UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

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FACULTAD DE QUÍMICA

ESTUDIOS SOBRE LA REGULACIÓN DE LA INMUNIDAD POR LOS MACROFAGOS

POR

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TESIS PARA OBTENER EL GRADO DE
DOCTOR EN CIENCIAS QUÍMICAS
(BIOQUÍMICA)

MÉXICO, D.F. 1979

Esta tesis es un resumen de las siguientes comunicaciones y revisiones.

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INTRODUCCION

En esta tesis presento un resumen de mi trabajo realizado sobre la participación de los macrófagos en la inducción inmune. Para ubicar estos resultados en el cuadro de funciones de esta célula haré una somera descripción de su fisiología. No presentaré un estudio de la extensa información bibliográfica sobre esta célula, solo referiré a los resultados experimentales más vinculados con nuestras observaciones e hipótesis de trabajo.

Los macrófagos o fagocitos mononucleares son elementos celulares que están implicados en la resistencia del huésped contra la infección y en la respuesta inflamatoria (1, 2). Originados de sus precursores que residen en la médula ósea circulan corto tiempo como monocitos y se adhieren en las paredes de los sinusoides, cavidades serosas y membranas alveolares en donde manifiestan cambios funcionales y en su estructura celular para transformarse en macrófagos.

Cultivados *in vitro* se adhieren firmemente a la superficie de la caja de cultivo y se expanden mediante prolongaciones y dobleces de su membrana plasmática, tomando una orientación polarizada. Su membrana celular es muy dinámica, ya que múltiples regiones se interiorizan contínuamente por invaginaciones para formar estructuras intracitoplásmicas que se conocen como vacuolas pinocíticas y fagosomas, sobre las cuales los lisosomas citoplasmáticos vierten su contenido enzimático para digerir las partículas endocitadas (3). No se conocen todavía los eventos biofísicos y bioquímicos de su membrana

plasmática durante la endocitosis, pero existe mayor información de las propiedades fisiológicas de sus receptores, como son aquellos que reconocen la región Fc de las inmunoglobulinas G, el complemento etc., sin embargo tampoco se sabe de la naturaleza química de estas moléculas receptoras.

El nivel intracelular de hidrolasas ácidas se incrementa a consecuencia de la endocitosis de solutos y partículas digeribles (4), estas enzimas se vierten de los lisosomas primarios a las vacuolas endocíticas y una fracción del 10 por ciento se secreta al medio extracelular. El macrófago secreta a otro grupo de enzimas, la lisozima (5), el activador del plasminógeno (6, 7), la elastasa (8) y la colagenasa (9, 10), de algunas de las cuales, hasta el 90% de su producción llega a secretarse al compartimento extracelular. También se ha descrito la secreción de varios polipéptidos y proteínas (11, 12, 13 y 14), el estudio de su naturaleza química se está realizando actualmente. Recientemente, varios investigadores, entre ellos nuestro grupo, hemos descrito en estas células la secreción de moléculas linfoestimuladoras (15).

Cuando los macrófagos endocitan un gran número de partículas, se llega a interiorizar hasta un 60% de membrana plasmática en el curso de una hora (16) las células se retraen y cesan su actividad pinocítica por 5 a 6 horas recobrándola posteriormente. Como resultado de este fenómeno, se genera un metabolismo aumentado y síntesis de nuevos productos de actividades biológicas definidas. A este estado fisiológico se le ha llamado macrófagos activados.

La introducción del antígeno a un organismo desencadena una serie de eventos que conocemos como respuesta inmune, que consiste en la interacción del antígeno con tres tipos celulares; los linfocitos derivados de la médula ósea (linfocitos B), los linfocitos derivados del timo (linfocitos T) y los macrófagos. Los dos primeros elementos celulares poseen en su membrana receptores específicos por el antígeno, en tanto que los macrófagos o fagocitos mononucleares son células inespecíficas las cuales no elaboran anticuerpos y sólo interaccionan con los antígenos, bien sea por adherencia a su membrana o por inmunoglobulinas específicas a través del receptor para Fc, fagocitando y catabolizando la mayor parte de estos antígenos capturados, y colaborando mediante algún mecanismo aún no definido en el proceso inductivo del reconocimiento inmune, tanto por la presentación de moléculas antigénicas que persisten en la membrana así como la secreción de moléculas linfoestimuladoras que potencian la respuesta inmune en los linfocitos.

La especificidad en el sistema inmune es una función exclusiva de los linfocitos implicados en la interacción con el antígeno. Antes del primer estímulo antigénico en un animal, existen linfocitos con receptores en su membrana (17), los cuales son inmunoglobulinas específicas por antígeno en los linfocitos B (18) en tanto que en los linfocitos T son proteínas estrechamente asociadas al grupo de antígenos de histocompatibilidad principal en cuya estructura contienen la región variable correspondiente a la cadena pesada de las inmunoglo-

bulinas, pero no la región constante (19, 20), por lo que la especificidad de una célula individual está restringida a un solo determinante antigénico. La respuesta inmune de los linfocitos solo se desarrolla en presencia de los macrófagos.

