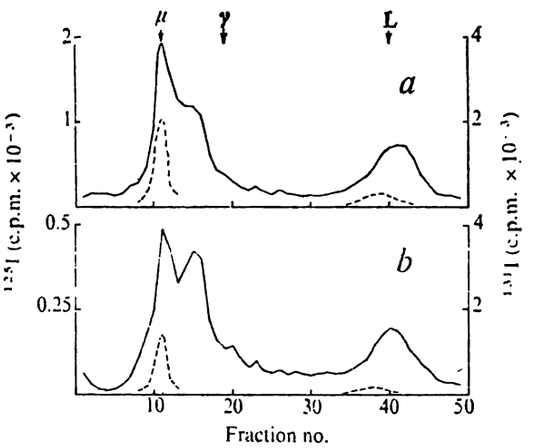


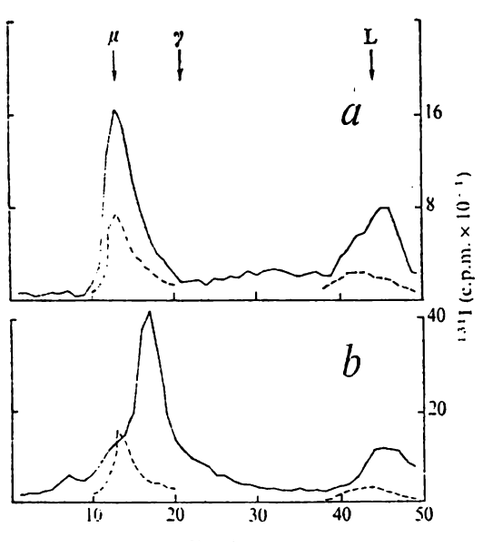

**Fig. 2** Heavy chain heterogeneity of surface immunoglobulin of murine splenic lymphocytes. Sections of the gel shown in Fig. 1 containing the $H_2L_2$ and HL components were eluted with 2% (w/v) sodium dodecyl sulphate (2mM dithiothreitol) 0.05 M sodium phosphate, $p$H 7.0. The eluate was heated for 15 min at 100° C in order to ensure reduction of all disulphide bridges, and then alkylated by addition of iodoacetamide to 10 mM. The resulting material was then applied to 10% (w/v) polyacrylamide gels containing sodium dodecyl sulphate to resolve the heavy chain components. In these conditions the $^{131}$I-$\mu_2L_2$ and μL originally added as internal markers for the first gel are also extracted and reduced, thereby providing internal markers for μ and L chains in the second separation in the 10% acrylamide gels. The gels were fractionated, counted and plotted as given in the legend to Fig. 2. The position of γ chains was assessed by running a mixture of totally reduced $^{131}$I-labelled mouse IgM (MOPC 104E) and IgG2a (Adj.PC5) on a parallel gel. *a*, Reduced, surface labelled $H_2L_2$; *b*, reduced, surface labelled HL. ——, $^{125}$I incorporated into cell-surface Ig; – – –, $^{131}$I-labelled internal marker.

Ig results from the presence of T-lymphocytes in the spleen cell suspension as similar results were obtained when spleen cells from nude mice were analysed by the same techniques. In addition, peripheral T-lymphocytes purified by passage of spleen cells through nylon wool columns[13] did not contain detectable amounts of surface immunoglobulin using the methods described above.

Reconstruction experiments were performed to check for degradation of μ chain-containing material during the preparation of labelled surface Ig. Both $^{125}$I-labelled $\mu_2L_2$ and μL, prepared by partial reduction of MOPC 104E IgM, could be recovered unchanged when added to spleen cells and then carried through the procedures outlined above for isolation of cell surface immunoglobulin. The HL subunits found on the surface of splenic lymphocytes, therefore, do not result from reductive depolymerisation of $\mu_2L_2$ during isolation. Reduction of the recovered $^{125}$I-$\mu_2L_2$ and $^{125}$I-μL yielded heavy chains entirely corresponding in size to untreated μ chain. We are therefore confident that the observed heavy chain size heterogeneity of cell surface immunoglobulin does not result from degradation of μ chain during the isolation procedure.

ssible explanation for the heterogeneity of surface globulin is that there is a precursor-product relation- ween the various species observed. Such a relationship ither reflect a biosynthetic pathway or metabolic of membrane-associated immunoglobulin. In the first HL subunit would be an intermediate in the bio- of $H_2L_2$, and the small heavy chain would be a glycosylated μ chain requiring the addition of further sidues for completion. In the alternative situation, the would be expected to be towards degradation of $H_2L_2$ nd proteolysis of the heavy chain. We have ruled out ossibility by culturing surface-labelled spleen cells for 0–12 h. The absence of any form of precursor- relationship was clearly indicated by the fact that the $H_2L_2$ to HL and large heavy chain (that is, a μ chain) heavy chain determined at time zero remained the cell surface and released immunoglobulin recovered at of incubation. This experiment demonstrated also half lives of all the immunoglobulin species present on surface are similar, a value of 10 h being determined.

en pursued the possibility that the small heavy chain ved from a class other than IgM by using antisera of lass specificity for isolation of surface immunoglobulin. ies specific for γ1, γ2a and α chains did not precipitate globulin from detergent-solubilised, surface-labelled ymphocytes. On the other hand, specific anti-μ chain-




wo different heavy chain classes present on the surface e splenic lymphocytes. Spleen cells were labelled with lescribed in the legend to Fig. 1. They were washed Eagle's medium containing 10% foetal calf serum, ded in the same medium at $2 \times 10^7$ cells $ml^{-1}$, and then d at 37° C in an atmosphere of 5% $CO_2$ in air for 6 h. nd of the incubation period, the cells were 95% viable blue exclusion). The medium, containing released cell immunoglobulin, was separated from the cells by ation (400*g*, 10 min), adjusted to contain 1% (w/v) P40, and dialysed against 1% (w/v) Nonidet P40 in e buffered saline. To the non-diffusate was added a rabbit anti-(mouse μ chain) serum (10 μl) and goat bit IgG) serum (100 μl). The resulting precipitate was by centrifugation, and the supernatant was further with polyspecific rabbit anti-(mouse Ig) (50 μl) by the appropriate amount of mouse Ig (40 μg) to mplete precipitation of mouse Ig. Both precipitates hed and analysed in 4.2% (w/v) polyacrylamide gels bed in the legend to Fig. 1. The $H_2L_2$ containing f both gels were eluted, reduced and alkylated, and on 10% (w/v) polyacrylamide gels as described in the Fig. 2. *a*, Reduced cell surface $H_2L_2$ precipitated with ti-(mouse μ chain); *b*, reduced cell surface $H_2L_2$ from the supernatant of the precipitation with rabbit se μ chain) by addition of rabbit anti-(mouse Ig). incorporated into cell-surface Ig; – – –, $^{131}I$-labelled internal marker.

precipitated $H_2L_2$ and HL containing heavy chain corresponding only in size to authentic, secreted μ chain (Fig. 3*a*). This material may therefore be identified as μ chain with certainty. When the supernatant from the anti-μ precipitation was further reacted with polyspecific anti-(mouse Ig), however, the $H_2L_2$ and HL species precipitated were predominantly composed of the small heavy chain (Fig. 3*b*). We may therefore conclude that there is present on the surface of murine splenic lymphocytes a heavy chain larger than the γ chain, yet smaller than the μ chain, which is not precipitated with antisera to γ, α or μ chains, but which is reactive with a polyspecific antiserum to mouse Ig. As the small heavy chain was not precipitated by anti-(μ chain), the possibility that it is an incompletely glycosylated μ chain[14] is ruled out.

The inescapable conclusion is that this heavy chain, which accounts for about 40% of heavy chain isolated from surface-labelled spleen cells, represents a hitherto undescribed class of immunoglobulin in the mouse; the most obvious surmise is that it corresponds to the human IgD class. To support this suggestion we note the similar mobility on SDS-polyacrylamide gels of human δ chain[2] and the novel heavy chain of mouse lymphocytes described above. This newly described mouse immunoglobulin, which we shall now refer to as IgD, is also similar to human IgD[2] in its marked susceptibility to proteolytic degradation (E.R.A. and R.M.E.P., unpublished). A similar conclusion has also been drawn by others (E. S. Vitetta and J. W. Uhr, unpublished).

Contrary to our expectation, however, was the finding of μ chain, but not δ chain, in foetal liver (16 d embryos) and neonatal spleen and liver. In addition, splenic lymphocytes of 6 week and 6 month old mice contained similar amounts of δ chain, the ratio of μ:δ being about 3:2. This could mean that the expression of IgD in the mouse is subsequent to the appearance of IgM. If this is not the case, then IgD-bearing lymphocytes must arise in organs other than spleen or foetal liver. Perhaps relevant to this point is the fact that IgD constitutes the major immunoglobulin class present on murine lymph node cells, where the μ:δ ratio was found to be 1:3.5–4.0.

It is certainly intriguing that spleen and lymph node cells differ markedly in terms of the relative amounts of μ and δ chains present on their surfaces. In this respect we may note that spleen cells, but not lymph node cells, respond to lipopolysaccharide (G. Janossy and R.M.E.P., unpublished), and that spleen cells secrete largely IgM, whereas the major product of lymph node cells is IgG[15]. The presence or absence, however, of a functional relationship between these separate observations remains to be determined. Whether the molecular heterogeneity of total cell surface Ig is reflected in a similar pattern on individual cells is not known. Nonetheless, a relationship between this observed heterogeneity and regulation of the B-lymphocyte response to antigen is suggested.

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# HETEROGENEITY OF SURFACE IMMUNOGLOBULIN ON MURINE B-LYMPHOCYTES

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## 1. Introduction

The distribution of IgD in Man has aroused considerable excitement recently. Present in normal human serum in very small amounts[1], IgD is found on the surface membrane of relatively large numbers of human B-lymphocytes[2,3,4,5,6,7]. It is therefore hardly surprising that a special (but as yet unknown) role for IgD has been the subject of speculation. In these circumstances the importance of establishing the existence of IgD in an animal system is obvious. To date, there have been two independent reports of an IgD-like molecule in the mouse [8,9,10], and we may confidently predict similar results in other mammalian species. An interesting question will be whether IgD occurs in vertebrates other than the mammals.

The results that we[8] obtained in the mouse were strictly in agreement with the work of others[9,10] and may be summarised as follows. Lymphocytes from spleen or lymph nodes were externally labelled with radioactive iodine using lactoperoxidase, and labelled immunoglobulin was selectively removed by precipitation with specific antibody to mouse Ig. When characterised by electrophoresis in dissociating gels, the surface Ig was predominantly a disulphide linked, $H_2L_2$ structure, although some HL subunits were also present. There was no 19S Ig. Using antisera specific for the mouse heavy chain classes we could not detect IgG or IgA. Precipitation with anti-$\mu$ chain accounted for some, but not all, of the cell surface Ig. The Ig remaining after precipitation with anti-$\mu$ chain was isolated by addition of anti-K chain, and after reduction yielded a heavy chain of size intermediate between $\mu$ and $\gamma$. We may therefore conclude that there are two heavy chain classes expressed on the surface of mouse lymphocyte. One is IgM and the other, since it does not react with anti-$\mu$, anti-$\alpha$ or anti-$\gamma$, appears to be a homologue of IgD. This candidate for IgD in the mouse resembles the human counterpart in heavy chain size, marked susceptibility to proteolysis and in its occurrence on lymphocyte surfaces. It is too small to be IgE. We discount the possibility that the heterogeneity of surface Ig results from the presence of T-lymphocytes in the cell suspensions since similar results were obtained with spleen cells from nude mice. In addition, peripheral T-lymphocytes purified by passage of spleen cells through nylon wool columns[11] did not contain Ig using the same methodology.

In the absence of sequence data this new Ig class in the mouse cannot be formally identified as IgD. However, for ease in presentation, and because of the similarities noted above, we shall henceforward refer to this molecule as IgD.

g spleen cells from 6 week old CBA or nude mice, we found 60% of the Ig as IgM; the remainder was IgD. Expression of IgD on the cell surface fore not dependent upon the presence of T-lymphocytes. In foetal liver, tal spleen and foetal liver, however, only IgM was detectable, indicating the mouse IgM precedes IgD in embryological development. This finding reement with recent results in the human[12]. Earlier assumptions that edes IgM in the human were based on comparisons between cord and adult , and appear to be incorrect. Interestingly, the amino acid sequence IgD is more homologous to IgE and IgG than IgM[13], which would suggest evolved some time after formation of the gene for μ chains. Thus IgD subsequent to IgM in both evolution and ontogeny.

ıtriguing observation was the marked difference between B-lymphocytes in spleen and lymph nodes. Although IgM and IgD were the only Ig classes both populations, the ratio of IgM/IgD was 1.5 in the spleen and 0.3 in ı nodes. This, taken together with the fact that IgD arises later than ıtogeny, suggests that the B-lymphocyte population in the lymph nodes more mature population than that present in the spleen. In this respect ɔte that spleen cells, but not lymph node cells, respond to lipopoly- ıe (G.Janossy and R.M.E.Parkhouse, unpublished), and that spleen cells argely IgM, whereas the major product of lymph node cells is IgG[14].

xperiments described above indicate heterogeneity of total immunoglobulin gD) found on the surfaces of murine B-lymphocyte populations. An obvious is the distribution of these two immunoglobulin classes on individual ytes. In order to answer the question in the absence of specific anti- urine IgD, we have based our strategy on the results of our $^{125}$I-labell- iments. The crucial assumption, which is supported by the labelling urselves[8] and others[9,10], is that IgM and IgD are the major immunoglobulins n lymphocyte surfaces. We are aware that claims have been made for the of IgG on the surface of B-lymphocytes in the mouse, but these will be d as artefacts due to the specificity of the reagents used. As an f one possible artefact we cite recent experiments in the human[15]. number of peripheral blood lymphocytes, which were positive for IgG scent staining with whole antibodies to Ig was dramatically reduced uorescent $F(ab)_2$ antibody was used. The authors conclude that most arently positive for IgG are in fact lacking in membrane Ig but do have ɔrs. We do not exclude the possibility of very low numbers of IgG ells, nor the presence of very small amounts of surface IgG. These ties, however, will not influence the experimental approach.

xperimental design depends on the known fact that IgM and IgD cap ıtly on human lymphocytes[3,5]. The protocol consisted of first capping a rhodamine-coupled anti-μ chain system and then reacting the cells with

a poly-specific, fluorescein-coupled anti-mouse Ig under non-capping conditions. Since we assume an absence of immunoglobulin other than IgM or IgD, the green rings visualised by the second reagent must represent IgD moieties on the cell surface. To control for capping of all IgM molecules in the first step, some cells were treated with fluorescein-coupled anti-μ chain instead of the poly-specific reagent. The approach is therefore identical to that used in order to identify IgD by radioactive labelling techniques.

2. Materials and Methods

Rabbits were immunised with purified MOPC 104E IgM ($\mu\lambda_1$) or the Fab portion of myeloma protein Adj PC5 ($\gamma_{2a}K$) as previously described [16,17]. The anti-IgM was absorbed by passage through Sepharose 4B coupled to a 7S fraction of normal mouse serum[18]. On Ouchterlony analysis, the absorbed anti-MOPC 104E precipitated MOPC 104E ($\mu\lambda_1$) and TEPC 183 ($\mu K$) IgM proteins, but not MOPC 25 ($\gamma_1 K$), Adj PC5 ($\gamma_{2a}K$), MOPC 195 ($\gamma_{2b}K$) or MOPC 315 ($\alpha\lambda_2$). In order to exclude traces of antibody activity towards immunoglobulin subunits other than μ chains, the absorbed anti-MOPC 104E was tested by a more sensitive radio-immunoprecipitation procedure. Myeloma proteins were labelled[19] with carrier free $^{125}I$ at about 1μCi/μg. The radioactive protein (5-10 ng) was mixed with the test antiserum (10 ul) and after 30 min. at room temperature, sufficient goat anti-rabbit IgG was added to ensure precipitation of the added rabbit Ig. When this was done, the absorbed anti-MOPC 104E was found to be negative for reactivity towards $IgG_1$, $IgG_{2a}$, $IgG_{2b}$ and IgA myeloma proteins. For brevity, this antiserum will henceforth be referred to as anti-μ chain. The anti-Fab reagent was polyspecific reacting against IgM, $IgG_1$, $IgG_{2a}$, $IgG_{2b}$ and IgA, but not other serum proteins, on Ouchterlony analysis and immunoelectrophoresis. The IgG fraction of both antisera was prepared, coupled to fluorescein isothiocyanate, and conjugates with fluorescein: protein ratios of 2-3:1 were selected by chromatography on Whatman DE 52[20].

A goat antiserum specific for rabbit IgG [16] was similarly treated to yield a rhodamine conjugate.

Spleen cell suspensions from six week old specific pathogen free, female CBA mice were prepared in veronal-buffered saline (Oxoid Ltd.) containing 1 mg/ml of bovine serum albumin (VBS-BSA) and the living cells were stained and prepared for examination as described by Raff [21]. Cells were incubated for 20 min. at room temperature with rabbit anti-μ chain or rabbit anti-Fab, washed x3 in VBS-BSA, and then suspended in purified, rhodamine-coupled goat anti-rabbit IgG (20 min. room temperature). The two antibody layers were used in order to ensure maximum capping. Samples were washed and then examined directly or transferred to VBS-BSA-0.03M sodium azide-20% (v/v) normal rabbit serum, for treatment with purified, fluorescein-coupled-anti-μ chain or anti-Fab (20 min., 0°). After three washes with VBS-BSA-0.03M sodium azide, cells were mounted in VBS-BSA-0.03M sodium azide, and examined under ultra-violet light with a Leitz Orthoplan research

microscope fitted with Ploem illumination. At least 60 stained lymphocytes were assessed for the presence of rhodamine and, or, fluorescein. All reagents were centrifuged (50,000 xg, 60 min.) on the day of use.

3. Results and Discussion

TABLE 1

Heterogeneity of surface immunoglobulin on murine lymphocytes

| Experiment | Total Cells | Cap | Green Ring | Red Cap-Green Ring |
|---|---|---|---|---|
| 1 | 240 | 23 (22) | 39 (37) | 43 (41) |
| 2 | 717 | 79 (29) | 109 (40) | 85 (31) |
| 3 | 135 | 11 (17) | 30 (45) | 25 (38) |
| 4 | 318 | 38 (27) | 52 (37) | 49 (35) |

Legend to the Table

CBA spleen cells were treated with rabbit anti-mouse μ chain followed by rhodamine-labelled goat anti-rabbit Ig under capping conditions. The cells were then treated with fluorescein-labelled rabbit anti-mouse Fab in the cold and in the presence of azide. The figures in parentheses express the number of cells observed as a percent of total stained cells.