Los macrófagos no desempeñan un papel instructor. La fagocitosis fue un mecanismo primordial de defensa mientras que los elementos específicos, los linfocitos, aparecieron posteriormente. Analizando a los macrófagos en términos evolutivos, en las especies invertebradas su función es la fagocitosis para eliminar el material extraño. Posteriormente se desarrolló la inmunidad específica como un elemento necesario para complementar y hacer más eficiente la función de reconocimiento y fagocitosis para la eliminación y destrucción de aquellos agentes extraños y nocivos y así, evitar una respuesta inmune contra sus propios tejidos.

INMUNOGENICIDAD DE LOS ANTIGENOS UNIDOS A LOS MACROFAGOS.

El atrapamiento del antígeno en los órganos linfoides propicia su inmunogenicidad. Se ha descrito una correlación entre la velocidad de desaparición de un antígeno del compartimento extracelular en animales durante la inmunización, con el grado inmunogénico de ese antígeno. Las proteínas polimerizadas son más inmunogénicas porque se adhieren más a los fagocitos mononucleares, mientras que su contraparte monomérica es tolerogénica, estado fisiológico que se define por una ausencia dinámica en la respuesta inmune.

Los experimentos para el estudio de la presentación del antígeno se han realizado utilizando dos sistemas. Uno en el cual se determina la formación de anticuerpos con requerimiento de la participación activa de los linfocitos T y B. En el segundo sistema se registra el estímulo en los linfocitos T al reconocer el antígeno asociado a los macrófagos, y no es necesaria la presencia de los linfocitos B. En ambos sistemas se han obtenido resultados que conducen a la misma interpretación sobre el papel de los macrófagos en la inducción inmune, pero también hay observaciones que sugieren diferentes mecanismos de presentación del antígeno y activación por los macrófagos.

En experimentos *in vivo* se ha establecido que el antígeno asociado a los macrófagos resulta superiormente inmunogénico que cuando se inyecta el antígeno en su estado libre (21, 22). Estos estudios consistieron en obtener poblaciones de macrófagos peritoneales, exponerlos con antígenos radiactivos y cuantificar la respuesta inmune hacia este antígeno asociado a los macrófagos. Su inmunogenicidad se analizó valorando los niveles de anticuerpos en los ratones irradiados, a los cuales se les habían transferido macrófagos con antígeno o el antígeno en forma libre, suplementados con linfocitos B y T como células respondedoras, obtenidas de los nódulos linfáticos o del bazo de un ratón normal (23, 24).

Los resultados de estos experimentos claramente establecieron que el antígeno asociado en los fagocitos mononucleares fue muy inmunogénico, es decir que comparado con el antí-

geno administrado en forma libre en molaridad equivalente, indujo un nivel de anticuerpos hasta de dos órdenes de magnitud superior.

Analizando las razones de la potenciación del antígeno asociado a estas células en cultivos *in vitro*, en los que también es posible observar una respuesta inmune de los linfocitos en mezcla con las células accesorias, se definió que durante esta interacción celular, el antígeno asociado a los fagocitos fue el responsable de esta potenciación inmune, y este sinergismo se anuló por la adición del antígeno soluble (25), interpretando que existen dos formas físicas del antígeno en relación a su capacidad de inducir síntesis de anticuerpo, una inmunogénica, asociada al macrófago y la otra no inmunogénica, la forma soluble. El antígeno conjugado a la superficie de la caja de cultivo o a la de fibroblastos a la misma dosis que en los fagocitos no produjo una respuesta significativa, sugiriendo que los macrófagos no solo participan como un sitio focal de cohesión del antígeno para que concurran los linfocitos durante el reconocimiento, sino que además han de participar otros eventos fisiológicos de esta célula en el proceso de potenciación.

En estudios sobre los requerimientos celulares para la respuesta inmune en los que se han usado antígenos sintéticos con determinantes antigénicos más definidos, se encontró que el copolímero D-glutámico-D-lisina-dinitrofenilado, antígeno tolerógeno porque induce en el ratón un estado refractario de la respuesta inmune cuando se administra en su forma libre,

cuando se administró asociado a los macrófagos indujo buena respuesta inmune tanto *in vivo* como *in vitro* (26), indicando que los fagocitos mononucleares bloquean la expresión de la propiedad tolerogénica de algunos antígenos, generando otra señal diferente a la de su estado libre.

Si el reconocimiento antigénico por los linfocitos resulta más efectivo cuando el antígeno es presentado por los macrófagos, y habiéndose demostrado que los fagocitos endocitan y digieren antígenos autólogos y heterólogos en la misma proporción en la velocidad de endocitosis y digestión, esta propiedad en el procesamiento de proteínas incorporadas, no parece estar directamente relacionada con la inmunogenicidad del antígeno extraño. La inducción inmune debe ocurrir cuando el antígeno aún conserva su estructura nativa, ya que con antígenos proteicos globulares la respuesta inmune está dirigida preferencialmente a las determinantes conformacionales (27) y en proteínas conjugadas con haptenos se ha determinado que la inducción de colaboración entre linfocitos T y B ocurre solo cuando sus determinantes antigénicos, los del hapteno y portador conservan su estructura nativa (28).

Para que el antígeno en los macrófagos presente a los linfocitos su estructura nativa, se ha propuesto que existe más de un mecanismo.

Se ha determinado que el antígeno unido a la superficie celular del fagocito mononuclear contribuye notablemente en la inducción de la síntesis de anticuerpo. Así, mediante el sistema de transferencia celular a ratones receptores, en la

que se ha determinado el grado en que el antígeno asociado a los macrófagos propicia la respuesta inmune (29, 30), solo una fracción menor del 5% del inicialmente capturado, que persiste en la membrana, es a la que se le ha atribuido la propiedad de inducción inmune en tanto que los demás son degradados al nivel de aminoácidos durante las primeras 24 horas de haberse endocitado.

Estudiando el destino del antígeno endocitado por los macrófagos, en nuestros experimentos determinamos que el 25% de la proteína capturada por los fagocitos, persiste en su forma macromolecular durante más de 24 horas (31, 32). Simultáneamente una fracción de esta forma molecular es liberada al medio extracelular en forma parcialmente hidrolizada pero preservando sus principales determinantes inmunogénicos. Estas moléculas que encontramos en el medio de cultivo no derivó de un desprendimiento de las moléculas inicialmente retenidas en la membrana, ya que esta liberación también se observó cuando estas células se trataron con tripsina para eliminar el antígeno expuesto en la superficie (31). Tampoco el 2% de desintegración celular que se observó consistentemente durante el experimento fue el origen de estas macromoléculas antigénicas del medio extracelular, ya que su contenido total no superaba el 10% del antígeno liberado.

En experimentos aún no publicados determinamos que el antígeno liberado por los fagocitos manifiesta actividad inmunogénica al inyectarse a ratones, a pesar de su fragmentación parcial por la hidrólisis. Estos resultados y los del

grupo de Rosenthal y cols. (33, 34), quienes observaron en macrófagos de cobayo, que no hubo reducción en la proliferación inducida en linfocitos T con los macrófagos que portando antígeno fueron tripsinizados previamente para eliminar la fracción de la membrana, siendo que la inducción mitogénica está mediada de manera específica por el antígeno asociado con el macrófago; sugieren que el antígeno intracelular, protegido de la acción proteolítica, es expuesto en algún momento para su reconocimiento, como puede ser mediante exocitosis del antígeno, proceso que observamos en nuestros experimentos (31).

INTERACCION FISIOLOGICA DE LOS MACROFAGOS CON LOS LINFOCITOS T

Partiendo de las observaciones de Mackaness y cols. (2, 37), J. David (35) y B.R. Bloom y B. Benett (36) en el que establecieron que la acumulación de macrófagos en las zonas de inflamación está mediada por la interacción del antígeno en los receptores de los linfocitos T, de los cuales se secretan un conjunto de moléculas agrupadas como linfocinas, entre ellas el factor inhibidor de la migración de macrófagos MIF, el cual activa metabólicamente a estas células, cuya especificidad es por un residuo de fucosa (38); y considerando que la distribución histológica de los linfocitos T siempre está en la zona medular de los nódulos linfáticos con los macrófagos, ello nos sugiere que existe una colaboración muy estrecha de ambas células en ciertos eventos fisiológicos.

Por lo tanto, el objetivo de nuestro estudio fue determinar si los macrófagos al interaccionar con los linfocitos T secretan mediadores, los cuales inducen cambios en la fisiolo-

gía de los linfocitos T y de las células B.

El procedimiento que utilizamos para analizar la presencia de productos linfoestimuladores secretados por los macrófagos, consistió en la formación de una monocapa de macrófagos del exudado peritoneal y la búsqueda de actividades biológicas en el caldo de cultivo de 24 horas, eliminando las células por centrifugación y determinación de la proliferación de linfocitos tumorales, del timo de ratones normales, así como la síntesis de anticuerpos por los linfocitos B (39, 40).

Inicialmente observamos que la fracción sobrenadante del medio de macrófagos cultivados inhibió la proliferación de las células leucémicas de ratón EL-4, las cuales recuperaron su velocidad de proliferación normal al ser lavadas e incubarse en medio nuevo normal. Esta actividad inhibidora residió en un componente dializable, de bajo peso molecular (39). Varios autores han encontrado esta misma actividad en otros sistemas celulares (41, 42) la cual también se ha atribuido a moléculas con pesos moleculares de un rango de 250 a 800 daltons. En estudios posteriores (43) definimos que la naturaleza química del inhibidor de la proliferación de las células EL-4 fue timidina, determinado por su migración en tres diferentes sistemas de cromatografía en capa delgada y en filtración por gel; que los macrófagos expuestos con el precursor ^{14}C-formato sintetizaron este inhibidor (timidina) de novo, esta timidina se sintetiza y libera constantemente de los fagocitos mononucleares. Hasta ahora no conocemos otra célula normal en biología que secrete nucleósidos. Por lo tanto la base del fenómeno de