Based on the double-staining protocol outlined above, lymphocytes with surface Ig could be classified into three groups (Table 1). A large number (31-41% of total Ig-bearing cells), which were capped with anti-μ, subsequently stained peripherally with anti-Fab under the non-capping conditions (i.e. surface IgM and IgD simultaneously present). The remaining stained cells were divided between those showing only caps (i.e. surface IgM only) (17-29% of Ig-bearing cells) and those showing only green rings (i.e. surface IgD only). (37-45% of Ig-bearing cells). Inspection of the doubly stained cells suggested a considerable variation in the relative intensities of the two fluorescent markers, suggesting a variation in the IgM to IgD ratio from cell to cell. Our assumption that peripheral staining with anti-Fab following capping of IgM is due to the presence of IgD, and not IgG, determinants is reinforced by the high numbers of peripherally stained lymphocytes observed. Also, the results agree with our general finding that the numbers of cells staining with anti-μ (25-30% total splenic lymphocytes) is always less than the numbers of cells staining with anti-Fab or anti-K (35-45% total splenic lymphocytes. Further, complete capping of IgM

in the first stage was evident since fluorescein-labelled anti-μ failed to reveal ring staining after the cells had been treated with the anti-μ under capping conditions. One possible objection, that the doubled staining is due to interaction of the fluorescein-coupled anti-Fab reagent with Fc receptors, is ruled out by two controls. First, addition of aggregated human IgG (5 mg/ml) failed to inhibit the second staining step with fluorescein-labelled anti-Fab. Second, cells capped with anti-Fab and rhodamine-labelled goat anti-IgG were not subsequently stained with the fluorescein-coupled anti-Fab.

Our findings in the mouse therefore, mirror those in the human[3,5]. In the mouse, there are fewer cells bearing both IgM and IgD, with correspondingly higher numbers of cells with exclusively IgM or IgD. The biological significance of these subpopulations of B-lymphocytes, although at present obscure, may now be revealed in the mouse system. Certainly IgM precedes IgD in ontogeny [8,10] and thus a developmental sequence is suggested. Thus, the B-lymphocyte, originally expressing only IgM, matures to a cell expressing only IgD via an intermediate cell type with both classes of immunoglobulin on its surface. One might therefore expect to find memory cells in the more mature cell type. The simultaneous expression in one cell of two heavy chain classes sharing the same variable regions [22] suggests a copy-choice model for V - C gene integration[23], and establishes a precedent for an Ig class switch by lymphoid cells.

If we accept that simultaneous expression of two heavy chain classes occurs at the level of transcription, then there is no reason not to postulate simultaneous integration of all heavy chain constant genes early in the ontogeny of a B-lymphocyte or its precursor. Questions relating to Ig class expression would then revolve entirely around differential gene activation and repression, and the requirement to account for a V - C gene integration event at this stage of differentiation would no longer exist.

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# Functional and Structural Characterization of Immunoglobulin on Murine B Lymphocytes

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There is no doubt that immunoglobulin is the receptor for antigen on B lymphocytes, but there is certainly some confusion regarding $C_H$ gene expression at the level of the lymphocyte surface. There are three very clear reasons for this confusion: First, much of the data comes from immunofluorescence, a technique plagued with technical problems, e.g., material binding nonspecifically or through Fc receptors. What is clear, however, is that the number of B lymphocytes recorded positive for IgG and IgA steadily decrease to a very low number (1–3%) as more care is taken to avoid these problems (Jones et al. 1975; Kurnick et al. 1975; Lobo et al. 1975; Winchester et al. 1975). Second, particularly in the mouse, when surface immunoglobulin is characterized by external labeling and immunochemical procedures, there is little, if any, IgG or IgA detected (Vitetta et al. 1971; Marchalonis et al. 1972; Abney and Parkhouse. 1974). Third, there is the problem of distinguishing between a precursor lymphocyte that has not been in contact with antigen with one that has, and, as a result, has embarked upon an irreversible differentiation pathway leading to the high-rate antibody-secreting cell. Indeed, there is evidence for a class of "triggered" memory cells within a few days of boosting (North and Askonas. 1976), and it is possible that a cell at this stage of differentiation could be stimulated into an abnormally extensive clonal expansion under certain experimental conditions, for example, by transfer into an irradiated host together with antigen.

At present, therefore, we are faced with the embarrassment of a total lack of correlation between isotypes expressed systemically and on lymphocytes. Since the number of IgG- and IgA-bearing lymphocytes is low, it is reasonable to ask whether this minor population of cells has biological significance. It is also a matter of urgency to define the immunoglobulin on the vast majority of B lymphocytes.

In answer to the first question we can say that it is entirely possible that a small population of, for example, IgG-bearing cells could be rapidly expanded by cell division in such a way as to account for all of the serum IgG. Indeed, there are experiments suggesting that IgG is present on the surface of some, but not necessarily all, precursors of IgG-secreting cells (Walters and Wigzell 1970; Herzen-

If most immunoglobulin on B lymphocytes is not found in serum, then what is it? For some time IgM has been recognized as a major component of B-lymphocyte membranes. External radiolabeling procedures showed that surface IgM differs from the secreted variety by being the monomeric ($H_2L_2$) subunit rather than the fully assembled $(H_2L_2)_5$ pentamer (Vitetta et al. 1971; Marchalonis et al. 1972). In these and other (Abney and Parkhouse 1974) similar studies, neither IgG nor IgA was detected (lower limit of detection about 5% of total cell-surface immunoglobulin). A major breakthrough came with the discovery that IgD was also present on many human peripheral B lymphocytes (van Boxel et al. 1972; Rowe et al. 1974[b]). This was quickly followed by the demonstration of its endogenous synthesis (Knapp et al. 1973; Rowe et al. 1973[a]) and occurrence on many human chronic lymphatic leukemia cells. Although some cells expressed either IgM or IgD, on many, both isotypes were simultaneously present (Knapp et al. 1973; Rowe et al. 1973[a]; Fu et al. 1974; Kubo et al. 1974). Using chronic lymphatic leukemia cells and an anti-idiotype raised against their IgM product, it was possible to show that where the two isotypes coexist on the same cell, they share the same idiotype (Salsano et al. 1974; Fu et al. 1975), and hence, presumably, V regions. The simultaneous expression by one cell of two isotypes sharing the same V region has implications for the mechanism of V-C gene integration. Because of the very long life of chronic lymphatic leukemia cells, it seems probable that in those cells expressing both IgM and IgD, the genome contains integrated genes for both heavy chains. To explain this, reiteration of the V gene or a copy-choice mechanism for V-C gene integration is required. These speculations are based on the long life of chronic lymphatic leukemia cells, and conclusive evidence (e.g., simultaneous transcription of mRNA for $\delta$ and $\mu$ chains) is lacking. However, having raised the possibility that two integrated heavy-chain genes exist in the chronic lymphatic leukemia cell, it does suggest the following hypothetical scheme. At some time in the development of a B lymphocyte there is integration of a unique V gene along with the constant-region genes of all heavy-chain isotypes. Multiple copies of the same V gene could be determined by germ line or somatically generated by making a series of copies (i.e., copy-choice) at the time of integration. The advantage of simultaneous integration of all $C_H$ genes early in the ontogeny of B lymphocytes is that questions relating to isotype expression would then revolve entirely around differential gene activation and repression; the need to account for V-C gene integration events at later stages of differentiation would no longer exist. paucity of information, however, it is fair to ask if this is the exception rather than the rule. The situation with IgA-secreting cell precursors is even more confused. These are reported to have or not have surface IgA, depending on the antiserum used to make the identification (Jones et al. 1974; Jones and Cebra 1974).

The paucity of serum IgD contrasts with its high frequency on B lymphocytes, and if a special role for this class of membrane immunoglobulin is to be considered, then the importance of establishing the existence of a similar molecule in an animal species is obvious. To date, there have been claims for a similar immunoglobulin in the mouse (Abney and Parkhouse 1974; Melcher et al. 1974; Vitetta et al. 1975b) and suspicions of one in the rabbit (Craig and Cebra 1975; Pernis et al. 1975).

In the mouse, the candidate for IgD is a disulfide-linked $H_2L_2$ molecule which can be precipitated from lysates of externally labeled nude or normal mouse B-lymphocytes with anti-light chain but not with antisera to any of the known mouse heavy-chain isotypes. It is therefore clearly an isotype hitherto undescribed in the mouse. This candidate for IgD resembles the human counterpart in heavy-chain size, marked susceptibility to proteolysis, and in its occurrence on lymphocyte surfaces. In the absence of sequence data, this new immunoglobulin class in the mouse cannot be formally identified as IgD. However, for ease in presentation, and because of the similarities noted above, we shall henceforth refer to this molecule as IgD. In addition, the probability that sequence studies will confirm this assignment appears to be very high.

Both in humans (Vossen and Hijmans 1975; Cooper et al., this volume) and in mice (Abney and Parkhouse, 1974; Vitetta et al. 1975b), IgM precedes IgD in ontogeny, IgD appearing about 2 weeks after birth in mice and sometime between 3 and 4 months of gestation in humans. Earlier assumptions that IgD precedes IgM in humans were based on comparisons between cord and adult blood and appear to be incorrect. Interestingly, what little we do know of the amino acid sequence of human IgD would suggest that it evolved some time after the gene for $\mu$ chains (Spiegelberg 1975). Thus IgD appears subsequent to IgM both in evolution and in ontogeny.

There is a marked difference between the relative amounts of IgM and IgD in murine spleen, lymph nodes, and Peyer's patches (Abney and Parkhouse 1974; Melcher et al. 1974; Vitetta et al. 1975a,b). By external labeling techniques, IgM and IgD were found in approximately equal amounts in the spleen; in lymph nodes and Peyer's patches, IgD accounted for 70% and 90%, respectively, of the total immunoglobulin. It is important to emphasize that, in these studies, IgM and IgD are the *only* immunoglobulins recovered.

The external labeling procedure can only give the total yield of IgM and IgD, not their distribution on individual cells. Based on the rarity of immunoglobulins other than IgM and IgD on the surface of mouse B-lymphocytes, fluorescent staining was used to indicate that in the mouse, as in the human, there are splenocytes bearing either IgM or IgD or both IgM and IgD (Parkhouse et al. 1976). In those experiments, IgM was first capped with rhodamine-labeled anti-$\mu$ chain, and then subsequent ring staining with fluorescein-labeled anti-Fab in the presence of sodium azide was taken as evidence for the presence of IgD. Recently, an antiserum specific for mouse IgD has been developed (Abney et al. 1976a), and the above result has been confirmed. Using this antiserum, it could be shown that the majority of B lymphocytes in lymph nodes and Peyer's patches were expressing mostly IgD, although some obvious doubles (IgM and IgD) and an occasional IgM-bearing cell were seen. In the spleen, all three categories of cells were present, and the considerable variation in relative intensities of the two fluorescent markers in doubles suggested a variation in IgM and IgD ratio from cell to cell. This, together with the observation of the three cell types (i.e., IgM, IgD, and IgM-IgD) and the fact that IgM precedes IgD in ontogeny, suggests a developmental sequence of immunoglobulin expression on B lymphocytes from IgM to IgD via an intermediate cell type with both isotypes. Thus the B lymphocytes of lymph nodes and Peyer's patches would, for the most part, be a more mature population of cells. Of interest in this context is the finding that large splenic lymphocytes, thought to be more immature, are enriched for IgD (Goodman et al. 1976).

The availability of an antiserum specific for mouse IgD has facilitated an enquiry into its biological role. In this paper we discuss the evidence for a structural homology between mouse and human IgD, our failure to demonstrate IgD on memory B cells, and some preliminary data relating to Ig class expression by developing lymphocytes. The last study is an ongoing collaboration between M. D. Cooper, J. F. Kearney and A. R. Lawton.

## MATERIALS AND METHODS

**Labeling and digestion of surface Ig.** Cell suspensions were prepared and labeled externally with $^{125}I$ or fluorescent antibodies. Radioactive cell-surface Ig was precipitated from Nonidet P-40 lysates with antibody and characterized by SDS gel electrophoresis using an internal marker of $^{131}I$-labeled proteins.

For the digestion experiments, trypsin was added (final conc., 40 $\mu$g/ml) to lysates of radiolabeled cells for various times at room temperature or 37°C. At the end of the incubation period, the sample was adjusted to contain a fivefold excess of soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 10 mM iodoacetamide. The proteolytic cleavage products of surface immunoglobulin were isolated using goat anti-rabbit IgG and sequential additions of rabbit antibodies to mouse K chain, $\mu$ chain and $\delta$ chain. Precipitates were then submitted to SDS gel analysis with or without a prior reduction step. In some experiments, and in order to reduce the complexity of gel patterns, radioactive cells lysates were passed through Sepharose-linked anti-$\mu$ or anti-$\delta$ prior to treatment with trypsin.

These procedures have already been described in detail elsewhere (Abney and Parkhouse 1974; Abney et al. 1976a; Parkhouse et al. 1976).

*Memory cells.* We used an anti-DNP clone (clone 5A of Klaus and Willcox 1975) 9–15 months after its original isolation. The clone was raised in CBA mice against DNP-ovalbumin by the procedure of Askonas et al. (1970) and consisted of three main spectrotypes, as determined by the method of Phillips and Dresser (1973); the most acid was IgG1, the adjacent one was IgG2a, and there was a poorly resolved "alkaline" collection which was largely IgG2a.

*Transfer and inhibition of the clonal response.* Washed spleen-cell suspensions from clonal cell donors ($40 \times 10^6$ cells per ml) were incubated for 20 minutes at room temperature with rabbit anti-mouse Ig antisera of various specificities in HEPES-buffered Eagle's medium containing 5% fetal calf serum (FCS). They were washed twice and then incubated for 2 hours at 37°C so as to give maximal shedding or endocytosis of capped surface Ig. The cells were then pelleted, washed once, cooled in an ice bath, and counted. They were then adjusted to the same cell concentration. Viabilities were also checked and were always above 90%. Samples were taken before and after the 37°C incubation and stained with rhodamine-conjugated goat anti-rabbit IgG to confirm the absence of cell-surface Ig. All class-specific rabbit antisera gave a strong precipitin line when tested against homologous Ig at 1 mg/ml by Ouchterlony analysis. They also brilliantly stained relevant, but not irrelevant, mouse plasma cell tumors. When incubated with mouse lymphocytes, the antisera were diluted so that the final concentration of antibody was 50–150 $\mu$g/ml.

The clonal cells ($10–15 \times 10^6$ cells per ml) were next incubated with $DNP_4$ tetanus toxoid (tet.tox.), 20 $\mu$g/ml, for 1 hour at 4°C in 10% FCS in 199 medium. They were then washed twice, and $2.5 \times 10^6$ were mixed with $10 \times 10^6$ washed spleen cells from mice immunized with tet.tox. and transferred intraperitoneally (i.p.) to recipients that had been irradiated (600 rad) 6 hours previously. The response was measured by isoelectric focusing and Farr methods. The carrier (tet.tox.)-primed mice were immunized with 7 L.f. alum-precipitated tet.tox., boosted 4 months later with 0.7 L.f. aqueous tet.tox., and used 1–3 months later. All of these procedures have been described in detail elsewhere (Abney et al. 1976b). In previous work, the cell type responsible for transferring responsiveness after binding the antigen was shown to be the B cell, since it was depleted by neither anti-Thy-1 serum and complement, cotton wool columns, nor carbonyl iron. The proportion of dead cells in the suspension also made very little difference, although much of the bound antigen is on dead cells.

## RESULTS

### Enzyme Digestion of Surface Ig: Similarity between Human IgD and Its Mouse Analog

These experiments were suggested by the unique susceptibility of human IgD to trypsin. Although unlike other Ig classes, human IgD is hydrolyzed to Fc and Fab very rapidly, further incubation with the enzyme results in degradation of Fab but not Fc. Moreover, the Fab fragment is degraded to a molecule consisting essentially of $V_H$ and $C_L$, which are disulfide-linked (Jefferis 1975).

Digestion of mouse IgD with trypsin follows a similar course. After 10 minutes at room temperature, the IgD is almost entirely cleaved to Fc and Fab. Surface IgM, on the other hand, is not degraded by this and even longer (1-hr) periods of digestion at room temperature. After 5 hours at 37°C, however, IgM is converted to $Fab_2$, Fab, and Fc. Under similar prolonged digestion conditions, most of the IgD Fc is undegraded. The IgD Fab, on the other hand, is partly degraded to give a heavy-chain fragment of molecular weight 15,000, which, by analogy with the data of Jefferis (1975), we assume to be an extended δ-chain $V_H$. These studies clearly confirm the immunoglobulin nature of the molecule tentatively identified as IgD in the mouse. In addition, the striking similarity in susceptibility to trypsin of human IgD and its murine counterpart is circumstantial evidence for a structural homology.

### Receptors on Memory Cells

As discussed in the introduction, the paucity of lymphocytes with surface IgG contrasts with the high number of cells bearing IgD and IgM. This, and the fact that IgM precedes IgD in ontogeny, has led to speculation that the precursors of some (or all) of the IgG-producing cells may have IgD receptors, although their ultimate origin is probably from IgM-bearing cells. A convenient way to examine the heavy-chain class of memory B cells is the selected antibody-forming cell-cloning system (Askonas et al. 1970). Such clones have the advantage thay they produce the same antibody species for many months (in response to antigen), which contrasts sharply with the heterogeneity and variability of conventional responses. We have therefore measured the responses elicited by brief exposure of the cells to antigen in vitro. Preincubation with appropriate anti-Ig, followed by capping and stripping, was done in order to selectively modulate a given Ig class with minimal, nonspecific effects upon antigen-binding by a second class. Following modulation with anti-Ig and exposure to antigen in vitro, the cells were transferred to irradiated recipients. Although the clone was raised against DNP-ovalbumin, in order to restrict antigen-binding to DNP-reactive cells, the carrier for presentation of DNP in vitro was tetanus toxoid.

Exposure to antigen in vitro boosted the IgG1 and the "alkaline" clones efficiently, whereas the IgG2a clone responded less vigorously (Table 1, Fig. 1). These responses were not affected by pretreatment with anti-HSA serum, whereas polyspecific anti-Fab serum greatly suppressed the responses of all three clones. The effects of specific anti-$\mu$ serum were striking: there was an almost complete suppression of the IgG1, and probably also of the IgG2a, clones, but only a partial inhibition of the alkaline ones.