inhibición de la proliferación que observamos consistió en un bloqueo de la conversión de citidilato a desoxicitidilato. Este mecanismo sobre el bloqueo de la proliferación por timidina en otros sistemas celulares ya se había descrito (44, 45 y 46) y se había establecido que las cantidades necesarias de timidina para lograr este bloqueo fueron del orden de 10^{-2} a 10^{-4} M. En nuestro sistema celular, con la línea EL-4, el bloqueo se logró con soluciones de 10^{-6} M de timidina, ¡concentración que fue tres a cuatro órdenes de magnitud inferior! Esta susceptibilidad en estas células tumorales particularmente alta, explica porqué los medios sobrenadantes del cultivo de macrófagos redujeron el crecimiento en esta línea tumoral. Como era de esperarse, el efecto de la timidina secretada por los macrófagos sobre las células EL-4 se arrestó mediante la adición de 2-desoxicitidina al medio. ¿Cómo secretan timidina los macrófagos? Nuestra explicación se basa en los experimentos de Green y cols. (47) quienes obtuvieron líneas del cultivo de tejidos que carecen de la enzima timidina cinasa, en ellas hubo liberación de timidina al medio. Recientemente Stadecker y cols. en experimentos aún no publicados, determinaron que los macrófagos carecen de timidina cinasa.

Otros autores describen en los fagocitos mononucleares actividades inhibidoras de la proliferación (48) que residen en compuestos de bajo peso molecular, pero diferentes a la timidina. Sin embargo para su identificación química se requiere de mejores estudios para establecer su significado en la fisiología de los macrófagos.

Cuando analizamos la actividad biológica en sobrenadantes de cultivos de macrófagos ya dializados, eliminando así estos componentes inhibidores; observamos que estos indujeron una mayor incorporación de timidina radiactiva en cultivos de timocitos, hasta de una proporción de 30 veces sobre el nivel basal correspondiente al cultivo testigo que no se le adicionó este medio condicionado. Una actividad similar ya se había reportado en macrófagos activados con endotoxina (Lipopolisacárido A) inicialmente por Gery y cols. (49). Se hicieron 35 diferentes experimentos determinando en cada uno de ellos la dosis óptima del sobrenadante en cultivos por triplicado. El objetivo de este extenso trabajo fue para definir la frecuencia de aparición de sobrenadantes de macrófagos con actividad mitogénica alta o baja. Como se muestra en la tabla I del trabajo, "The modulation of lymphocyte functions by molecules secreted by macrophages, I Description and partial biochemical analysis", J. Exp. Med. 142: 151, 1975, se observó que los niveles de actividad mitogénica fueron muy variables, pero consistentes en su efecto. Posteriormente demostramos que el nivel de síntesis y secreción de este principio activo mitogénico se pudo inducir en los macrófagos mediante la activación de su metabolismo (50). Observamos que la exposición de partículas, como eritrocitos de carnero opsonizados, microorganismos de *Listeria monocytogenes*, a monocapas celulares de macrófagos, estas partículas fueron fagocitadas en gran abundancia, y como consecuencia de esta intensiva endocitosis, apareció en los sobrenadantes correspondientes un nivel de actividad linfoestimuladora de 5 veces superior cuando los monocapas celu-

lares se expusieron a los eritrocitos opsonizados y cerca de 20 veces mayor cuando tuvieron bacterias de Listeria, en relación con el cultivo testigo, donde no se promovió la fagocitosis.

Esta activación fue equivalente al aumento de secreción de la enzima activadora del plasminógeno (10) de elastasa (8) y colagenasa (9) en macrófagos activados por una carga fagocítica previa; sugiriendo que estas células, cuya función es la búsqueda y atrapamiento de antígenos, son activables cuando están sujetas a una intensiva endocitosis, induciéndose así una mayor secreción de principios activos los cuales por sus actividades biológicas modifican el medio ambiente extracelular vecino, repercutiendo especialmente en las células que tienen una estrecha interacción fisiológica, como son los linfocitos T. En condiciones basales cuando el metabolismo no está activado en los fagocitos, los niveles de secreción de la actividad mitogénica son muy bajos.

Definimos algunas características fisicoquímicas de este principio activo mitogénico. Mediante filtración en Sephadex resultó tener un peso molecular aparente de 15,000 daltons, fue termolabil, digerible por quimotripsina y papaína, sugiriéndonos que su estructura es una proteína catiónica por su movilidad electroforética (15). No hemos logrado determinar si esta molécula posee alguna actividad enzimática.

Con el propósito de valorar la secreción de esta molécula mitogénica en macrófagos activados *in vivo*, es decir obtenidos de ratones a los cuales se les había administrado 10 días antes una dosis subletal de 2×10^3 microorganismos de

Listeria intraperitonealmente, se tituló en el medio de cultivo una actividad mitogénica sobre timocitos hasta de 150 veces superior en relación al control. Las bases celulares de esta activación metabólica ya se habían definido anteriormente (51), es decir que los linfocitos T son los responsables de la activación de los fagocitos mononucleares. Por estas razones diseñamos una serie de experimentos para probar si los linfocitos T inmunes al interaccionar con el Ag, inducían en los macrófagos una mayor secreción de esta actividad linfoestimuladora. El protocolo experimental para este estudio consistió en lo siguiente: se adicionaron linfocitos T de ratón normal o ratones inmunes hacia el antígeno hemocianina de hemolinfa de lapa (KLH) conjugada con isotiocianato de fluoresceina (F-KLH), a capas de cultivo conteniendo macrófagos o ninguna célula adherente, incubándose esta mezcla celular durante 24 horas en presencia o ausencia del antígeno (F-KLH) y colectándose al término el caldo de cultivo de cada condición experimental para ser dializado. Se determinó la actividad mitogénica de todos estos sobrenadantes sobre cultivos de timocitos, cuantificando su incorporación de timidina tritiada. Los resultados mostrados en la tabla III del trabajo, "The modulation of lymphocyte functions by molecules secreted by macrophages. II Conditions leading to increased secretion", J. Exp. Med. 144: 155, 1976, indicaron que los linfocitos T en presencia del antígeno indujeron en el macrófago una mayor secreción del componente mitogénico para timocitos. Esta molécula tuvo su origen en los fagocitos mononucleares, ya que no se encontró en el sobrenadante del cultivo que contenía linfocitos T ex-

clusivamente. Este estímulo por los linfocitos T requiere de un contacto íntimo de célula a célula, gobernado por los antígenos de histocompatibilidad de ambas células en interacción, como lo describieron frecuentemente Farr y Unanue (52).

También analizamos si existía una relación entre la secreción de esta actividad que estimula la proliferación en linfocitos T, con la magnitud de la respuesta inmune; es decir, con la generación de células formadoras de anticuerpos, determinada en la respuesta inmune secundaria en cultivos *in vitro* por el método de Mishell y Dutton (53). Para ello determinamos si este producto de los macrófagos incrementaba la acción cooperativa de los linfocitos T sobre los B, amplificando la respuesta inmune secundaria contra el determinante antigénico que fue la fluoresceína, conjugado a dos portadores proteínicos diferentes entre sí, en los que había memoria inmunológica en los linfocitos hacia uno de ellos, la hemocianina (KLH).

En estos experimentos *in vitro*, que consistieron en el cultivo de linfocitos de ratones inmunes a la proteína KLH, incubados durante 4 días en presencia o en ausencia de este antígeno y conteniendo diferentes dosis de estos productos secretados por los macrófagos, se valoró el número de células formadoras de anticuerpos con especificidad por la fluoresceína, mediante el método de placas líticas de Jerne (54), usando eritrocitos de carnero conjugados con isotiocianato de fluoresceína. Estos resultados que se encuentran en la Figura 1 en el trabajo publicado en el J. Exp. Med. 142: 151, 1975,

mostraron un aumento en la generación de las células formadoras de anticuerpo, tanto en la producción de IgG como IgM. Este incremento fue superior cuando se usó el antígeno portador proteico KLH al cual había memoria inmunológica en las células T, ya que cuando otros cultivos equivalentes se utilizaron linfocitos tratados con suero inmune anti theta (el antígeno marcador de la membrana de linfocitos T) en presencia de complemento, se abatió la respuesta a niveles basales. También determinamos que esta actividad estimuladora de la síntesis de anticuerpos residió en una proteína de 15000 daltons. La demostración de si es la misma entidad molecular con la que estimula la proliferación en timocitos no se ha hecho hasta ahora.

Considerando la importancia de los linfocitos T como elementos mediadores para permitir la expresión biológica de esta molécula de 15000 daltons de los macrófagos, en el estímulo de la síntesis de anticuerpos; estudiamos el efecto de estas moléculas en los cultivos de células esplénicas de ratones atímicos Nu/nu, las cuales ya se sabía que no generaban respuesta inmune primaria *in vitro*. Encontramos que la adición de sobrenadantes de cultivo de macrófagos en su forma dializada y el antígeno eritrocitos de carnero, se indujo una marcada respuesta progresiva en el número de células formadoras de placa de la clase IgM con relación a la dosis utilizada, y en menor proporción también hubo un incremento en la clase de anticuerpos IgG, sugiriendo estos resultados que por acción directa de este factor linfoestimulador sobre las células B es posible magnificar

la respuesta inmune, sin embargo, la posibilidad de activación de linfocitos precursores no definidos pero presentes en esta línea de ratón, también es un posible mecanismo.

Estos resultados demuestran que los macrófagos ejercen actividades que regulan la fisiología de los linfocitos, tanto en la diferenciación celular independiente del antígeno (55) como en las funciones dependientes del antígeno en la que los fagocitos mononucleares presentan el antígeno en alguna forma más adecuada pero haciendo más efectivo su reconocimiento. Estas actividades de los macrófagos también están sujetos a la acción reguladora de los linfocitos. El ejemplo más claro de este fenómeno es el incremento de la secreción de actividades linfoestimuladoras por acción de los linfocitos T inmunes en presencia de antígeno.

No se ha establecido si todas las funciones referidas a los macrófagos son manifestación en una célula individual o de una población heterogénea de los macrófagos. Es posible también que la misma célula en su proceso de diferenciación exprese funciones individuales en cada etapa de su maduración. Para resolver estas dudas, es necesario primero identificar marcadores moleculares de la membrana celular y analizar si toda la población de fagocitos mononucleares los contienen, y de esta menra clasificar y aislar subpoblaciones a los que se les pueda determinar sus actividades biológicas en forma individual.

RESUMEN

En nuestro trabajo experimental determinamos que los fagocitos mononucleares secretan compuestos químicos que modifican la fisología de los linfocitos. El nivel de esta secreción depende de la actividad de fagocitosis así como de su interacción con linfocitos T activados específicamente por el antígeno, los cuales inducen en los macrófagos mayor producción de factores mitogénicos.