**Table 1. Blockade of Clonal Receptors with Anti-$\mu$ and Anti-Fab Antisera**

| Group | Clonal cells[a] | Pretreatment | Antigen in vitro | Anti-DNP antibody responses: day 10 ABC[b] | day 27 ABC[b] | day 34 I.E |
|---|---|---|---|---|---|---|
| 4 | spleen | – | + | 39.2 (1.2) | 590 (1.65) | 4/4 |
| 1 | spleen | anti-HSA | + | 63.7 (1.7) | 473 (2.8) | 3/4 |
| 2 | spleen | anti-$\mu$ | + | 4.7 (1.35) | 99.5 (1.5) | 4/4 |
| 3 | spleen | anti-Fab | + | 0.8 (1.9) | 46.6 (2.2) | 4/4 |
| 5 | spleen | – | – | 0.14 (1.7) | 16.6 (1.75) | 3/5 |
| 6 | l. n. | – | + | 7.9 (2.6) | 419 (3.9) | 3/3 |
| 7 | l. n. | – | – | 0.1 (1.0) | 16.9 (2.1) | 3/4 |

*Protocol:* Spleen or lymph-node cell suspensions from third transfer generation clonal donors were preincubated with the appropri antisera under capping conditions, washed, and then incubated at 37°C for 2 hr. They were then washed and incubated at 4°C with 20 $\mu$g DNP, tet.tox. for 1 hr and washed again. They were then mixed with spleen cells from tet.tox-primed mice (10$^7$ per recipient) and tr ferred i.p. to irradiated mice, which were bled on days 10 and 19. They were boosted with 1 $\mu$g aqueous DNP tet.tox. on day 20 and

[a] Clonal cells: $2.5 \times 10^6$ spleen; $2.5 \times 10^6$ l. n. (lymph node).

[b] ABC (antigen-binding capacities) of recipients' sera measured in Farr assay versus D-[$^{125}$I]-P-DNP-Lys. ABC (mM/ml $\times 10^{-6}$) expres as geometric mean (± standard deviation).

[c] (Number of mice)/(total in group) showing IgG1 and IgG2a clones on isoelectric focusing (I.E.F.).

The titers of anti-DNP antibody correlated well with the isoelectric focusing (I.E.F.) patterns (Table 1). The overall suppression after treatment with anti-$\mu$ serum was about tenfold, but the effect on the IgG1 clone was still greater: if we compare undiluted sera in this group with sera of the positive control groups diluted 1:7, there is still a large difference in hapten-binding intensity on I.E.F. (see Fig. 1). Evidently there was a selective effect of the anti-$\mu$ serum on the IgG1 clone.

These antisera almost certainly acted by blocking or removing receptors and not by crudely killing B cells; when the recipients in this experiment were boosted with antigen in vivo on day 20, the anti-Ig and anti-$\mu$ pretreated groups responded even better than the controls initially given no antigen at all (group 5, Table 1). This confirms that the antisera cannot have killed all the clonal cells. It also suggests that the antigen in vitro may have given a very limited stimulus even to the anti-Ig pretreated cells, as these eventually responded more strongly than group 5. In other words, the blockade may not have been complete.

Finally, the strong IgG1 clonal response of the lymph node cells to the in vitro boost (Fig. 1, Table 1) makes it unlikely that macrophages were playing an essential role in binding the antigen in vitro, since such cell suspensions contain low numbers of macrophages.

In other experiments, cells were preincubated with anti-$\gamma_1$, anti-$\gamma_{2a}$, and anti-$\delta$ antisera. In this case, however, only the polyspecific anti-Fab and the anti-$\mu$ sera blocked; again, the effect of the anti-$\mu$ was selectively on the IgG1 and IgG2a clones, whereas that of the polyspecific anti-Fab was nonselective.

Separate aliquots of the anti-$\mu$ and anti-Fab pretreated cells were allowed to resynthesize their receptors in vivo before they were exposed to antigen; antigen challenge was delayed until two days after transfer in these groups. As expected, neither the anti-$\mu$ nor the anti-Fab had any effect in this situation, confirming that the antisera were not simply killing all the cells. As a further confirmation of the specificity of action of the anti-$\mu$ serum, some of it was absorbed on an IgM-Sepharose column; after that, it no longer blocked the IgG1 clonal response significantly.

### Immunoglobulin Class Distribution

In collaboration with M. D. Cooper, J. F. Kearney and A. R. Lawton, we have begun a systematic study of heavy-chain class expression in the mouse. Several major points worthy of comment have already emerged.

First, and as might have been expected from the already existing data, IgD-bearing lymphocytes must arise from cells expressing surface IgM. This conclusion is derived from the absence of IgD-positive cells in mice treated from birth with anti-$\mu$-chain antibodies. In normal mice, however, lymphocytes with IgD, as well as those with IgG and IgA, begin to appear in the first week after birth, whereas IgM is detectable in the 16–17-day fetus. Unlike all other Ig classes, plasma cells containing IgD could not be found after an extensive search in normal and lipopolysaccharide (LPS)-activated mouse lymphoid tissue. This finding, while not defining an exact role, emphasizes the unique association of IgD with the lymphocyte membrane. Furthermore, upon stimulation of B lymphocytes with LPS, IgD disappears from the membrane; meanwhile, for an individual cell, the Ig located in the cytoplasm, be it IgM or IgG, is also displayed on the cell membrane.

Perhaps the most interesting results relate to the many cells with at least two different classes expressed in the membrane. It is by analysis of these cell types that the ontogeny of surface Ig will probably be defined and understood. At present, it is clear than in addition to IgD, cells expressing IgG and IgA are derived from precursors bearing IgM (reviewed in Lawton et al. 1975). The question to be considered, therefore, is the developmental sequence among all these classes at the level of the lymphocyte and its progeny. One interesting point is that surface IgG is invariably associated with IgM in young mice but can occur together with IgD in older mice. Thus IgG- and IgA-bearing cells may well develop directly from cells bearing IgM, but, under certain conditions, there may be an intervening cell type expressing IgD.

## DISCUSSION

At the outset we should confess that the role of IgD in lymphocyte responses remains undefined. Indeed, the very experiment designed to demonstrate a role for IgD, far from achieving this, instead implicated IgM as the functional receptor for antigen on memory B lymphocytes. Furthermore, in a series of experiments of the "try it and see" variety, our antibody to IgD failed to influence the fate of normal or antigen-stimulated spleen cell cultures (R. Maizels, unpubl.).

An extremely crucial point was the observation that, in young mice, IgG-bearing lymphocytes also have surface IgM, but not IgD. Although lymphocytes of older mice can have IgG and IgD coexisting on their membranes, it seems as if the switch from IgM to IgG does not necessarily proceed via a cell that expresses IgD. At least this observation can be reconciled with the fact that anti-$\mu$, but not anti-$\delta$, inhibited the antigen-stimulated differentiation of memory cells to IgG-secreting cells.

It is possible that the sequence of $C_H$-gene expression on lymphocyte surfaces is influenced by antigen. For example, the switch to IgG is via IgD when antigen-driven, but the necessity to express IgD is by-passed in antigen-independent differentia-

Let us now consider the experiments done in the cloned memory-cell system in more detail. The basic observation of inhibition with anti-$\mu$ argues strongly that receptors of the IgM class are maintained on at least some memory cells committed to differentiate into IgG1 and IgG2a secretion. The lack of effect of anti-$\gamma_1$, anti-$\gamma_2$, and anti-$\delta$ is also striking and points to the same conclusion. Our evidence cannot rule out a minority population of IgG or IgD receptors in addition to the IgM, but the completeness of the suppression by anti-$\mu$ indicates that most of the functional receptor was IgM. If the clones we have studied are representative, we can reasonably conclude either that memory B cells have no receptor IgD at all, or that it does not mediate their reactivation. Therefore, our results clearly rule out any simplistic model in which IgD is uniquely associated with antigenic triggering, whereas tolerance is induced via IgM receptors (Vitetta and Uhr 1975).

However, not every clone necessarily behaves like the IgG1 and IgGa clones, since a fraction of the alkaline clones clearly behaved differently. They were apparently only partially suppressed by anti-$\mu$ pretreatment, whereas they were much more completely and consistently suppressed by the anti-Fab serum. Hence some of their memory-cell receptors cannot have been IgM. If they were indeed a mixture of several clones, bearing Ig of different classes and subclasses, an antiserum to any one of these by itself would not have given a significant suppression. To achieve that, one would need a *combination* of anti-$\gamma$ or anti-$\delta$ antisera *plus* anti-$\mu$.

Heterogeneity among B cells may help to resolve the apparent disagreement between other results (Walters and Wigzell 1970; Herzenberg et al., this volume) and our own. It is possible that, in these other systems, a minority subpopulation could have been overlooked or, conversely, constituted the major subject of the study. For example, recent priming can elicit a more terminally differentiated memory B cell with class restricted receptors and a short life-span (Pierce et al. 1972a,b; North and Askonas 1976). This type of cell could have been continually generated in the experiments of Walters and Wigzell, where priming of memory cells was effected by giving antigen in a depot of adjuvant.

These considerations raise a major point that we would like to stress: there is likely to be more than one type of memory B cell, and the developmental sequence between antigen-triggered cells and their secreting progeny probably contains cells expressing a variety of Ig classes on their membranes. In the face of this, it seems absurd to be dogmatic and insist on Ig class restriction for all antigen-sensitive lymphocytes. More important is to define the control and evolution of Ig expression in the population, an objective which is feasible through experimental manipulation of selected antibody-forming cell clones.

## CONCLUSIONS

Evidence for a structural homology between human IgD and its presumed mouse counterpart has been provided by the remarkably similar profiles of fragments resulting from tryptic digestion of the human and mouse proteins. More definitive homology awaits sequence determination.

The memory cells of two antibody-forming cell clones had receptors of the IgM class, even though the clones had been producing IgG1 or IgG2a anti-DNP antibodies for 9–15 months previously (on antigenic stimulation). Thus a phenotypic switch in heavy-chain constant region evidently occurred after reexposure of these memory cells to antigen. These conclusions were drawn because removal of IgM, but not IgG or IgD, from the memory-cell population inhibited antigen recognition and subsequent differentiation to IgG-secreting cells. Thus surface IgM cannot be solely responsible for tolerance induction; neither can IgD be the unique triggering receptor for antigen on memory B cells. Instead, there may be a heterogeneity of Ig receptors on memory (and other) B lymphocytes which reflects the presence of subpopulations arising in the presence and absence of antigenic selection. Preliminary evidence does suggest that the immediate precursors of IgG-bearing cells may express either IgM or IgD. Thus IgG expression can occur independently of IgD, which would be in accord with our finding that at least some memory B cells recognize antigen via surface IgM. The details of the control and evolution of Ig expression remain to be elucidated, as does a defined role for IgD. The cells which bear IgD, however, are certainly derived from IgM-positive precursor lymphocytes, and the high frequency of lymphocytes expressing two Ig classes has profound implications for the mechanism of V-C gene integration.

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gure 1. Blockade of clonal receptors with anti-$\mu$ and anti-Fab antisera. Isoelectric focusing patterns of anti-DNP antiies of 18-day sera of recipients in the experiment summarized in Table 1. Individual sera were loaded, at the dilutions own (undiluted or 1/7), on pH 4–8 gels, in the arrowed positions. After focusing, the gels were overlaid with D-[$^{131}$I]-*P*-NP-Lys, washed, and autoradiographed, to reveal anti-DNP antibodies. Protocol and group numbers are the same as in able 1.

$\alpha$-HSA, $\alpha$-$\mu$, and $\alpha$-Fab refer to pretreatment of clonal cells with anti-HSA, anti-$\mu$, and anti-Fab sera, respectively. Lymph node cells (l.n.) and spleen cells are both from clonal donors; marker serum (M) is from third transfer generation one-bearing mouse.

# MOUSE IMMUNOGLOBULIN RECEPTORS ON LYMPHOCYTES : IDENTIFICATION OF IgMs AND IgD MOLECULES BY TRYPTIC CLEAVAGE AND A POSTULATED ROLE FOR CELL SURFACE IgD

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## INTRODUCTION

Studies of mouse B-lymphocyte receptors by isotopic labelling techniques showed little, if any, IgA or IgG. Instead, the surface immunoglobulin (Ig) consists of monomeric IgM (IgMs), and an Ig which has been described as the murine equivalent of the human IgD[1,2,3,4]. In order to confirm this assumption, we compared, in the present paper, the susceptibility to tryptic digestion, and the tryptic products of the surface Ig, to those of human immunoglobulins. These experiments were suggested by the unique susceptibility of human IgD to trypsin[5]. Unlike other classes, human IgD is hydrolysed to Fc and Fab very rapidly; further incubation with the enzyme results in degradation of Fab, but not Fc. Furthermore the Fab fragment is degraded to a molecule consisting essentially of $V_H$ and $C_L$ which are disulphide-linked. The striking similarity in susceptibility to trypsin of human IgD and its murine counterpart that was observed is circumstantial evidence for a structural homology. In addition, the extremely rapid enzyme hydrolysis of the murine IgD equivalent ("IgD") suggests a possible unique role for cell surface IgD, namely the elicitation of anti-idiotype responses which play a crucial controlling role in the genesis of the immune response.

## 2. MATERIALS AND METHODS

### 2.1 Preparation of iodinated membrane lysate

Spleen cell suspensions of 4 - 6 week old CBA mice were labelled with $^{125}I$ by the lactoperoxydase catalysed procedure[6], washed once in phosphate buffered saline (PBS) and then lysed for 10 minutes at 0°C in 1% (w/v) Nonidet P40 (Shell) in PBS. This lysate was passed over Sephadex G25 equilibrated with the same solution and then centrifuged (12,000g x 10min

### 2.2 Removal of IgMs or "IgD" molecules

The "IgD" molecules were removed by specific precipitation with a purified immunoglobulin fraction of rabbit anti mouse δ chain[4] followed by goat immunoglobulins (purified fraction) anti rabbit immunoglobulins (the respective final concentrations were .2 mg/ml and 2 mg/ml).

Alternatively, IgM molecules were removed prior to trypsin treatment by passage of the radioactive cell lysate through a column of purified rabbit anti mouse μ chain antibodies coupled to sepharose 4B[7].

### 2.3 Trypsination and isolation of the tryptic fragments

To the IgD or IgM-depleted lysate was added TPCK (L-1-tosyl-amido-2 phenyl-ethyl chloromethyl ketone) treated Trypsin (Worthington) (final concentration of .1 mg/ml). After the times and temperatures indicated, soy bean trypsin inhibitor, phenylmethyl-sulfonyl fluoride and iodoacetamide were added to final concentrations respectively of 1 mg/ml, 2 mM and 20 mM. The sample was centrifuged (12,000g x 10 min) and tryptic products were isolated by precipitation with specific rabbit antisera (anti-μ chain, anti-δ chain or anti-κ chain) and goat anti-rabbit Igserum. The precipitates were washed thre times with ice cold 0.5% (w/v) Nonidet P40 in PBS, once with PBS and then dissolved in 50mM sodium phosphate, pH 7,- 2% (w/v) sodium dodecyl sulphate.

.4 Analysis of the tryptic fragments

Direct analysis was performed in 4.5% (w/v) polyacrylamide gel containing 0.1% (w/v) sodium dodecyl sulphate[8]. Alternatively samples were reduced in 2 mM dithioerythritol - 50 mM sodium phosphate, pH 7 - 2% (w/v) sodium dodecyl sulphate (Sigma) at 100°C for 10 min, alkylated by addition of M iodoacetamide to 100 mM and then resolved on 7.5% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS. Various internal $^{131}I$-labelled[9] markers were added: A = unreduced mouse Ig (150,000); B = reduced transferrin (80,000); C = reduced bovine serum albumin (68,000); D = reduced creatine phosphokinase (40,000); E = reduced human myeloma λ chain (24,000); F = reduced cytochrome C (12,000). After electrophoresis the gels were sliced into 1 mm segments and radioactivity was determined. Values were corrected for cross-channel spill and plotted with the top of the gel on the left hand side of the figure. The proteins used for molecular weight standards were reduced by treatment with 2 mM dithioerythritol in 50 mM sodium phosphate, pH 7 - 2% (w/v) sodium dodecyl sulphate (10 min, 100°) and then alkylated by addition of M iodoacetamide to 100 mM.

## 3. RESULTS

### 3.1 Cleavage of surface IgM

At room temperature even 5 hr incubation with trypsin resulted in little or no conversion of IgMs to Fc and Fab, and at 37°C several hours (3 - 5 hr) were necessary to complete the tryptic cleavage of IgMs. Digestion for 5 hr yielded three fragments, designated $M_1$, $M_2$ and $M_3$. Fragment $M_1$ was precipitated with $M_2$ by an anti L chain serum (figure 1a), and with $M_3$ by anti μ chain serum (figure 1b). Using the supernatant of the anti μ chain precipitation, it was possible to precipitate $M_2$ alone by the anti L chain serum. The reduction products of the isolated $M_2$ were MR2 and MR4 (figure 1e). Similarly $M_3$ alone was precipitated by the anti μ chain serum from the anti L supernatant. Reduction of the isolated $M_3$ yielded fragment MR3 (figure 1f). It was then possible to deduce that the reduction products of $M_1$ are MR1 and MR4 (figure 1b and 1d). Determination of the molecular weight by using internal markers, and the specific recognition by the anti L chain and anti μ chain sera, allowed an identification of the fragments by comparison with the tryptic products of human IgM[10] (Table I). Thus $M_1$, $M_2$ and $M_3$ were $F(ab')_{2\mu}$, Fabμ and Fcμ respectively (nomenclature from ref. 22).

### 3.2 Cleavage of surface IgD

The surface IgD was, on the other hand, markedly susceptible to tryptic digestion. An 80% conversion to Fab and Fc occurred in 10 min at 0°, and 10 min at 25° was sufficient for complete digestion, producing fragments $D_1$ (figure 2a) and $D_2$ (figure 2c), which were precipitated by an anti-L chain and an anti-H chain of IgD ("δ ") respectively. Thus

$D_1$ and $D_2$ are Fab$\delta$ and Fc$\delta$ respeccctively. The reduction products of Fab ($D_1$) were DR1 (Fd$\delta$) and DR3 (L chain), but when cleavage was continued for several hours at 25°C an additional fragment (DR4) was obtained (figure 2b). The reduction of $D_2$ produced fragment DR2 (figure 2d). These results, and an estimation of the molecular weight of the fragments, clearly showed a similarity with the results obtained following tryptic cleavage of human IgD[5] (Table I). In addition, fragment DR4 has a molecular weight (15,000) similar to that of a tryptic fragment obtained from human IgD digested for several hours at room temperature[5].

TABLE 1 : Analysis of the tryptic products of mouse immunoglobulin receptors

| Tryptic fragments | Precipitated by [a] | | [b] Molecular weight | Human equivalent | Reduction products | [c] Molecular weight | Human equivalent |
|---|---|---|---|---|---|---|---|
| | α L chain | α H chain | | | | | |
| M1 | + | + | 125,000 | $(Fab')_{2\mu}$ [d] | MR1<br>MR4 | 44,000<br>23,000 | Fd'μ<br>L |
| M2 | + | - | 50,000 | Fab μ [d] | MR2<br>MR4 | 34,000<br>23,000 | Fd μ<br>L |
| M3 | - | + | 50,000 | Fc μ [d] | MR3 | 27,000 | reduced Fc μ |
| D1 | + | - | 55,000 | Fab δ [e] | DR1<br>DR3 | 33,000<br>22,000 | Fdδ<br>L |
| D2 | - | + | 55,000 | Fc δ [e] | DR2 | 28,000 | reduced Fcδ |

a) An anti μ chain is used for fragments M1, M2 and M3 and an anti δ chain for fragments D1 and D2. These sera do not recognise the $V_H$ and $C_H1$ domains

b) Molecular weights determined in 4.5% SDS polyacrylamide gels with a precision of 10%

c) Molecular weights determined in 7.5% SDS polyacrylamide gels with a precision of 5%

d) Human IgM tryptic fragments obtained at 37°C Nomenclature for $(Fab')_{2\mu}$ and $Fab_\mu$ from refer. 22

e) Human IgD tryptic fragments obtained at room temperature

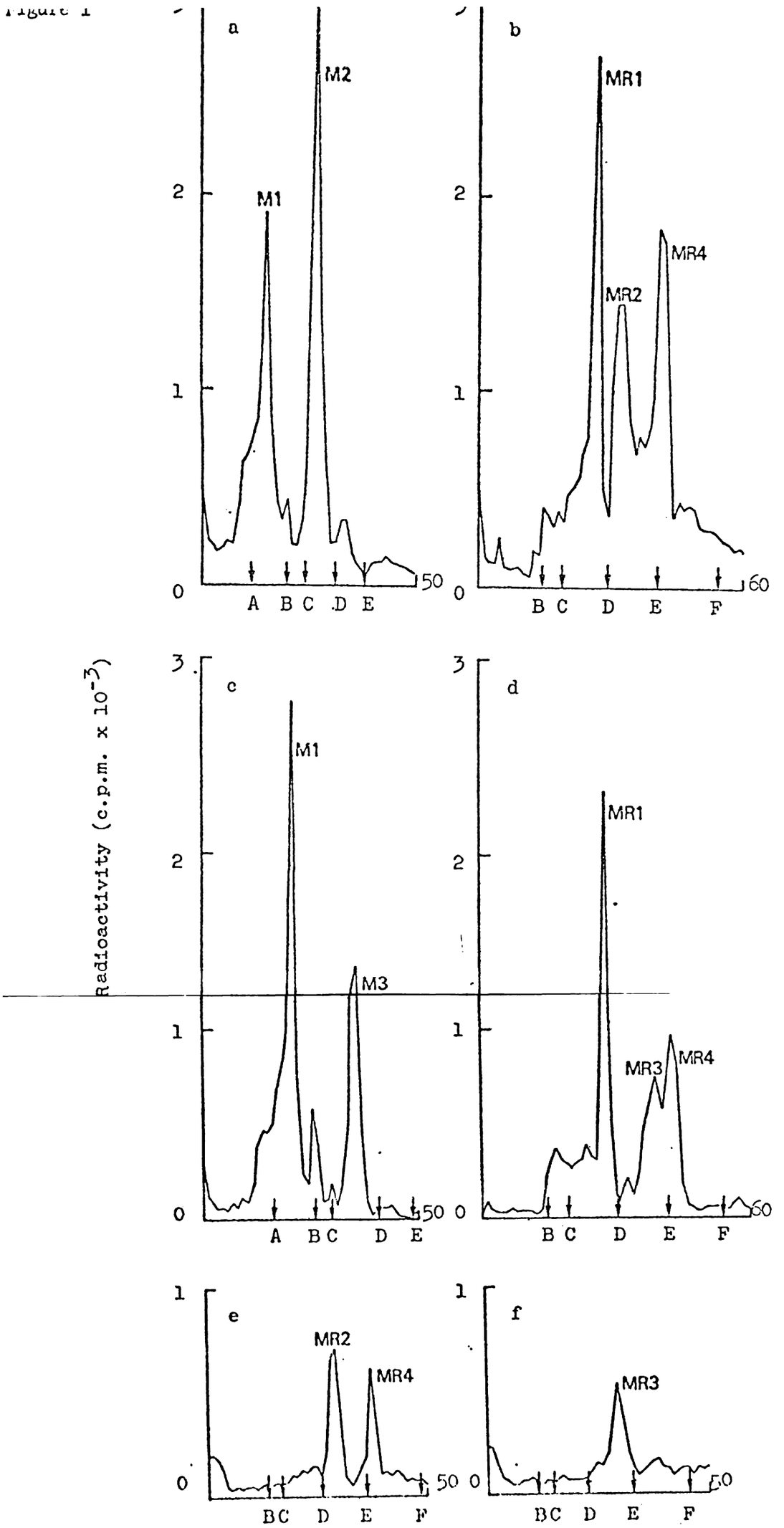
## 4. DISCUSSION

Different fragments were released from mouse IgM and "IgD" molecules by tryptic cleavage and a considerably higher proteolytic susceptibility of the "IgD" was observed: in 10 minutes an 80% split at 0°C, and a complete cleavage at 25°C were obtained, whereas to cleave $IgM_s$, digestion for several hours at 37°C was necessary. The tryptic susceptibility of the mouse $IgM_s$ and "IgD" is comparable to that of human IgM[10] and IgD[5] respectively and the fragments released have a similar molecular weight in both species (except for the pentameric Fc released from the 19S human IgM, which has a proportionally higher molecular weight). Therefore, the tryptic digestion patterns obtained are those expected from $IgM_s$ and IgD molecules, and are different from those of IgG, IgA and IgE molecules[11,12,13]. It is worth emphasising that under prolonged digestion conditions the IgD Fab is partly degraded to give a heavy chain fragment of molecular weight 15,000, which by analogy with the data of Jefferis for human IgD[5], we assume to be an extended $\delta$-chain $V_H$ fragment. remarkably similar profile of tryptic digestion fragments from human IgD and its presumed human counterpart is, therefore, evidence for a structural homology.

Our results emphasise the exceptional susceptibility of IgD molecules to be cleaved into Fab and Fc fragments, and it is reasonable to propose that the biochemical properties of the IgD and IgM immunoglobulins are related to their respective roles. It has been proposed[14] that proteolysis of IgD on the cell occurs after interaction with the antigen (Ag) and produces the exposure on the Fc fragment, of a site necessary for cellular triggering. An alternative hypothesis is that the Fab-Ag

complex liberated by the cleavage plays the main role. T-cells could recognise not only the antigen on this complex, but also the idiotypic determinants carried by the Fab fragment. Then the effector molecule (activating or suppressing) eventually produced by the T-cells would recognise the cells bearing those idiotypes. This allows recognition of a cell able to produce a specific antibody not only by its antibody properties, but also by its antigenic (idiotypic) determinants. Such a system must favour the synthesis of antibodies with cross-reacting idiotypes, even if those molecules are directed toward different determinants of the same antigen, or are not specific for the antigen. Cazenave and colleagues found such molecules in the serum[15,16,17] and on lymphocytes[18]. It has been suggested[19,20,21] that IgD and IgM on the surface of one cell bear the same variable regions. It is then conceivable that while the IgD allows the production ofthe Fab-Ag complex, the IgM maintains the presence on the cell surface of the idiotypes recognised by the T-cell factor. The major role of IgD then would be the elicitation of a regulatory anti-idiotype response.

Figure 1


a
M1
M2
b
MR1
MR2
MR4
c
M1
M3
d
MR1
MR3
MR4
e
MR2
MR4
f
MR3
Radioactivity (c.p.m. x 10⁻³)
0
1
2
3
50
60
A B C D E
B C D E F
BC D E F

LEGEND TO FIGURE 1

Tryptic products of mouse surface IgM. Spleen cell suspensions were labelled with $^{125}I$ by the lactoperoxidase procedure and the surface IgD was removed by precipitation with rabbit anti-mouse IgD and goat anti-rabbit IgG. The remaining IgM was digested by trypsin (5 hr, 37°C). Tryptic products were then isolated by specific precipitation using anti-L or H-chain antisera and analysed by polyacrylamide gel electrophoresis. Samples were analysed unreduced on 4.5% (w/v) gels (a and c) or reduced on 7.5% (w/v) gels (b, d, e and f). Internal $^{131}I$-labelled markers were included with the test sample and are indicated on the figure by the arrows (A, unreduced mouse IgG, mol.wt. 150,000; B, reduced transferrin mol.wt. 80,000; C, reduced bovine serum albumin, mol.wt. 68,000; D, reduced creatine phosphokinase, mol.wt. 40,000; E, reduced human myeloma $\lambda$ chain, mol.wt. 24,000; F, reduced cytochrome C, mol.wt. 12,000.) Other details as in materials and methods.

a) $IgM_s$ tryptic products precipitated at 0°C from 200 µl of lysate (from $10^7$ cells), by addition of rabbit anti mouse L chain serum (10 µl) and goat anti rabbit Ig serum (100 µl).

b) Reduction of the precipitate described in (a).

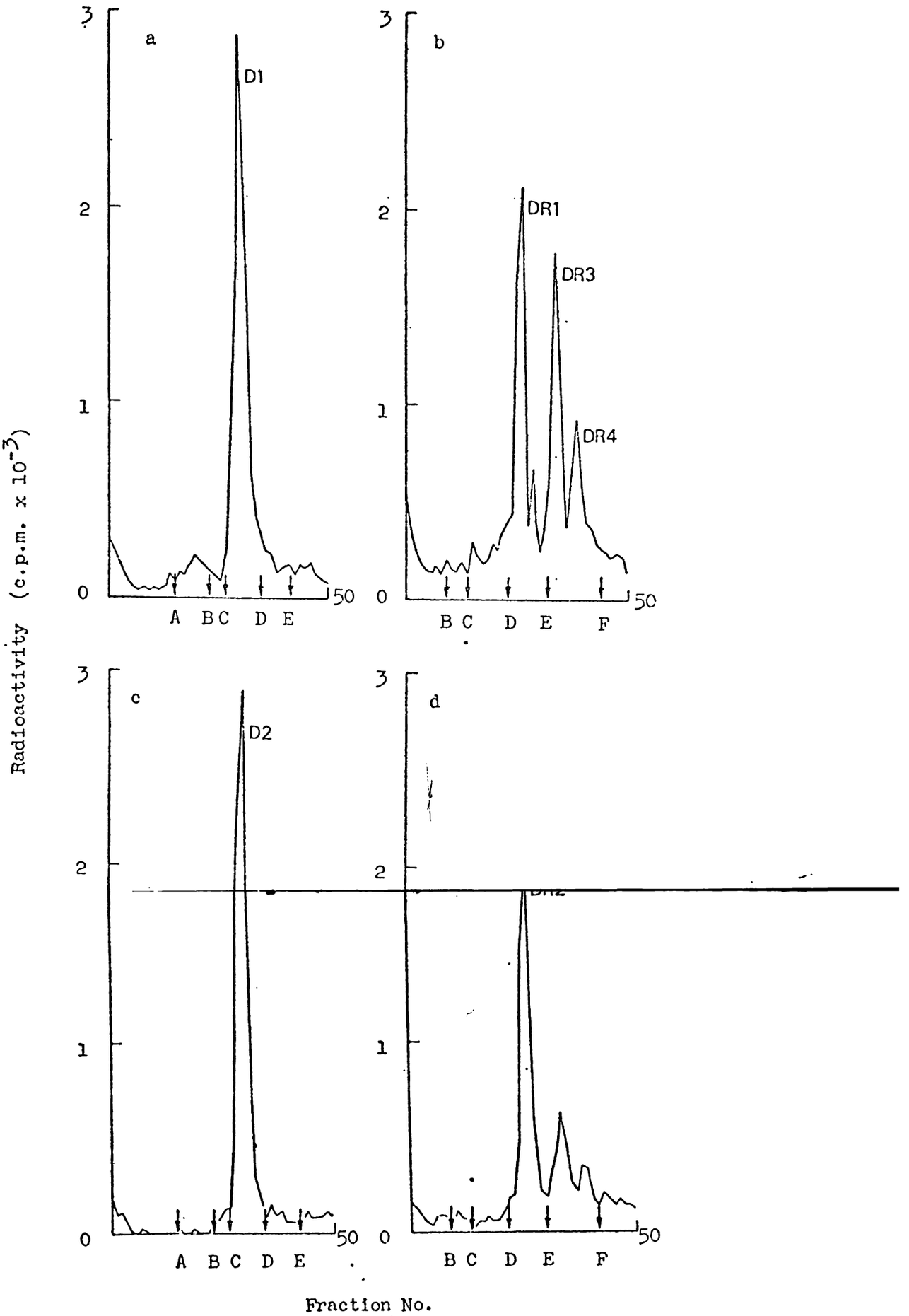
c) $IgM_s$ tryptic products precipitated at 0°C, from 200 µl of lysate, by addition of rabbit anti mouse µ chain serum (10 µl) and goat anti rabbit Ig serum (100 µl).

d) Reduction of the precipitate described in (c).

e) Reduction of the isolated M2 fragment.

f) Reduction of the isolated M3 fragment.

Figure 2


a
D1
b
DR1
DR3
DR4
c
D2
d
A B C D E
B C D E F
Radioactivity (c.p.m. x 10⁻³)
Fraction No.

LEGEND TO FIGURE 2

Tryptic products of surface "IgD". Procedure as described in Figure 1 with the following modifications; the "IgD" molecules were not removed. Instead, the IgM molecules were removed prior to trypsin treatment by passage of the radioactive cell lysate through a column of purified anti mouse μ chain antibodies coupled to Sepharose 4B[7]. The tryptic digestion was performed at 25°C for 5 hours. Samples were analysed unreduced on 4.5% (w/v) gels (a and c) or reduced on 7.5% (w/v) gels (b and d). Other details as in Materials and Methods and the legend to figure 1.

a) "IgD" tryptic products precipitated at 0°C, from 200 μl of lysate by addition of rabbit anti mouse L chain serum (10 μl) and goat anti rabbit Ig serum (100 μl).

b) Reduction of the precipitate described in (a).

c) "IgD" tryptic products precipitated at 0°C, from 200 μl of lysate by addition of rabbit anti mouse "δ" chain serum[4] (10 μl) and goat anti rabbit Ig serum (100 μl).

d) Reduction of the precipitate described in (c).

SUMMARY

The two mouse immunoglobulin receptors (IgM and IgD-like) were individually digested by trypsin. Their tryptic susceptibility, and the products released, were similar to those of their human counterparts. Evidence for a structural homology between human IgD and its presumed mouse counterpart has been provided by the remarkably similar profile of fragments resulting from digestion. More definitive homology awaits sequence determination. The extreme susceptibility of surface IgD to proteolysis contrasted with the resistance of surface IgM. We therefore propose that the major role of IgD is to release a fragment (Fab$\delta$ ) following exposure to antigen and then elicit a regulatory anti-idiotype response which acts through recognition of the protease resistant IgM idiotype remaining on the cell surface.

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Ref iv

# Heterogeneity of surface immunoglobulin on murine B lymphocytes

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**Summary.** Surface IgM on murine splenic lymphocytes was capped using a rhodamine-coupled anti-$\mu$ chain system. The cells were then reacted with a polyspecific, fluorescein-coupled anti-mouse immunoglobulin (Ig) under non-capping conditions. A large number (31–41 per cent of total Ig-bearing cells) which gave red caps with anti-$\mu$ subsequently stained green peripherally with anti-Fab under the non-capping conditions. The remaining stained cells were divided between those showing only red caps (17–29 per cent of Ig-bearing cells) and those showing only green rings (37–45 per cent of Ig-bearing cells). These data are interpreted by assuming that most of the staining with the second reagent (fluorescein-coupled anti-mouse Ig) is due to the presence of surface associated IgD.

## INTRODUCTION

The majority of normal (Knapp, Bolhuis, Rádl and Hijmans, 1973; Rowe, Hug, Forni and Pernis, 1973) and leukaemic human B lymphocytes (Fu, Winchester and Kunkel, 1974; Kubo, Grey and Pirofsky, 1974) simultaneously bear both IgM and IgD on their surfaces. There are in addition some cells which appear to be restricted to either IgM or IgD.

Recently, we (Abney and Parkhouse, 1974) and others (Melcher, Vitetta, McWilliams, Lamm, Philips-Quagliata and Uhr, 1974; Vitetta, Melcher, McWilliams, Lamm, Philips-Quagliata and Uhr, 1975) have provided evidence for the presence of an immunoglobulin similar to IgD on the surface of mouse B lymphocytes. By surface labelling of splenic lymphocytes with radioactive iodine and subsequent immunochemical procedures, this candidate for IgD in the mouse was found to account for 40 per cent of the total recoverable surface immunoglobulin. The remainder was IgM, and IgG was never found in detectable amounts (Abney and Parkhouse, 1974; Melcher *et al.*, 1974; Vitetta *et al.*, 1975). An obvious question is the distribution of these two immunoglobulin classes on individual B lymphocytes. In order to answer the question, in the absence of a specific antibody to murine IgD, we have based our strategy on the documented absence of immunoglobulins other than IgM and IgD on the surface of murine B lymphocytes (Abney and Parkhouse, 1974; Melcher *et al.*, 1974; Vitetta *et al.*, 1975). We are aware that claims have been made for the presence of IgG on the surface of B lymphocytes in the mouse, but the frequency of such cells is low. We do not exclude the possibility of very low numbers of IgG-bearing cells, nor the presence of very small amounts of surface IgG. These possibilities, however, will not influence the experimental approach.