Las moléculas linfoestimuladoras secretadas por los macrófagos manifiestan varias actividades: 1) incrementan la síntesis de DNA en los linfocitos T, 2) inducen diferenciación en los linfocitos B hacia células sintetizadoras de anticuerpos y 3) promueven la maduración de los timocitos hacia linfocitos T maduros. Aislamos y caracterizamos parcialmente algunas de estas actividades. Identificamos a una proteína de 15000 daltons como mitogénica y estimuladora de la síntesis de anticuerpos en cultivos *in vitro*. Proponemos que la secreción de estos productos por estas células es una función importante en la inducción de la respuesta inmune.

Identificamos la secreción de timidina por los macrófagos y su acción de bloqueo en la proliferación de las células tumorales EL-4. En cuanto al destino del antígeno en los fagocitos mononucleares, determinamos que de un 5 al 20 por ciento del material endocitado es exocitado posteriormente en forma macromolecular, y proponemos que este evento puede ser otra forma de exposición del antígeno a los linfocitos para su reconocimiento.

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THE JOURNAL OF IMMUNOLOGY
Vol. 112, No. 5, May 1974

THE RELEASE OF ANTIGEN MOLECULES FROM MACROPHAGES: CHARACTERIZATION OF THE PHENOMENA[1]

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Accepted for publication December 17, 1973

Macrophages were pulsed with ^{125}I-labeled hemocyanin and then cultured in media devoid of any radioactive material. A great part of the cell-bound ^{125}I was released into the culture fluid in a form not bound to protein; however, a small amount (3 to 7%) was released as protein-bound ^{125}I. The released protein-bound ^{125}I was associated with live, viable macrophages and did not derive from dishes nor from lysis of dead cells. About ¼ of the released protein was immunochemically reactive with anti-keyhole limpet hemocyanin (KLH); the remaining ¾ most likely represents KLH in which the immunoreactive groups lost their original conformation. Trypsin treatment of macrophages did not affect the release of ^{125}I-KLH, suggesting that the product derives from the cell interior. A great majority of the ^{125}I KLH tends to be of smaller size than the original molecule. The release of KLH decreases with time of culture but can be reactivated if fresh medium is added. We conclude that the cell conserves a small pool of intracellular antigen in a form or in a compartment which is slowly catabolized; from this pool a small amount is slowly released into the cell exterior, probably by a process akin to endocytosis but in reverse.

The lymphoid cells that participate in an immune process can be separated into two groups: in one group are the lymphocytes of the T and B class, endowed with specific receptors restricted to one antigenic determinant and capable of proliferating and differentiating upon antigen stimulation; in the other are the macrophages, characterized by their capacity to handle many antigens in a nonrestricted manner and/or by participating in the inflammatory response characteristic of many immune processes. The handling of many antigens by macrophages represents an important—and positive—step favoring immune induction. On a molar basis, antigens taken up by macrophages are more effective than soluble antigen molecules. Indeed, macrophages appear to play an important regulatory role in immunity, in part because of their capacity to take up and handle antigens, therefore, determining the size of the immunogenic stimulus (1). In trying to understand the complex interactions which take place among antigen, macrophages, and lymphocytes, it is necessary to have a complete knowledge of the manner in which antigen molecules are handled by macrophages. Previous work indicated that the majority of antigen molecules taken up by macrophages were endocytosed and then subjected to extensive degradation with subsequent elimination (2-5). The catabolized products were not immunogenic (5). However, a few molecules remained for a finite period associated with the surface membrane in a form resembling their native molecules (6, 7). These surface molecules were immunogenic and explained in great part the immunogenicity of macrophage-bound antigen molecules (7, 8).

During the course of tissue cultures studies on antigen metabolism by macrophages, a second possible route of presentation of antigen to lymphocytes was envisioned (8-11). Culture supernatants from macrophages pulsed with radioactive antigens disclosed a consistent, albeit small, amount of material being released in a soluble form into the culture media. Such a release of antigens has not been adequately

[1] This work was supported by National Institutes of Health Grant AI 10091.

[2] Dr. Calderon is supported by an International Exchange Fellowship of the Public Health Service.

[3] Dr. Unanue is a recipient of a Research Career Award from the National Institutes of Health.

explained. Furthermore, it was not observed consistently with all protein or particulate antigens studied so far. For example, Cohn's group did not observe it with peroxidase (3); in the studies from our laboratory it was noted to differing extent among proteins like hemocyanins, albumins, or immunoglobulins. The present paper reports on a series of experiments which attempts to characterize this phenomenon of antigen release. We have used as antigen the large molecular weight protein hemocyanin that binds well to macrophage membranes. This protein is catabolized extensively after endocytosis, but a small amount is consistently released into the culture media. The experiments consisted of an analysis of fluids from cultures of macrophages exposed briefly to radioiodinated hemocyanin. Our experiments indicate a small and persistent release of hemocyanin molecules, some of which have a different size from the native antigen. This release appears to depend on actively metabolic cells.