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## MATERIALS AND METHODS

*Preparation of antisera and fluorescent conjugates*

Rabbits were immunized with purified MOPC 104E IgM ($\mu\lambda$1) (Parkhouse and Askonas, 1969)

Fab portion of myeloma protein Adj PC5 (Knopf, Parkhouse and Lennox, 1967) as ısly described (Parkhouse and Askonas, The anti-IgM was absorbed by passage ı Sepharose 4B-coupled to a 7S fraction of mouse serum (Axen. Porath and Ernback, On Ouchterlony analysis, the absorbed anti- 104E precipitated MOPC 104E ($\mu\lambda_1$) and 183 ($\mu$K) IgM proteins, but not MOPC 25 Adj PC5 ($\gamma$2aK), MOPC 195 ($\gamma$2bK) or 315 ($\alpha\lambda_2$). In order to exclude traces of ly activity towards immunoglobulin subunits ıan $\mu$ chains, the absorbed anti-MOPC 104E sted by a more sensitive radio immuno- .ation procedure. Myeloma proteins were l (Hunter and Greenwood, 1962) with free $^{125}$I at about 1 $\mu$Ci/$\mu$g. The radioactive (5–10 ng) was mixed with the test antiserum and after 30 min at room temperature, ıt goat anti-rabbit IgG was added to ensure .ation of the added rabbit Ig. When this was he absorbed anti-MOPC 104E was found to ative for reactivity towards IgG1, IgG2a, and IgA myeloma proteins. For brevity, this ım will henceforth be referred to as anti-$\mu$ The anti-Fab reagent was polyspecific, ; against IgM, IgG1, IgG2a, IgG2b and ıt not other serum proteins, on Ouchterlony :. The IgG fraction of both antisera was d, coupled to fluorescein isothiocyanate, and .tes with fluorescein: protein ratios of 2–3:1 lected by chromatography on Whatman DE ıra and Goldstein, 1965).

at antiserum specific for rabbit IgG (Park- ınd Askonas, 1969) was similarly treated to ı rhodamine conjugate. Rabbit antisera for $\alpha$, $\gamma$1, $\gamma$2a and $\gamma$2b heavy chains were d by injection of purified myeloma proteins, ı by absorbtion with appropriate myeloma ; coupled to Sepharose. The sera were as monospecific by the criteria described

*tion and staining of cell suspensions*

cell suspensions from 6-week-old specific :n-free, female CBA mice were prepared in -buffered saline (Oxoid Ltd) containing l of bovine serum albumin (VBS–BSA) and ng cells were stained and prepared for ıtion as described by Raff (1970). Cells were ed for 20 min at room temperature with rabbit anti-$\mu$ chain or rabbit anti-Fab, washed three times in VBS–BSA, and then suspended in purified, rhodamine-coupled goat anti-rabbit IgG (20 min at room temperature). The two antibody layers were used in order to ensure maximum capping. Samples were washed and then examined directly or transferred to VBS–BSA–0·03 M sodium azide-20 per cent (v/v) normal rabbit serum, for treatment with purified, fluorescein-coupled-anti-$\mu$ chain or anti-Fab (20 min at 0°). After three washes with VBS–BSA–0·03 M sodium azide, cells were mounted in VBS–BSA–0·03 M sodium azide, and examined under ultraviolet light with a Leitz Orthoplan research microscope fitted with Ploem illumination. At least 60 stained lymphocytes were assessed for the presence of rhodamine and a fluorescein. All reagents were centrifuged 50,000 *g* for 60 min) on the day of use.

## RESULTS AND DISCUSSION

The experimental design depends on the known fact that IgM and IgD cap independently on human lymphocytes (Knapp *et al.*, 1973; Rowe *et al.*, 1973). The protocol consisted of first capping IgM using a rhodamine-coupled anti-$\mu$ chain system and then reacting the cells with a polyspecific, fluorescein-coupled anti-mouse Ig under non-capping conditions. Since we assume an absence of immunoglobulin other than IgM or IgD, the green rings visualized by the second reagent must represent IgD moieties on the cell surface. To control for capping of all IgM

**Table 1.** Heterogeneity of surface immunoglobulin on murine lymphocytes

| Experiment | Total cells | Cap | Green ring | Red cap–green ring |
|---|---|---|---|---|
| 1 | 240 | 23 (22) | 39 (37) | 43 (41) |
| 2 | 717 | 79 (29) | 109 (40) | 85 (31) |
| 3 | 135 | 11 (17) | 30 (45) | 25 (38) |
| 4 | 318 | 38 (27) | 52 (37) | 49 (35) |

CBA spleen cells were treated with rabbit anti-mouse $\mu$ chain followed by rhodamine-labelled goat anti-rabbit Ig under capping conditions. The cells were then treated with fluorescein-labelled rabbit anti-mouse Fab in the cold and in the presence of azide. Details in the text.

The figures in parentheses express the number of cells observed as a percentage of total stained cells.

molecules in the first step, some cells were treated with fluorescein-coupled anti-$\mu$ chain instead of the polyspecific reagent.

Based on the double-staining protocol outlined above, lymphocytes with surface Ig could be classified into three groups (Table 1). A large number (31–41 per cent of total Ig-bearing cells), which were capped with anti-$\mu$, subsequently stained peripherally with anti-Fab under the non-capping conditions, i.e. surface IgM and IgD simultaneously present. The remaining stained cells were divided between those showing only caps, i.e. surface IgM only (17–29 per cent of Ig-bearing cells) and those showing only green rings, i.e. surface IgD only (37–45 per cent of Ig-bearing cells). Inspection of the doubly stained cells suggested a considerable variation in the relative intensities of the two fluorescent markers, suggesting a variation in the IgM to IgD ratio from cell to cell. Our assumption that peripheral staining with anti-Fab following capping of IgM is due to the presence of IgD, and not IgG, determinants is reinforced by the high numbers of peripherally stained lymphocytes observed. Also, the results agree with our general finding that the numbers of cells staining with anti-$\mu$ (25–30 per cent total splenic lymphocytes) is always less than the numbers of cells staining with anti-Fab or anti-$\kappa$ (35–45 per cent total splenic lymphocytes). Furthermore, staining with specific rabbit antibodies to mouse $\alpha$, $\gamma$1, $\gamma$2a and $\gamma$2b heavy chains revealed very few positive cells (0·5–1 per cent of splenic lymphocytes).

Complete capping of IgM in the first stage was evident since fluorescein-labelled anti-$\mu$ failed to reveal ring staining after the cells had been treated with the anti-$\mu$ under capping conditions.

One possible objection, that the doubled staining is due to interaction of the fluorescein-coupled anti-Fab reagent with Fc receptors, is ruled out by two controls. First, addition of aggregated human IgG (5 mg/ml) failed to inhibit the second staining step with fluorescein-labelled anti-Fab. Secondly, cells capped with anti-Fab and rhodamine-labelled goat anti-IgG were not subsequently stained with the fluorescein-coupled anti-Fab.

Our findings in the mouse therefore, mirror those in the human (Knapp *et al.*, 1973; Rowe *et al.*, 1973). In the mouse, there are fewer cells bearing both IgM and IgD, with correspondingly higher numbers of cells with exclusively IgM or IgD. The biological significance of these subpopulations of B lymphocytes, although at present obscure, may now be revealed in the mouse system. Certainly in the mouse IgM precedes IgD in ontogeny (Abney and Parkhouse, 1974; Vitetta *et al.*, 1975), and thus a developmental sequence is suggested. The simultaneous expression in one cell of two heavy chain classes sharing the same variable regions (Salsano *et al.*, 1974) suggests a copy-choice model for V–C gene integration (Williamson, 1971), and establishes a precedent for an Ig class switch by lymphoid cells.

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# ion and characterisation iserum to the ndidate for immunoglobulin D

of mouse B-lymphocyte surface immunoglobulin topic labelling techniques reveals little, if any, i. Instead, the surface Ig consists of monomeric in Ig similar in physicochemical properties to )[1–3]. In the absence of sequence comparison man IgD and its assumed mouse counterpart, ient is tentative. With this proviso, however, and ce of clarity in presentation, we refer to this bulin as IgD. During development, IgM-bearing e first to arise and the ratio of IgM–IgD deter- labelled adult lymphocytes is higher in the spleen iph nodes or Peyer's patches[1–4]. These findings, ve the total yield of the two Ig classes and not ution on individual lymphocytes. On the basis of of immunoglobulins other than IgM or IgD on the surface of B lymphocytes, we indicated the presence of cells bearing IgM or IgD, or IgM and IgD in mouse spleen cell suspensions[5,6]. In these experiments, IgM was first capped with rhodamine-labelled anti-$\mu$ and subsequent ring staining with fluorescein-labelled anti-Fab in the presence of sodium azide was taken as evidence for the presence of IgD. We report here the preparation of an antibody specific for mouse IgD, and its use in the characterisation of mouse B-lymphocyte surface Ig.

Plasma cell membranes were prepared from the spleens of 1,750 BALB/c mice (wet weight 160 g) to yield 130 mg of protein. The membranes were dissolved in 25 ml of phosphate-buffered saline (PBS) containing 1% (w/v) Nonidet P-40 and 1 mM phenylmethylsulphonyl fluoride, centrifuged at $10^5 g$ for 3 h, and then passed successively through three columns of Sepharose 4B: (1) normal rabbit Ig (6.0 mg)–Sepharose (3.0 ml); (2) rabbit anti-mouse $\mu$ (3.6 mg)–Sepharose (1.8 ml); (3) rabbit anti-mouse Fab (5 mg)–Sepharose (2.5 ml). The rabbit anti-mouse $\mu$ was prepared by applying a specific rabbit anti-$\mu$ serum to IgM ($\lambda^1$) (MOPC 104E)–Sepharose, and then eluting the antibody with molar acetic acid. Anti-Fab was prepared similarly and was a mixture of the following acid eluates: rabbit anti-whole $IgG_1(K)$(MPC 21) retained by $IgG_{2a}(K)$(Adj PC5)–Sepharose, rabbit anti-whole $IgG_{2a}(K)$(Adj PC5) retained by $IgG_1(K)$(MPC 21)–Sepharose and rabbit anti-whole IgM(K)(TEPC 183) retained by $IgG_{2a}(K)$(Adj PC5)–Sepharose. Purified antibody was coupled to Sepharose[8] to give high capacity immunoabsorbants. After passage of the sample, the columns were washed with phosphate-buffered saline (PBS), emulsified in complete Freund's adjuvant and injected into rabbits[9]. Each column was divided between two rabbits. Serum from a rabbit receiving the third column, the anti-Fab, was passed over $IgG_{2a}(K)$(Adj PC5)–Sepharose and then IgM(K)(TEPC 183)–Sepharose. The absorbed serum was tested by radioimmunoassay[5] and found to be unreactive with $^{125}$I-labelled IgM(K)(TEPC 183), $IgM(\lambda_1)$(MOPC 104E), $IgA(\lambda_2)$(MOPC 315), $IgG_1(K)$(MPC 21), $IgG_{2a}(K)$(Adj PC5), $IgG_{2b}(K)$(MOPC 195), $IgG_3$(FLOPC 21), $\lambda_1$ chains (RPC 20), K chain (MOPC 41) and mouse $\alpha_2$-macroglobulin. The radioimmunoassay will detect less than 1 $\mu$g of antibody in 1 ml of serum.

When used in conjunction with rhodamine-labelled goat anti-rabbit Ig (ref. 10), the absorbed antiserum stained[11]

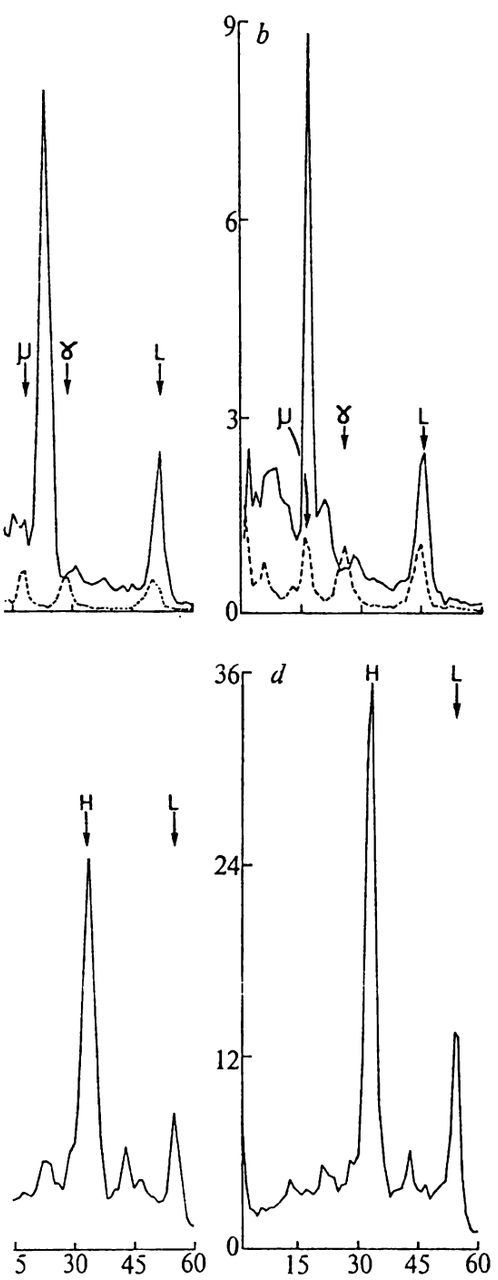


**Fig. 1** Surface immunoglobulin of murine splenic lymphocytes. Spleen cell suspensions of 3–6-month-old, specific pathogen-free, female CBA mice were labelled with $^{125}$I by the lactoperoxidase-catalysed procedure[18], washed once in ice-cold PBS and then lysed for 10 min at 0 °C in 1% (w/v) Nonidet P-40 in PBS containing 1 mM phenylmethylsulphonyl fluoride and 100 mM recrystallised iodoacetamide. The centrifuged lysate (30,000$g$ × 15 min) was passed over Sephadex G-25 equilibrated with the solution used for lysis of the cells. Labelled IgD was precipitated at 0 °C by addition of rabbit antibody (10 μl) and a goat anti-rabbit Ig (100 μl). The precipitate was removed by precipitation and the supernatant was further reacted with rabbit anti-mouse μ chain (10 μl) and goat anti-rabbit IgG (100 μl). Both precipitates were washed three times with ice cold 0.5% (w/v) Nonidet P-40 in PBS, once with 50 mM sodium phosphate, $p$H 7.0, and then dissolved in 50 mM sodium phosphate, $p$H 7.0, containing 2% (w/v) sodium dodecyl sulphate (SDS) and 1 mM dithiothreitol. The precipitates were heated at 100 °C for 10 min, and then iodoacetamide was added to 100 mM. Internal $^{131}$I-labelled[19] markers were added to the labelled cell surface Ig samples, which were then resolved by SDS-polyacrylamide gel electrophoresis[1]. After electrophoresis the gels were sliced into 1-mm segments and radioactivity was determined. Values were corrected for cross-channel spill and plotted with the top of the gel on the left hand side of the figure. Reduced cell surface Ig precipitated with the anti-IgD (*a* and *c*) and subsequently anti-μ (*b* and *d*) were electrophoresed on 7.5% (*a* and *b*) and 4.2% (*c* and *d*) polyacrylamide gels. ——, $^{125}$I incorporated into cell surface Ig; . . . ., $^{131}$I-labelled internal marker.

Table 1 Frequency of IgM and IgD-bearing cells in mouse lymphoid tissue

| | Organ | Single staining Anti-Ig | Anti-μ | Anti-δ | Double staining μ | μ+δ | δ |
|---|---|---|---|---|---|---|---|
| 1 | Peripheral nodes | 14.9 | 3.1 | 10.5 | 0.7 | 1.5 | 8.1 |
| | Mesenteric nodes | 14.4 | 4.1 | 10.5 | 1.1 | 1.8 | 7.2 |
| | Peyer's patches | 23.1 | 8.5 | 26.8 | 1.6 | 8.5 | 19.3 |
| | Spleen | 35.6 | 19.1 | 23.8 | 12.1 | 13.8 | 12.6 |
| 2 | Peripheral nodes | 15.6 | 3.1 | 9.1 | 0.5 | 2.7 | 11.1 |
| | Mesenteric nodes | 14.7 | 3.6 | 12.6 | 0.8 | 2.8 | 11.6 |
| | Peyer's patches | 23.2 | 9.4 | 22.5 | 0.8 | 8.2 | 14.0 |
| | Spleen | 32.2 | 17.8 | 25.9 | 8.7 | 11.9 | 14.3 |

nsions were prepared from 7-month-old, specific pathogen-free, female CBA mice by teasing the organs with veronal-buffered l), containing bovine serum albumin (1 mg $ml^{-1}$) (VBS-BSA), and the living cells were stained and prepared for examination as Raff[11]. Peripheral nodes collected were superficial cervicals, axillary, brachial and inguinal. For single staining the cells were incumin at room temperature with rabbit antiserum, washed three times with VBS-BSA and then suspended in rhodamine-labelled bit IgG (20 min, room temperature). Samples were washed, and then mounted in 50% (w/v) glycerol in (PBS)-0.03 M sodium ouble staining, cells were treated with anti-mouse IgD and fluorescein-labelled goat anti-rabbit IgG as described above. The two ssential to ensure capping of all the IgD positive cells. The cells were then incubated in the cold for 20 min with rhodamine-labelled ouse μ chain and with sodium azide at 0.03 M. After washing with cold VBS-BSA-0.03 M sodium azide the cells were mounted d under ultraviolet light with a Leitz Orthoplan research microscope fitted with Ploem illumination. At least 500 lymphocytes l for staining and the results are given as percentages of total lymphoid cells observed. All reagents were centrifuged (50,000*g*, ne day of use. On the basis of the double staining protocol, lymphocytes could be classified into three groups, those with green sitive), those with peripheral red staining (IgM positive) and those with green caps and red rings (IgM and IgD positive). The rabbit g was prepared by immunisation of rabbits with the Fab portion of myeloma protein Adj PC5 ($\gamma_{2a}$K) (ref. 20) as described before[9] specific, reacting against IgM, $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$ and IgA (but not other serum proteins) on Ouchterlony analysis. Rabbit chain was prepared by immunisation with myeloma protein MOPC 104E ($\mu\lambda_1$)[9] and absorbing the serum with a 7S fraction of e serum coupled to Sepharose 4-B. The resulting serum precipitated IgM on Ouchterlony analysis, but did not react with any other ss when tested by radioimmunoassay against $^{125}$I-labelled immunoglobulins (see the text and ref. 5). Preparation of rabbit antidescribed in the text. Fluorochrome conjugates were prepared using DEAE-purified Ig fractions, and conjugates with fluorochrome: s of 1.5–2.5 were selected by chromatography on DEAE[10].

of CBA strain spleen cells, but not thymocytes C57BL, DBA/2, C3H, AKR and BALB/c mice. . the antiserum was also negative for peripheral ared by the nylon wool method[12].

evaluated the antiserum by testing with $^{125}$I-lled spleen cells as described before[1], and found precipitated corresponded to IgD (Fig. 1*a*, refs ilar profile resulted when 100 μl of normal mouse incubated with 10 μl of the rabbit antiserum (30 temperature) before addition of the radioactive as circulating IgD is likely to be present in very nts in the mouse. Recovery of IgM was possible pernatant of the first precipitation by addition Fig. 1*b*). The radioactive material which runs after the μ and δ chains also occurs in non-cipitates. Furthermore, the higher molecular erial largely disappears when less concentrated ide gels are used (Fig. 1*c* and *d*), and thus may n aggregation artefact. The disadvantage of the trated polyacrylamide gels is that there is no between the μ and δ chains. When unreduced cell surface Ig were electrophoresed on 4.2% crylamide gels, the material then coincided with -linked 7S IgM internal marker[1].

asis of these results, we were therefore confident ntiserum was specific for mouse IgD, and thus studies using fluorescent reagents prepared by omatography[10] to stain live cells[11]. With 1–2-BA mouse splenocytes, we were unable to find numbers of cells reacting with the anti-IgD, i-mouse μ chain revealed 8–14% of the cells as lls bearing IgD were, however, readily apparent ns of 6-week-old mice (~20% total lymphocytes). of the antiserum with foetal liver cells lls per ml of serum) did not remove its capacity th spleen cells, whereas absorption with adult did ($1.9\times10^{10}$ cells per ml of serum). The finding precedes IgD in ontogeny[1,3] is therefore con-

determined the distribution of IgM and IgD on from various lymphoid organs (Table 1). Single staining was done by the indirect procedure. Double staining was achieved by capping IgD with a fluorescein-labelled system followed by staining for IgM with a rhodamine-labelled rabbit anti-mouse μ chain under non-capping conditions. From the single staining results it is immediately clear that IgD constitutes the major cell surface immunoglobulin of lymph nodes and Peyer's patches, as would be suggested by biochemical investigations[1–4]. Double staining of lymphocytes from these organs not surprisingly revealed most of the Ig-positive cells as having only IgD, although some doubles (IgM and IgD) and occasional cells positive for IgM alone were also present. In the spleen, however, all three categories of cells were represented in similar proportions.

Our findings in the mouse therefore mirror those in the human[13,14]. In the mouse, there are fewer cells bearing both IgM and IgD, with correspondingly higher numbers of cells with exclusively IgM or IgD. These studies in the human, however, have been made with peripheral blood and so the comparison is not necessarily valid. The biological significance of these subpopulations of B cells should now be open to investigation. Since IgM precedes IgD in ontogeny, we favour the idea that in B-lymphocyte maturation there is differentiation from cells expressing only IgM to those expressing only IgD (ref. 5). An intervening cell type expresses both Ig classes. Thus the B lymphocytes of lymph nodes and Peyer's patches would in the main constitute a more mature population, possibly enriched with memory cells and precursors of cells destined to secrete IgG and IgA. The finding that IgA precursor cells of rabbit Peyer's patches are IgM and IgA negative, but certainly do have surface immunoglobulin[15], suggests that these cells bear the rabbit equivalent of IgD.

The simultaneous expression of two Ig classes on the cell surface has implications for "switch" and *V–C* gene integration mechanisms. For example, the two constant region genes for μ and δ chains are known to share the same V region[16], and if this reflects the simultaneous presence of two integrated genes (that is *VH–Cμ* and *VH–Cδ*), then a copy-choice mechanism for *V–C* gene integration[17] is suggested.

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# Immunoglobulin M receptors on memory cells of immunoglobulin G antibody-forming cell clones

The memory cells of two antibody-forming cell clones had receptors of the IgM class, even though the clones had been producing $IgG_1$ or $IgG_{2a}$ anti-2,4-dinitrophenyl antibodies for 9–15 months previously (on exposure to antigen). Thus a phenotypic switch in heavy chain constant region evidently occurred *after* re-exposure of these memory cells to antigen.

To show that, we first removed the clonal cells' surface immunoglobulins by "capping" and "stripping", with class- or subclass-specific antisera. Then, to assay their remaining receptor activity, the cells were incubated with antigen *in vitro*, washed and transferred (together with carrier-primed cells) to irradiated recipients, and their antibody responses to this *in vitro* boost were assayed by isoelectric focusing. Pretreatment with anti-$\mu$ serum, as well as with anti-Fab($\kappa$), prevented the responses of the $IgG_1$ and $IgG_{2a}$ clones to an *in vitro* boost, while anti-$\gamma_1$, and anti-$\gamma_{2a}$ antisera had no effect. An anti-serum to the putative mouse IgD also had no effect. The anti-$\mu$ serum failed to react with the $IgG_1$ and $IgG_{2a}$ clonal serum antibodies in the test tube. Some other contaminating clones were suppressed completely only by the anti-Fab serum.

This result strongly suggests that switching in class commitment may occur during the differentiation of memory cells to antibody producers, and may therefore be antigen-dependent. It also implies that some apparently naïve cells with surface IgM may, in reality, be B memory cells.

ion

y, ontogeny and in the development of single B here is very frequently (or perhaps always) a early production of IgM to the later production s of other classes such as IgG or IgA ([1–7], re-, 9]). However, in the vast majority of cases, nly secrete antibodies of one class at any one 0]). Hence, though the precursors of these cells be uncommitted with respect to heavy chain y must subsequently become restricted to the immunoglobulin of a single class.

inding of a relatively high proportion of B cells M and IgD on their surface strengthens this evi-tching in the class, and therefore of the constant e immunoglobulin product. Moreover, such cells occur in the mouse* [11, 12] as well as in man (*e.g.* [13–15], reviewed in [16]). However, there is probably no concomitant switching in the variable region, since the IgM and IgD appear to share the same idiotype [17] and combining specificity [18]. Earlier work on rabbit idiotypes [2] and on patients with double myelomata [3] likewise showed that the same variable regions might be shared between immunoglobulins of different classes.

The IgD-bearing cells appear relatively late in development [11, 19] and therefore do not seem to be primitive B cell progenitors. Their high frequency contrasts sharply with the low incidence of cells bearing surface IgG and is grossly out of proportion with the relative serum concentrations of IgG and IgD. Furthermore, estimates of the frequency of surface IgG-bearing cells have recently decreased to about 5 % or less owing to technical refinements (*e.g.* [20, 21]). Most B cells can, in fact, be assigned to one of three populations of roughly equal size (in mouse spleen) which have either IgM, or IgD, or both, on their surfaces [22, 23]. All these considerations have led to speculation that the precursors of some (or all) of the IgG-producing cells may have IgD receptors, and may ultimately originate from IgM-bearing cells.

An obvious question, therefore, is the position of B memory cells along these pathways of differentiation: in other words, what is the heavy chain class of their receptors for antigen? A convenient means of exploring that is the selected antibody-forming cell cloning system of Askonas, Williamson and Wright



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: ABC: Antigen-binding capacity B cell: Bone marrow-ocyte BSA: Bovine serum albumin DIP-DNP-lys: o-4-hydroxyphenacetyl)-ϵ-N-(2,4-dinitrophenyl)-L-: 2,4-Dinitrofluorobenzene DNP: 2,4-Dinitrophenyl ic mean HOP-DNP-lys: α-N-(4-Hydroxyphenacetyl)-ϵ-phenyl)-L-lysine HSA: Human serum albumin IEF: using i.p.: Intraperitoneally l.n.: Lymph node LPS: aride OVA: Hen egg ovalbumin PBS: Phosphate : SD: Standard deviation SRBC: Sheep red blood cell(s) s-processed lymphocyte tet. tox.: Tetanus toxoid f serum

* The mouse candidate for IgD resembles its human counterpart in heavy chain molecular weight, marked susceptibility to proteolysis and a selective location on B lymphocytes [11, 12] but, in the absence of sequence comparisons with human IgD, the assignment is provisional. Nevertheless, for brevity, we shall refer to this mouse immunoglobulin as IgD.

such "clones" can be propagated *in vivo* and can be iden- by the characteristic isoelectric spectra ("spectrotypes") r antibody products. They have the advantage that they ce the same antibody species over many months (in re- to antigen) – which contrasts sharply with the hetero- y and variability of conventional responses. Also, muta- o yield variant products must be rare, if they occur at have studied the receptors of such cloned cell popula- y measuring the responses elicited by brief exposure cells to antigen *in vitro* – the *in vitro* boosting system d by Mitchison [25]. The cells make antibodies after g and transfer to irradiated recipients. Preincubation nti-Ig antisera, either in the cold [25, 26] or under capping ions (this paper), blocks this response, and we now report of such experiments on clones using appropriate class- antisera. We have used the "capping" and "stripping" [27] because receptors of a given class can be selec- emoved with minimal nonspecific effects on antigen by other receptors.

## erials and methods

### imals, immunization and irradiation

CBA/Ca mice, bred under specific pathogen-free con- , were used throughout at 4–6 months of age. Carrier- donors were immunized with 7 Lf. alum-precipitated toxoid (tet. tox.), boosted four weeks later with 0.7 Lf. s tet. tox., and used 1–3 months later. Recipients were ed with 600 r from a $^{60}$Co source.

### tigens, iodinations

d tet. tox. was kindly provided by P.A. Knight, Well- aboratories, Beckenham, Kent, and was dinitrophenyl- the method of Benacerraf and Levine [28], but using trofluorobenzene (DNFB), to yield a preparation with (2,4-dinitrophenyl) groups per 140 000 Daltons. -Hydroxyphenacetyl)-ε-N-(2,4-dinitrophenyl)-L-lysine )NP-lys) was provided by Dr. P.C. Montgomery and thesized as described in [29]. Iodinations were per- by the method of Hunter and Greenwood [30].

### ti-immunoglobulin antisera

paration of specific rabbit anti-mouse Ig, and goat bit Ig antisera has been described previously [22]. In e anti-mouse μ-chain serum was raised against purified 04 E IgM (λ) myeloma protein [31], was absorbed mouse Ig coupled to Sepharose 4B, and was found nreactive with $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$ and IgA mye- oteins, in a sensitive radioimmunoassay. The anti-Fab m (raised against an $IgG_{2a}$ (κ) protein) precipitated ese proteins including IgM. The subclass-specific anti- e raised against mouse myeloma proteins, and absorbed oluble myeloma proteins of different classes and sub- and their specificity was confirmed by radioimmuno- he preparation and characterization of the specific nti-mouse δ-chain antiserum has also been described in 3].

-human serum albumin (HSA) antiserum was kindly d by Dr. J.H. Humphrey, and had been raised by im- munizing rabbits with three injections of 12.5 mg purified HSA in Freund's complete adjuvant. All antisera were thus prepared using the same adjuvant, and before use all were absorbed thrice with $10^8$ normal mouse thymocytes per ml of serum.

Some of the anti-μ antiserum was absorbed by passage over a column of purified MOPC 104 E myeloma protein coupled to Sepharose 4B. An IgG fraction of the goat anti-rabbit IgG serum was coupled with tetramethylrhodamine isothiocyanate, and a conjugate with a rhodamine-to-protein ratio of 2:1 was selected [32].

### 2.4. Antibody assays

Farr titrations were performed as described by Mitchison [33], using the radiolabeled DNP hapten D$^{125}$IP-DNP-lys at $10^{-8}$ M. Antigen-binding capacities were calculated as in [33]. Isoelectric focusing (IEF) was done as described by Williamson [34], using D$^{131}$IP-DNP-lys to overlay the gels before they were washed and autoradiographed.

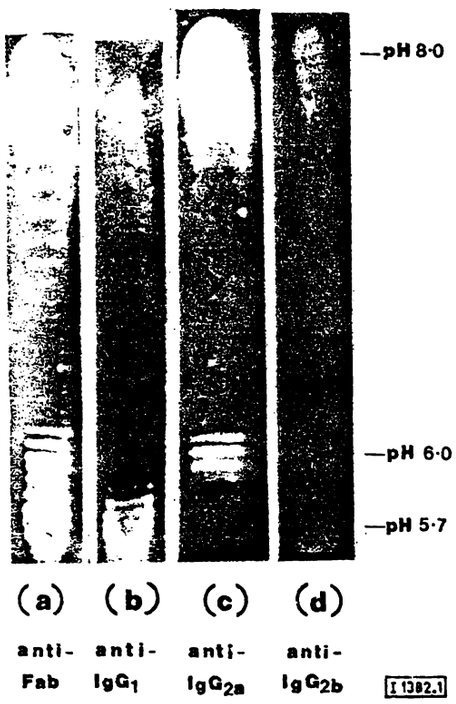
For the indirect precipitation assays (Section 3.3.1.), 50 μl $^{125}$I-labeled $DNP_{17}BSA$ (bovine serum albumin) at 2 μCi/μg, diluted to 0.5 μg/ml in 1 % (w/v) BSA in phosphate buffered saline (PBS), was incubated with 2–5 μl of test clonal serum at 37 °C for 30 min. Then enough class-specific rabbit anti-mouse Ig serum was added to give maximal binding and incubated at 37 °C for 30 min. Soluble immune complexes were then precipitated with polyspecific goat anti-rabbit Ig serum for 30 min at 37 °C followed by 1 h at 4 °C. The precipitates and supernatants were counted after centrifugation, and the percentage precipitation determined.

### 2.5. Cell transfer experiments

#### 2.5.1. The 5A clones

The origin of this clonal cell line was described by Klaus and Willcox [35]. In the present work, donors from the positive control groups in the experiments described in Sections 3.4. and 3.5. of [35] were used *9–15 months after the clone was originally raised.* They had been immunized exclusively with DNP-hen egg ovalbumin (OVA) and were boosted 4–8 weeks before use.

This "clone" was not homogeneous. The subclasses of the three main spectrotypes seen on IEF were determined by the method of Phillips and Dresser [36]; IEF gels were overlayed with DNP-sensitized SRBC and subclass-specific developing anti-Ig sera (kindly provided by Dr. D.W. Dresser [37]) and complement were subsequently added. It was clear (Fig. 1) that the very acidic spectrotype was $IgG_1$, the adjacent one was $IgG_{2a}$, and the antibodies with high isoelectric points were predominantly $IgG_{2a}$. These will henceforward be called the "$IgG_1$", "$IgG_{2a}$" and "alkaline" spectrotypes, respectively. The alkaline spectrotypes focused poorly, even when the most alkaline gradients were used, and were probably heterogeneous. They appeared to represent large amounts of low-affinity antibodies and only appeared in response to antigen: they were therefore neither "background" antibodies nor artefacts due to hapten binding by hemoglobin.




ermination of subclasses of 5A component clonal anti-
:tion by overlay of IEF gels with haptenated SRBC, fol-
class-specific developing antisera and complement.
-e same clonal serum were focused in parallel on five
gels (pH 5–9), which were then overlaid with DNP-Fab-
BC, and developed by adding various subclass-specific
umented in [37]) and complement. On a control plate,
-eveloping serum, there was no lysis. The anti-Fab serum
ı $IgG_1$ and $IgG_{2a}$ antibodies. Antisera were kindly pro-
•D.W. Dresser. The weak bands apparently developed by
e probably artefactual, as normal serum may yield similar

'ng and stripping with anti-Ig sera [27]

en cell suspensions from clonal cell donors
:lls/ml) were incubated for 20 min at room tem-
h rabbit anti-mouse Ig antisera of various speci-
•0–150 μg of antibody*/ml) in HEPES-buffered
um containing 5 % fetal calf serum (FCS). They
ashed twice and incubated for 2 h at 37 °C so as
mal shedding or endocytosis of capped surface
were then pelleted, washed once, cooled in an
counted. They were then adjusted to the same
ration. Viabilities were also checked and were
ɜ 90 %. Samples were taken before and after the
ation and stained with rhodamine-conjugated goat
y to rabbit IgG [38], to confirm the absence of
g.

ation with antigen

ells ($10 \times 10^6 - 50 \times 10^6$ cells/ml) were next in-
ı DNP-tet. tox. (20 μg/ml) for one h at 4 °C in
199 medium. They were then washed twice,
washed spleen cells from mice immunized with
x $10^6$/recipient), and transferred i.p. to recipients

ception was the anti-δ antiserum which, for obvious
not be titrated in this way.

which had been irradiated with 600 r 6 h in advance. The doses of clonal cells are given in the table or figure legends.

In previous work the cell type responsible for transferring responsiveness after binding the antigen was shown to be the B cell, since it was depleted neither by anti-Θ serum and complement, nor by cotton wool columns, nor by carbonyl iron [39]. The proportion of dead cells in the suspensions also made very little difference, although much of the bound antigen is on dead cells [40].

## 3. Results

### 3.1. Experimental protocol: the *in vitro* boosting system

In all experiments, spleen cells from clone-bearing mice (previously immunized exclusively with DNP-OVA) were pretreated with class-specific anti-Ig sera under conditions previously found to give maximal capping and subsequent clearing of surface Ig, and minimal secondary effects on antigen binding. They were then washed and incubated in the cold with DNP-tet. tox. and, after further washing, were mixed with tet. tox.-primed spleen cells (not incubated with antigen), and transferred i.p. to irradiated recipients, which were bled subsequently. The clonal anti-DNP antibody responses to the antigen bound *in vitro* were measured by IEF and Farr methods (Section 2.4.). The carrier chosen was tet. tox. rather than OVA, in order to restrict binding to DNP-specific cells and minimize any potential attachment to OVA-reactive ones.

#### 3.2.1. Effect of anti-μ serum on clonal receptors

Exposure to antigen *in vitro* boosted the $IgG_1$ and the "alkaline" clones efficiently, while the $IgG_{2a}$ clone responded less vigorously (Table 1, Fig. 2). These responses were not affected by pretreatment with anti-HSA serum, whereas poly-specific anti-Fab serum greatly suppressed the responses of all three clones. The effects of specific anti-μ serum were striking: there was an almost complete suppression of the $IgG_1$ and probably also of the $IgG_{2a}$ clones, but only a partial inhibition of the alkaline ones.

The titers of anti-DNP antibody correlated well with the IEF patterns (Table 1). The overall suppression after treatment with anti-μ serum was about ten-fold, but the effect on the $IgG_1$ clone was still greater: if we compare undiluted sera in this group with sera of the positive control groups diluted 1:7, there is still a large difference in hapten-binding intensity on IEF (see Fig. 2). Evidently there was a selective effect of the anti-μ serum on the $IgG_1$ clone.

These antisera almost certainly acted by blocking or removing receptors, and not by crudely killing B cells; when the recipients in this experiment were boosted with antigen *in vivo* on day 20, the anti-Ig and anti-μ pretreated groups responded even better than the controls initially given no antigen at all (group 5, Table 1). This confirms that the antisera cannot have killed all the clonal cells. It also suggests that the antigen *in vitro* may have given a very limited stimulus even to the anti-Ig pretreated cells, as these eventually responded more strongly than group 5. In other words, the blockade may not have been complete.