MATERIALS AND METHODS

Macrophages. Macrophages were harvested aseptically from the peritoneal cavity of mice injected with 1.5 ml of 10% proteose-peptone (Difco Laboratories, Detroit, Mich.) i.p. The mice donating the macrophages were of the inbred A/St strain (West Seneca Lab., Buffalo, N. Y.) or outbred Swiss (Charles River Company, Boston, Mass.), of either sex, usually about 12 weeks of age. Three days after injection the mice were killed; the peritoneal cells were harvested by injecting i.p. 3 ml of minimal essential medium (MEM)[4] (Microbiological Associates, Catalogue No. 12-683) containing 10 units of heparin per milliliter and 10% (v/v) heat inactivated fetal calf serum (FCS) (Associated Biomedics, Buffalo, N. Y.) and withdrawing the fluid after a few minutes; the cells were centrifuged at 500 × G for 6 min, washed once (with MEM containing 20% FCS, 2 mM glutamine and 50 units of both penicillin and streptomycin) and suspended at a concentration of 10^7/ml.

[4] Abbreviations used in this paper: MEM, minimal essential medium; FCS, fetal calf serum; KLH, keyhole limpet hemocyanin; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; LDH, lactic dehydrogenase.

Antigen. We used the hemocyanin from *Megathura crenulata* (keyhole limpets) (KLH) obtained by ultracentrifugation of the hemolymph from the limpets. KLH was maintained sterile at 6°C and occasionally ultracentrifuged (in a Beckman Model L3-50 ultracentrifuge at 35,000 rpm for 2 hr in a 40 rotor) in order to remove "dissociated" material. When necessary, KLH was dissociated by alkaline pH (12).

Iodination. KLH and L-tyrosine were radioiodinated by a chloramine T method (13) with ^{125}I-Na (New England Nuclear Corporation, Boston, Mass.). After iodination, KLH was filtered through a Sephadex G-25 column in order to remove non-protein-bound ^{125}I and used immediately. More than 96% of the iodinated KLH was precipitable in 10% trichloroacetic acid (TCA). Specific activities were about 3 to 10 μCi per microgram. After iodination the solution of ^{125}I tyrosine was passed through an AG-1-XP anion exchange column (of 18 x 1.2 cm). L-Tyrosine was eluted with 0.1 M glycine-HCl buffer, pH 2.2. The iodinated product was analyzed on thin layer chromatography with MN cellulose 300 polygram plates (14). More than 98% of the iodinated product had an Rf value (0.29) corresponding to tyrosine. Isotope counting was done in a Packard gamma ray spectrometer at the appropriate settings; background counts for ^{125}I were about 30 cpm. The efficiency of our counter for ^{125}I was 65% which allowed us to detect as little as 5 to 10 pg of iodinated KLH material.

Culture of macrophages. 10^7 Cells in 1 ml of media were planted in plastic dishes (Falcon; of 35 x 10 mm) and incubated at 37°C in a 5% CO_2 in air incubator. The medium was MEM containing 20% FCS and supplemented with 2 mM L-glutamine and 50 units of penicillin and streptomycin. After 24 hr of incubation, the dishes were washed twice (this time with MEM buffered with 20 mM HEPES—from Microbiological Associates, Catalogue No. 17-737—in order to maintain a stable neutral pH outside the incubator). At this time, the dishes contained ¼ of the original number of cells planted (this was estimated on the basis of Folin analysis). More than 98% of the cells were typical well-spread macrophages forming an interrupted monolayer. After the washes, the dishes were incubated with ^{125}I (usually 10 to 20 μg for 1 hr at 37°C), then washed seven times with media and incubated at 37°C for various pe-

riods of time (after the fifth wash the release of counts was negligible). The release of macromolecular ^{125}I was studied in two time periods: an *early period*—consisting of the first 2 to 4 hr after pulsing with KLH; and a *late period*—consisting of the 24- to 32-hr period after pulsing with antigen; in these instances the macrophages were exposed to KLH, cultured for 24 hr, then the medium was collected, the cells were washed, and then cultured 8 more hr. When necessary, cells were trypsinized by incubation in 1.0 ml of MEM containing 100 μg of trypsin/ml (trypsin-TPCK, Worthington Biochemical Corporation, N.J.) for 20 min at 37°C and then washed with medium containing 20% FCS.

Examination of ^{125}I-labeled products. ^{125}I material was examined from culture supernatants or cells. Culture supernatants were spun at 500 × G for 6 min in order to remove any possible cells, and precipitated in 10% TCA; the precipitates were washed once in 10% TCA. ^{125}I material in precipitates and supernatant fluid was assumed to represent protein-bound and non-protein-bound I. The radioactivity associated with the cells (cell-associated I) was obtained after addition of 0.5 ml of 0.1 N NaOH to the cell monolayers; the cellular material was harvested after gently scraping it from the dish with a rubber policeman. On occasion, the cell-associated material was brought to pH 7 and then precipitated in 10% TCA in order to determine amounts of protein-bound and non-protein-bound ^{125}I.