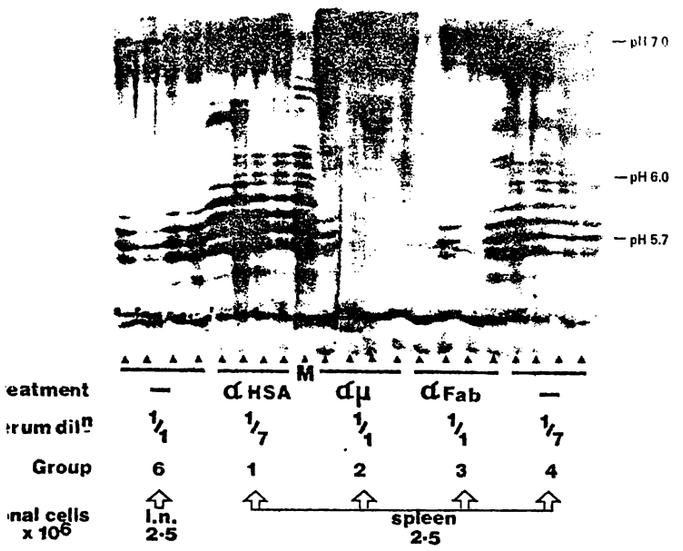



*Figure 2.* Blockade of clonal receptors with anti-$\mu$ and anti-Fab antisera. IEF patterns of anti-DNP antibodies of 18-day sera of recipients in the experiment summarized in Table 1. Individual sera were loaded, in the arrowed positions, at the dilutions shown (undiluted or 1/7) on pH 4–8 gels. After focusing, the gels were overlaid with $D^{131}IP$-DNP-lys, washed, and autoradiographed, to reveal anti-DNP antibodies. Protocol and group numbers same as in Table 1. αHSA, α$\mu$, etc. refers to pretreatment of clonal cells with anti-HSA, anti-$\mu$ sera, etc. l.n. = lymph node cells; spleen = spleen cells – both from clonal donors; M = marker serum from 3rd transfer generation clone-bearing mouse.

lockade of clonal receptors with anti-$\mu$ and anti-Fab antisera[a])

| Clonal cells ($2.5 \times 10^6$) | Pretreatment | Antigen *in vitro* | Anti-DNP antibody responses Day 10 ABC[b]) GM ($\bar{x}$ SD) | Day 27 ABC[b]) GM ($\bar{x}$ SD) | Day 34 IEF[c]) no. positive/total |
|---|---|---|---|---|---|
| Spleen | — | + | 39.2 (1.2) | .590 (1.65) | 4/4 |
| Spleen | anti-HSA | + | 63.7 (1.7) | 473 (2.8) | 3/4 |
| Spleen | anti-$\mu$ | + | 4.7 (1.35) | 99.5 (1.5) | 4/4 |
| Spleen | anti-Fab | + | 0.8 (1.9) | 46.6 (2.2) | 4/4 |
| Spleen | — | — | 0.14 (1.7) | 16.6 (1.75) | 3/5 |
| Lymph node | — | + | 7.9 (2.6) | 419 (3.9) | 3/3 |
| Lymph node | — | — | 0.1 (1.0) | 16.9 (2.1) | 3/4 |

or lymph node cell suspensions from third transfer generation clonal donors were preincubated with the appropriate under capping conditions, washed, and then incubated at 37 °C for 2 h (see Section 2.). They were then washed and ed at 4 °C with 20 $\mu$g/ml DNP4tet.tox. for 1 h, and washed again. They were then mixed with spleen cells from tet.tox- mice ($10^7$/recipient) and transferred i.p. to irradiated mice, which were bled on days 10 and 19. They were boosted with ieous DNP-tet.tox. on day 20, and bled again, 4 mice/group. Same group numbers as in Fig. 2.
recipients' sera measured in Farr assay vs. $D^{125}IP$-DNP-lys.ABC expressed as mM/ml x $10^{-8}$. GM ($\bar{x}$ standard deviation).
of mice/total in group showing $IgG_1$ and $IgG_{2a}$ clones on IEF.

ne strong $IgG_1$ clonal response of the lymph node e *in vitro* boost (Fig. 2, Table 1) makes it unlikely ophages were playing an essential role in binding n *in vitro*, since such cell suspensions contain low f macrophages (see also Section 2.5.3.).

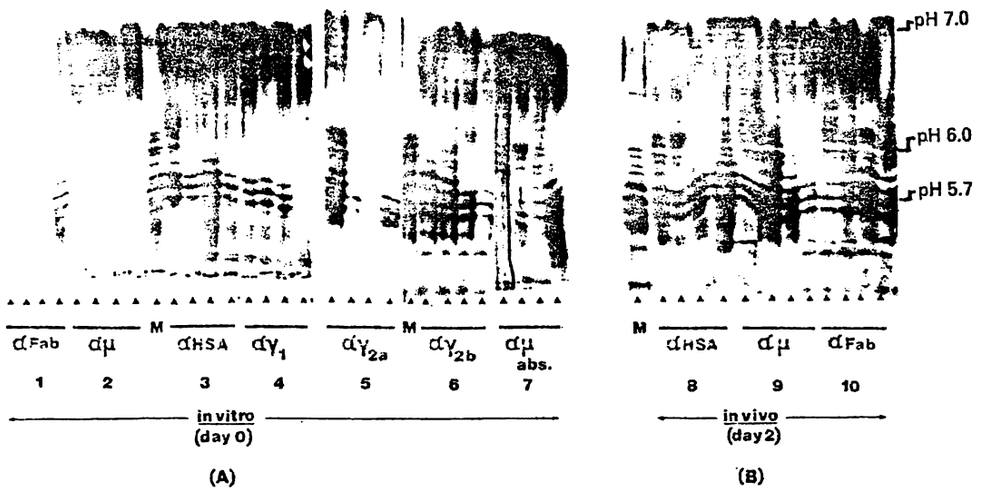
### k of effect of subclass-specific antisera

d experiment, additional batches of cells were pre- with anti-$\gamma_1$ and anti-$\gamma_{2a}$ antisera. In this case, nly the polyspecific anti-Fab and the anti-$\mu$ sera hile the anti-$\gamma_1$ and anti-$\gamma_{2a}$ sera had no effect e $IgG_1$ clone (Fig. 3A). Again, the effect of the selectively on the $IgG_1$ and $IgG_{2a}$ clones, whereas polyspecific anti-Fab was unselective. In this ex- the contribution of the "alkaline" clones to the nse was so great that only the anti-Fab serum af- Farr titers (data not shown). The anti-$\mu$ serum re- n only marginally – which confirms its selective he $IgG_1$ and $IgG_{2a}$ clones.

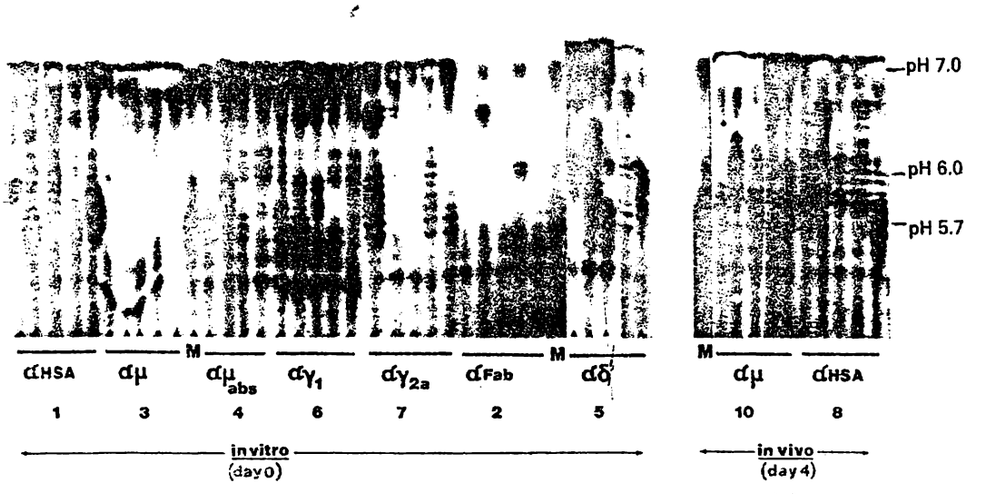
Separate aliquots of the anti-$\mu$ and anti-Fab pretreated cells were allowed to resynthesize their receptors *in vivo* before they were exposed to antigen; antigen challenge was delayed until two days after transfer in these groups. As expected, neither the anti-$\mu$ nor the anti-Fab had any effect in this situation (Fig. 3B), confirming that the antisera were not simply killing all the cells. As a further confirmation of the specificity of action of the anti-$\mu$ serum, some of it was absorbed on an IgM Sepharose column; after that it no longer blocked the $IgG_1$ clonal response significantly (Fig. 3A). This was also confirmed in the next experiment.

### 3.2.3. Repeat experiment

A third experiment confirmed the above findings, in that anti-$\mu$ serum again selectively blocked the $IgG_1$ and $IgG_{2a}$ clonal responses to an *in vitro* boost, and the anti-Fab impartially inhibited all the clones. The subclass-specific antisera, and the anti-$\mu$ serum absorbed with IgM, again had no effect. Also, the anti-$\mu$ and anti-Fab pretreatment had no ef-




ockade of clonal receptors with anti-Ig antisera. IEF patterns of anti-DNP antibodies of 25-day sera of recipients of ient described in Section 3.2.2. Sera all loaded undiluted. Focusing and development as in Fig. 2. Protocol in prin- able 1: clonal spleen cell suspensions (5 x $10^6$ cells/mouse) from third and fourth transfer generation donors were vith the indicated anti-Ig antisera. They were then incubated with 20 μg/ml DNP-tet.tox. (part A) or with medium B), washed, and transferred together with carrier-primed cells ($10^7$/recipient). The mice in groups 8–10 were boosted aqueous DNP-tet.tox. on day 2 (to allow the treated cells to resynthesize their receptors). The cells for groups 1 and , and 3 and 8, were each pretreated together as a single batch with antisera. Anti-μ abs: anti-μ serum absorbed with na protein coupled to Sepharose 4B. *N.B.* The dark zones closest to the arrows represent binding of labeled hapten




ockade of clonal receptors with anti-Ig sera. IEF patterns of anti-DNP antibodies of 25-day sera of recipients in the ummarized in Table 2. Sera all loaded undiluted. Focusing and development as in Figs. 2 and 3. Group numbers able 2.

responses to an *in vivo* boost on day 4 (Fig. 4, e clonal responses were somewhat variable in this , as rather weak donors had to be used.

y, in this experiment, pretreatment with a putative δ-chain antiserum [23] had no effect on responses *in vitro* or an *in vivo* boost. This result has been n one other experiment with these clones, in nti-Fab (though exceptionally not the anti-μ) cessfully in parallel groups (not shown).

l experiments

re of anti-μ to bind to the clonal serum antibodies

eriments were done to test whether the anti-μ -reacts (*e.g.* via idiotypic determinants) with the clonal serum antibodies in the test tube. Sera from selected recipients showing predominantly either the $IgG_1$ or the $IgG_{2a}$ clonal antibodies were therefore chosen and were preincubated with $^{125}$I-labeled DNP-BSA. The complexes these antibodies formed with this antigen were then precipitated with appropriate (rabbit anti-mouse) class-specific anti-Ig antisera, followed by polyspecific goat anti-rabbit Ig serum.

The results (Table 3) show that the anti-μ serum failed to precipitate a significant amount of antibody in the clonal sera, whereas it did precipitate the IgM antibodies elicited by DNP-Ficoll as efficiently as did the anti-Fab serum. The clonal antibodies were instead precipitated by the appropriate subclass-specific antisera. In a further experiment, preincubation with anti-μ serum also failed to block the binding of antigen by the $IgG_1$ clonal antibody, as subsequently detected by pre-

ct of class-specific antisera on responses to *in vitro* or ing[a)]

| retreatment | Antigen | Anti-DNP response ABC day 10 (GM ± SD) | |
|---|---|---|---|
| | *in vitro* | | |
| nti-HSA | day 0 | 12.8 | (1.4) |
| nti-Fab | day 0 | 0.6 | (1.7) |
| nti-μ | day 0 | 3.3 | (1.8) |
| nti-μ (abs.)[b)] | day 0 | 9.6 | (1.1) |
| nti-δ | day 0 | 10.1 | (1.7) |
| nti-$\gamma_1$ | day 0 | 11.7 | (1.3) |
| nti-$\gamma_{2a}$ | day 0 | 6.8 | (2.5) |
| | *in vivo* | | |
| nti-HSA | day 4 | 3.2 | (1.7) |
| nti-Fab | day 4 | 3.9 | (1.7) |
| nti-μ | day 4 | 5.2 | (1.8) |
| nti-δ | day 4 | 4.8 | (1.4) |
| nti-HSA | None | 0.1 | (1.0) |

s in Table 1, (Footnote a) and Section 3.2.3., except that re from fourth transfer generation, and the clonal spleen as 10 x $10^6$/recipient. Also groups 1 and 8, 2 and 9, 3 and 11 were each pretreated as a single batch: they were vided, half of each being incubated with antigen *in vitro*, alf only being given antigen *in vivo* 4 days after transfer P-tet. tox./mouse i.p.). Same group numbers as in Fig. 4. = anti-μ serum absorbed with IgM myeloma protein Sepharose 4B.

th anti-$\gamma_1$ antiserum, whereas the anti-μ serum IgM anti-DNP-Ficoll antibody (data not shown).

conclude that the very anti-μ serum that blocked of the $IgG_1$ clone memory cells did not bind the duct of their progeny detectably and therefore with idiotypic determinants.

### nti-Ig pretreatment affect total amount of antigen ?

ble that the anti-Ig acts by preventing antibody cells (*e.g.* macrophages or dead cells) from bind- n, rather than by selectively blocking the me- eptors. The resulting decrease in the amount of d would indirectly reduce the boost to the clonal r, a final control experiment, using $^{125}$I-labeled showed, in this case, that neither antiserum significantly affected the total amount of antigen bound by a spleen cell suspension prepared from clonal donor mice (Table 4).

Table 4. Lack of effect of anti-Ig sera on total amount of $^{125}$I-labeled DNP-tet.tox. bound by clonal spleen cell suspension[a)]

| Pretreatment | Antigen bound (%) |
|---|---|
| anti-HSA | 0.63 |
| anti-μ | 0.54 |
| anti-Fab | 0.71 |

a) Clonal spleen cells ($10^7$) were pretreated with anti-Ig sera as in Section 2.5.2. and then incubated with 20 μg/ml $^{125}$I-labeled DNP-tet.tox. (1.6 μCi/μg) as in Section 2.5.3. After standard washing, the cells were counted in a Packard autogamma counter, and the counts were corrected for cell recovery.

## 4. Discussion

The results we have presented argue strongly that receptors of the IgM class are maintained on memory cells of *some* clones, even several months* after the differentiated clonal cells have begun producing $IgG_1$ or $IgG_{2a}$ clonal antibodies. This argues that re-exposure to antigen causes a switch in heavy chain constant region. Before accepting this conclusion, we should briefly answer some possible objections to the evidence.

We have already presented control data (Section 3.2.) showing that our anti-Ig sera were not simply cytotoxic, and that the anti-μ serum was acting specifically (Figs. 3 and 4). Also, the anti-μ serum failed to bind $\alpha_2$-macroglobulin (which is present on B cell membranes, [42]), when tested in a sensitive radioimmunoassay (not shown). Furthermore, cross-reactions between the anti-μ and the $IgG_1$ or $IgG_{2a}$ clonal antibodies appear to be an unlikely explanation (Section 3.3.1.). We have also shown (Section 3.3.2.) that the anti-μ and anti-Fab antisera did not significantly decrease the gross amount of antigen bound by the donor cells, and thereby reduce the stimulus they might receive from antigen nonspecifically adsorbed to macrophages or dead cells.

* A slow turnover of surface IgM [41] is unlikely to be the sole explanation of its persistence on these clonal memory cells, in view of this very long time lag.

of anti-μ to bind clonal serum antibodies in test tube

| -Test mouse anti-DNP antibodies[a)] | Anti-DNP ABC[b)] | % precipitation of DNP-BSA $^{125}$I-labeled anti-μ | anti-$\gamma_1$ | anti-$\gamma_{2a}$ | anti-Fab |
|---|---|---|---|---|---|
| $gG_1$ + $IgG_{2a}$ clonal spectrotypes | 5.8 | 2.6 | 19.4 | 10.7 | 31.2 |
| $IgG_{2a}$ clonal spectrotypes | 8.5 | 5.0 | 5.0 | 77.7 | 80.0 |
| ›ll serum | 0.2 | 30.3 | 13.8 | 12.9 | 28.6 |

.ntisera were preincubated with $^{125}$I-labeled DNP-BSA: soluble antigen-antibody com- hen precipitated with the indicated rabbit anti-mouse class-specific antisera, followed fic goat anti-rabbit Ig. Low-titer clonal sera (A) and (B) were chosen because they showed ly the $IgG_1$ or $IgG_{2a}$ spectrotypes. A subtraction of 8 % has been made for binding by .e serum.

ned by Farr assay vs. $D^{125}$ IP-DNP-lys.

ossibility is that some of the anti-$\mu$ antibodies might e helper cells and thereby prevent them from recog- carrier. This seems extremely unlikely for many g. because of the experimental design (see Section use the anti-$\mu$ serum had relatively little effect on e clones, and because this anti-$\mu$ serum failed to M-like molecules in solubilized membranes of thy- r peripheral T lymphocytes [11, 43].

ıard to escape the conclusion that the $IgG_{2a}$ and y the $IgG_1$ clones had mostly or entirely IgM re- their memory cells. The lack of effect of anti-$\gamma_1$, d anti-$\delta$ antisera is also striking and points to the lusion. Our evidence cannot rule out a minority ı of IgG or IgD receptors in addition to the IgM but the completeness of the suppression by anti-$\mu$ ut of four experiments) – even of very strong re- indicates that the bulk of the functional receptors the clones we have studied are representative, we ably conclude either that B memory cells have no D at all, or that it does not mediate their reactiva-

t is possible that the $IgG_1$ and $IgG_{2a}$ clones were l in this respect, especially as the alkaline clones ɔn thereof) clearly behaved differently. Apparent- e only partially suppressed by anti-$\mu$ pretreatment 2, Figs. 3 and 4), whereas they were much more and consistently suppressed by the anti-Fab serum. ɔulk of their memory cell receptors cannot have f they were indeed a mixture of several clones, ɔf different classes and subclasses, an antiserum of these, by itself, would not have given a signi- ression. To achieve that, one would need a *combi-* ıti-$\gamma$ or anti-$\delta$ antisera *plus* anti-$\mu$.

y of B cell subpopulations is now becoming ob- t is quite likely that the clones we have studied esentative of only one of these subsets: it will ıg to see how great is their heterogeneity and ɔn. Different subpopulations are being defined ɔ their Fc or C3 receptors (*e.g.* [44]), and their ponsiveness [45, 46] as well as their Ig product, yet clear how these classifications are interrelated. ɔne of the great advantages of the cloning system is ı use it to isolate members of different subpopula- udy their properties in a series of experiments over ths.

ty among B cells may also help to resolve the agreement between previous results [47, 48] and alters and Wigzell [47] found that the primed pre- $IgG_1$ or $IgG_{2a}$ antibody-forming cells could be se- vented from binding to antigen columns by the subclass-specific antisera; and Herzenberg et al. that the 1 % of B cells bearing surface IgG in- ally all of the IgG memory cells*. In either system M receptors might have been present in addition ty that was not tested – or, alternatively, a mi- ɔpulation of, say, 20–30 % of IgG precursor, M receptors might have escaped detection. It is also possible that these systems may have been assaying a different cell type from ours. For example, Walters and Wigzell used donors primed with antigen in Freund's complete adjuvant, after which there may continually be a high proportion of recently activated and more terminal B cells present which may differ from the memory cells we have studied. Indeed, there is independent evidence for the appearance of such "triggered" memory cells within a few days of boosting [49], and it would therefore be interesting to study the class of their receptors, which may more usually resemble the final product in class.

These ideas might also explain the somewhat similar results of Pierce et al. [4, 5]. They showed that *in vitro* primary IgM, IgG and IgA responses to SRBC could be blocked by the addition of anti-$\mu$ to the cultures, which they confirmed was acting on the B cells. Early after priming there was a phase in which the $IgG_1$ and $IgG_{2a}$ responses could only be blocked by anti-$\gamma_1$ or anti-$\gamma_{2a}$, respectively, whereas later on anti-$\mu$ again blocked them. Finally, the IgA response, although small, clearly remained suppressible by anti-$\mu$ at all times tested. Here again, the recent priming may have elicited a more terminally differentiated B memory cell with class-restricted receptors, and a short life span. It therefore appears entirely possible that many resting memory cells will be proved to have IgM as their surface receptor for antigen, and conversely that a proportion of the apparently naïve B cell population with membrane IgM may, in fact, be quiescent memory cells.

These results strongly reinforce the concept of an antigen-dependent switch, as, indeed, do those of Kearney and Lawton (*e.g.* [50]) who showed that LPS-induced IgG responses can be inhibited by anti-$\mu$ antibody, although, as Lawton et al. point out [8], antigen-independent switching also remains plausible. Our results do nothing to solve the challenging problem of the function of the cell surface IgD The results with the anti-$\delta$ antiserum may have been negative for technical reasons, or because the IgD is confined to different B cell subpopulations or stages of differentiation, or conceivably because the memory cells have a mixture of IgD and other classes. Whatever the reason, it is clear that a positive method of isolating the IgD-bearing cells is required to identify their functions, and also that selected antibody-forming cell clones will provide invaluable raw material.

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# GENERATION OF IMMUNOGLOBULIN CLASS DIVERSITY IN B CELLS: A DISCUSSION WITH EMPHASIS ON IgD DEVELOPMENT

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The earliest B cells to appear during development express only IgM. The appearance of other Ig classes in their progeny involves sequential expression (switching) of $C_H$ genes. There is no concomitant switching of variable region or light chain genes. Thus malignant clones of B cells synthesizing more than one class of immunoglobulins may be restricted to one unique $V_H$ and light chain, and a similar situation is also observed in clones of normal B cells [5, 12]. The clearest demonstration that all B lymphocytes are ultimately derived from IgM-bearing cells is provided by experiments involving blockade with anti-$\mu$ antibodies [13, 14, 16]. The most useful approach to solving the problems of the responsible genetic mechanism, regulatory factors and biologic significance of the Ig-class switch is likely to come from detailed study of alterations in expression of Ig class during the early stages of B-cell development.

## Sequential expression of IgM, IgG and IgA

More is known about the orderly expression of $\mu$, $\gamma$ and $\alpha$ chains, and we will briefly review this information before concentrating on the developmental acquisition of IgD.

In mammals, IgM is initially expressed in the cytoplasm of large lymphoid precursors of small B lymphocytes bearing membrane-associated IgM, which appear later. The interval between the two levels of IgM expression is 3-5 days for the mouse embryo [24] and appears to be around two weeks for human foetuses [7]. Lymphocytes bearing surface(s) IgG or sIgA are developed later than $sIgM^+$ cells [7, 15, 29]. All of the $sIgG^+$

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and sIgA+ lymphocytes in young human and mouse foetuses also express sIgM (unpublished observations).

In heterozygous rabbits sIgM has been found on the surface of plasma blasts or plasma cells synthesizing cytoplasmic(c) IgG of the same $V_H$ allotype [20]. Surface IgM may also be observed on mouse spleen cells induced by lipopolysaccharide (LPS) to begin $cIgG_2$ synthesis [10]. These observations indicate at a cellular level the direct conversion of synthesis from sIgM to surface and then cytoplasmic synthesis of other Ig classes, and are supported by evidence that anti-$\mu$ antibodies can inhibit differentiation of unprimed precursors of IgG- and IgA-secreting cells *in vitro* [21]. Thus under normal circumstances switching of immunoglobulin class synthesis occurs at the level of the B lymphocyte rather than in more mature antibody-secreting cells. B cells which express more than one Ig class fall into two categories: small lymphocytes bearing two classes of sIg and more mature cells containing cytoplasmic Ig of one class and sIg of another. Mature plasma cells simultaneously synthesizing two classes of cIg are rare. Before expanding this concept, we will discuss the evidence that expression of non-$\mu$ immunoglobulins on B lymphocytes is relevant in terms of receptor activity.

Because sIgG and sIgA are found on small percentages of B lymphocytes by immunofluorescence, and generally are not detected by immunochemical analysis of radioiodinated membrane proteins, doubt has been raised as to whether or not these two classes of antibodies exist as functional receptors on B lymphocytes [28]. The problems of cytophilic IgG and unwanted antibody contaminations of anti-$\gamma$ and anti-$\alpha$ preparations have been raised as possible sources of artifactual demonstration of sIgG and sIgA [30]. However, several lines of evidence unambiguously demonstrate that B lymphocytes can produce the surface IgG or IgA which they bear and imply that these may serve as functional antibody receptors, at least on primed B cells. (1) Treatment of chick embryos or of mice from birth with anti-$\alpha$ antibodies or of mice with anti-$\gamma$ antibodies will suppress the development of cells making the corresponding class of immunoglobulins [13, 14, 16]. (2) IgG antibody responses of precursor lymphocytes from the spleens of recently-primed mice are inhibitable by anti-$\gamma$ antibodies but not by anti-$\mu$ antibodies [21]. (3) In rabbit Peyer's patches, the lymphocyte precursors of IgM-producing plasma cells bear sIgM, while lymphocyte precursors of IgA-producing plasma cells bear sIgA and not sIgM [9]. (4) Memory cells selected on the basis of expression of an allotype, Ig-1b, of the $IgG_{2a}$ class of mouse immunoglobulin serve only as precursors for antibody-forming cells of that class [8]. (5) Malignant clones of B lymphocytes from selected patients with chronic lymphocytic leukemia (CLL) have been shown to produce the sIgG which they bear; similarly,

---

C = cytoplasmic
CLL = chronic lymphocytic leukemia
LPS = lipopolysaccharide
s = surface

rare patients with CLL have malignant clones of sIgA-producing lymphocytes [27]. Occasionally, IgG or IgA accumulates as crystals in the cytoplasm of CLL lymphocytes bearing the corresponding class of sIg ([27] and unpublished observations).

The fact that Ig-class heterogeneity is generated initially at the B-lymphocyte level and prior to further B-cell maturation is clearly illustrated by observations in individuals with panhypogammaglobulinemia due to an arrest in B-lymphocyte differentiation: despite their lack of mature B cells, the B lymphocytes in such patients frequently display sIg of all classes in percentages indistinguishable from normals [4, 27].

Clearly, antigens and helper T cells regulate the differentiation of B lymphocytes to mature plasma cells. Since T-cell help is especially important in antigen-induced responses of IgG- and IgA-producing plasma cells, many have considered antigens and T cells to be essential regulators for the switch from expression of IgM to IgG and IgA. Since the changes in expression of $C_H$ genes are already apparent at the precursor B-lymphocyte stage, it seems more pertinent to question the role of antigens and T cells in the initial surface expression of non-IgM classes. While a role for antigens and T cells in development of Ig-class heterogeneity at the B-lymphocyte level cannot be excluded, several observations mitigate against this possibility. First, normal proportions of IgM-, IgG- and IgA-bearing B lymphocytes are developed very early in human foetal life [7, 15], and are present in germ-free [13] and congenitally athymic mice [3] and humans [4]. Second, precursors for cells synthesizing IgG and IgA develop in cultures of mouse foetal liver established well before T cells could have been seeded to this site [17, 18].

Next we turn to the expression of the various classes of Ig during the terminal differentiation of $sIg^+$ lymphocytes to mature B cells producing large amounts of cytoplasmic immunoglobulin for secretion. Using sensitive immunofluorescence techniques to detect surface Ig most cells producing cytoplasmic IgM can also be shown to bear sIgM. In malignant clones of IgM-producing cells, as in patients with Waldenström's macroglobulinemia and in certain IgM-producing malignancies of mice, representatives of the malignant clone show persistent differentiation from $sIgM^+$ lymphoid cells to mature cells simultaneously expressing surface and cytoplasmic IgM [23]. All of these observations are consonant with the anti-μ-mediated inhibition of both primed and unprimed normal precursors of IgM-producing cells.

IgM may also be found on the surface of normal and abnormal clones of IgG-secreting cells [20, 27]. This may reflect the fact that « virgin » precursors of IgG-plasma cells continue to express sIgM and are thus inhibitable by anti-μ antibodies. In newborn mouse spleens, a small proportion of $sIgM^+$ cells also bear $sIgG_2$, and all of the $sIgG_2^+$ cells also stain for sIgM [10]. When cultures of such cells are stimulated with LPS, the proportion of $sIgG_2^+$, $sIgM^+$ doubles drops drastically over the first 3 days in culture and the numbers of $sIgG_2^+$ « singles » begins to rise. Many of the later cells also begin to express cytoplasmic $IgG_2$. This corresponds with the

loss of inhibition of cIgG$_2$ cells by anti-$\mu$ treatment after the third day of culture. The existence of a population of B lymphocytes expressing both $\mu$ and $\gamma$ receptors at the initiation of culture suggests that in this system switching may involve the loss of IgM-synthetic capacity by cells already precommitted to IgG synthesis rather than the development of IgG-synthetic capacity by cells bearing only sIgM.

Two alternate pathways have been proposed for the switch from IgM precursors to cells expressing IgA: a direct switch from IgM to IgA and an intermediate stage of IgG expression. The following findings are compatible with the first possibility. (1) In most experiments, anti-$\gamma$ treatment only inhibits development of IgG-producing cells [13, 16]. (2) All of the sIgA$^+$ cells in human foetuses and newborn mice also bear sIgM (unpublished). (Unfortunately, so far sIgG has not been sought on these cells). (3) IgM$\lambda$ has been observed on a malignant population of plasma cells containing IgA$\lambda$ [27]. (4) In a single patient IgM- and IgA-myeloma components in the serum were shown to share light chains, idiotypic determinants and identical amino acid sequences for the first few N-terminal amino acids [31]. Evidence compatible with an intermediate stage of IgG expression includes the following. (*a*) In one of several experiments, anti-$\gamma$ treatment of young mice caused profound suppression of IgG synthesis in half of the recipients; those mice which were suppressed for IgG synthesis were also suppressed for IgA. In other experiments it has not been possible to achieve complete suppression of IgG synthesis [13]. (*b*) During ontogeny in humans, mice and chickens, IgG$^+$ cells are developed before IgA$^+$ cells and, in the chicken, cells committed to the synthesis of IgG are seeded from the bursa before cells capable of IgA synthesis [11]. (*c*) In a CLL patient, dual populations of lymphocytes bearing either IgG$\kappa$ or IgA$\kappa$ were associated with a single serum monoclonal IgG of the same idiotype [26]. Surface IgG was found on cells with either cytoplasmic IgG or IgA, whereas surface IgA was present on cells with cytoplasmic IgA but not on those with cytoplasmic IgG, thus suggesting that the direction of the switch was from IgG to IgA synthesis.

## Developmental relations of IgD

Although poorly represented in serum, IgD is present on the surface of a large proportion of B lymphocytes, where it has been postulated to play an important role in antigen triggering of B-cell maturation [2, 19, 25, 28]. So far sIgD, or its homologue, has been demonstrated on B lymphocytes only in man, monkey and the mouse; mature IgD-secreting plasma cells have been found only in man. The vast majority of IgD serum proteins in man, but not of sIgD molecules, have $\lambda$ light chains. Several years ago, it was shown that most sIgM$^+$ lymphocytes bear sIgD and vice versa, but the developmental, relationships between sIgD and other classes of Ig have until recently remained unclear.

Since the monkey lacks serum IgD, and anti-human $\delta$ reacts with sIgD

on monkey lymphocytes, Pernis and associates were able to test the effects of anti-δ antiserum on monkey lymphocytes [19]. Following the injection of anti-human IgD, monkeys responded with an increase in serum IgM which was followed about a week later by a 5 to 10-fold increase in serum IgG. The increase in serum immunoglobulins was associated with the presence of antibodies against the serum proteins of rabbits, the species in which the anti-δ antibodies were made. The simplest interpretations of these results are that (1) antigens and anti-δ antibodies synergistically trigger the maturation of appropriate clones of B lymphocytes, and (2) IgD is present on the precursors for both IgM and IgG responses.

Using differential immunofluorescence, we have begun to examine these relationships at a cellular level in humans [7] and mice (unpublished observations). Such studies in mice have been made possible by the successful isolation of sufficient sIgD to produce a specific anti-δ antiserum in rabbits [1]. These investigations are still in progress, but the preliminary results appear to be of sufficient interest and potential importance to recount here. 1 Surface IgD first appears on a small proportion of $sIgM^+$ lymphocytes in 12-week human foetuses (*i. e.*, more than 2 weeks after $sIgM^+$ cells appear) and in newborn mice (*i. e.*, approximately 5 days after $sIgM^+$ cells appear). (2) B lymphocytes bearing either sIgG or sIgA appear at about the same time as those bearing sIgD. In fact, B lymphocyts bearing $sIgG_3$ and $sIgG_2$ in mice and sIgG in humans may precede the appearance of $sIgD^+$ cells. (3) Whereas all sIgG- and sIgA-bearing lymphocytes in very young human foetuses and neonatal mice also bear sIgM, very few of the $sIgG^+$ cells in newborn mice bear detectable sIgD. (4) Later in human and mouse development sIgD can be found in combination with sIg of all other classes. (5) The percentage of μ-, γ- and α-bearing cells that also express sIgD varies not only with age but with tissue distribution of B cells. Even in adult mice and humans, a relatively small proportion of $sIgM^+$ bone marrow cells are $sIgD^+$ (approximately 30-60 %), while in peripheral tissues usually more than 80 % of the sIgM cells bear sIgD. (6) No IgD-containing plasma cells were found in the mouse. (7) No $sIgD^+$ cells were detectable in mice treated from birth with anti-μ antibodies. Other results indicate that stimulated B lymphocytes and maturing B cells containing cytoplasmic IgM, IgG or IgA cease to express sIgD in significant amounts. For example, mouse spleen cells induced by LPS to synthesize large amounts of these cytoplasmic immunoglobulins no longer bear sIgD. As another example, virtually all of the many $sIgA^+$ cells in the highly-stimulated Peyer's patches bear neither sIgM nor sIgD in detectable amounts.

Complementary observations have been made in monoclonal human lymphoid malignancies (reviewed *in* [5]). CLL cells very often express both sIgM and sIgD. In Waldenström's macroglobulinemia, the lymphoid members of the clone bear both sIgM and sIgD, whereas the mature IgM-secreting cell members continue to express sIgM in easily detectable amounts but little or no sIgD [19 A]. In examples of both types of malignancies the sIgD and sIgM have been shown to bind the same anti-

gens [19 A] or to have idiotypic identity [6]. In the cases we studied, IgG- and IgA-producing clones of CLL cells or of malignant cells resembling those in patients with Waldenström's macroglobulinemia lacked detectable sIgD ([22] and unpublished observations). However the apparent combination of sIgA and sIgD has been observed on CLL cells from one patient (S. Kanner and D. Bull, personal communication) and that of $\gamma$ and $\delta$ chains on cells from a patient with $\gamma$ chain disease (unpublished observation).

## Tentative model of B-cell generation of Ig-class diversity

Taken together, these observations suggest a tentative model for the development of Ig-class diversity within B-cell clones in mammals. The first stage of B-cell development begins with expression of IgM at a cytoplasmic level to be followed later in development by surface expression of IgM. (It seems likely that clonal diversity of V-region genes will prove to be generated during the stage of cIgM expression only). The $sIgM^+$ cells give rise to cells committed to synthesis of IgM, IgG and IgA classes. All of these cells express their commitment to their respective Ig classes and subclasses by synthesis of the appropriate surface immunoglobulins while continuing to express sIgM. Around this time, sIgD as an additional antigen receptor begins to be expressed on the surface of all subclasses of B lymphocytes. When triggered by antigens, T-cell help and/or B-cell mitogens, the stimulated B cells are induced to become mature plasma cells or to divide and give rise to memory cells. During these proliferative and maturational processes, sIgD appears to be lost, sIgM synthesis may or may not be continued, depending upon the number of cell divisions, and continued synthesis of surface and cytoplasmic immunoglobulins will reflect the primary Ig commitment of the activated B cell.

The role of sIgD-antibody receptors thus would appear to be exerted primarily during the initial triggering of the unprimed B lymphocyte, and to a lesser extent, if at all, at the memory cell stage.

A genetic model whereby multiple copies of the selected V-region gene are made and inserted in front of each $C_H$ region and the switch in Ig-class expression determined by a programmed regulatory mechanism may be the simplest hypothesis to explain the intraclonal switches in $C_H$-gene expression. This is especially attractive in view of the instances in which two or more Ig classes with the same specificity are expressed by the same cell. As an alternative, stable mRNA could account for continued synthesis of $C\mu$ or $C\gamma$ after transcription may have switched to $C\gamma$ or $C\alpha$ respectively. For all of these classes, a single copy of the $V_H$ gene could suffice if sequential translocation occurred. This latter possibility invoking the expression of a single $V_H$ copy per chromosome does not easily explain the apparent simultaneous and continued synthesis of $C\delta$ in concert with $C\mu$

and perhaps Cγ and Cα as well, unless the IgD is transcribed from the homologous chromosome.

KEY-WORDS: Immunoglobulin, IgD, B lymphocyte, Immunogenetics; Developmental relations, Ig-class diversity, Switching.

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