Immunochemical studies. Two procedures were followed in order to study the immunochemical specificity of KLH present in culture supernatants: 1) was a coprecipitation method with supernatants from experiments in which macrophages were exposed to ^{125}I KLH to obtain specific or nonspecific immune complexes. This was done by incubating about 60 ng of ^{125}I KLH or the ^{125}I product from macrophage cultures with a solution containing antigen and an excess of antibody under conditions leading to the production of an insoluble precipitate. One solution had the specific complex and contained 80 μg of dissociated KLH and 200 μl of rabbit anti-KLH serum; the other solution, the control, contained a nonspecific complex made up of 60 μl of mouse serum and 170 μl of rabbit anti-mouse globulin. Solutions were incubated for 24 hr at 4°C after which they were spun and the precipitates were washed three times with cold phosphate-buffered saline (PBS); 2) in the second method supernatants from macrophages pulsed with non-radioactive KLH were studied for the KLH content by their capacity to block a standard ^{125}I KLH-anti-KLH reaction. Details of the procedure have been detailed (15). In brief, a standard curve was first set by incubating various amounts of dissociated KLH (10 to 400 ng) with 50 μl of a mouse hyperimmune anti-KLH serum (in a total volume of 400 μl) for 16 hr; aliquots of 200 μl of this first reaction were then incubated with 5 ng of ^{125}I KLH for 15 min at 37°C and 8 hr at 4°C; after this time 200 μl of rabbit anti-mouse Ig were added in order to precipitate all mouse Ig. The radioactivity associated with the precipitated globulin represented the ^{125}I KLH bound to antibody. The amount of anti-KLH serum employed binds about ⅓ of the ^{125}I KLH; this amount of ^{125}I KLH is blocked to different degrees by the addition of the various standard amounts of KLH. The capacity of the culture supernatant to inhibit the ^{125}I KLH-anti-KLH reaction was then studied and the inhibition, if present, compared to that produced by the known amounts of standard KLH. For both these procedures, the culture supernatants were first dialyzed against PBS and then concentrated about 10-fold by ultrafiltration.

Enzyme studies. Culture supernatants and cell extracts were assayed for their contents of lactic dehydrogenase (LDH) by the procedure of Wroblewski *et al.* (16), with pyruvate and NADH (Sigma, St. Louis, Mo.). For releasing the enzyme from the cell monolayers, these were treated with 0.2% Triton X-100 (Sigma) for 2 hr at 4°C after which the fluid was harvested and centrifuged at 11,740 × G for 15 min. Control studies indicated that the treatment with Triton did not affect the LDH values; also, LDH activity in culture fluids was not changed for at least 6 hr. The sum of the values of LDH in supernatants and cells was taken as the total (100%) content.

Analysis of ^{125}I products. The size of the ^{125}I products in culture supernatants or associated with macrophages was studied by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. Samples were incubated in 1% SDS at 37°C for 30 min and applied on a 12-cm long glass column containing 5% polyacrylamide gel. The material was run at 8 mA per gel for 4 hr.

The gels containing ^{125}I were frozen on dry ice, cut in 1.5-mm thick slices, and counted. Calculations of molecular sizes were done with standard proteins of known molecular weights (17).

RESULTS

The release of protein-bound ^{125}I. Macrophages were cultured for 1 hr at 37°C in media containing ^{125}I KLH, washed in order to remove the soluble KLH not bound to cells, and then cultured for various periods of time. Figure 1 shows the data of a representative experiment in which both culture fluids and cells were examined for their content of protein-bound and non-protein-bound ^{125}I. There is a progressive loss of radioactivity from the cell and a concomitant increase in the amounts of non-protein-bound ^{125}I in the culture fluids. Two points are worthy of emphasis: first is the persistence with time in the cell of protein-bound ^{125}I; indeed, at 24 hr the cell still has 11% of ^{125}I in macromolecular form; second is the appearance of protein bound ^{125}I in the culture fluid, in the experiment up to 3.70% of the initial cell-bound ^{125}I. The bulk of the radioactivity released as protein-bound ^{125}I (about ¾ of it) was already present during the first 2 hr of culture. This release of protein-bound ^{125}I into the culture fluids was noted consistently in all experiments, most of which examined the culture fluids at one or two time periods, usually 2 to 4 hr after exposure to ^{125}I KLH. The results of several experiments can be seen in the different Tables (Table II and Figs. 3-5).

Figure 2 and Table I show an interesting observation concerning the release of protein bound ^{125}I and for which we have, as yet, no explanation. In the same experiment of Figure 1 we also ran a series of dishes in which the media were changed after various periods of time and the culture was allowed to proceed for further time (in the group depicted in Figure 1, the dishes were cultured for the entire time periods without changing the media). Note that in conditions where the media were not changed, the release of protein-bound ^{125}I decreases markedly after 2 hr. However, the release persists if the old media (containing the radioactive products) are removed and fresh media are now added. This is clearly seen in the time periods between 2 and 8 hr where the media were changed every 2 to 4 hr. After 12 hr the media were not changed until 12 hr later, and the above results are not apparent. In the period from 2 to 4 hr of culture the rate of release (as cpm per hour) is 521 when fresh media were introduced at 2 hr; no new release was depicted in the culture in which the media were not changed. This increased release of protein-bound ^{125}I was not simply the result of handling the dishes in and out of the incubator for the purpose of changing the media. A control experiment was set up as follows: Macrophages were exposed to ^{125}I KLH for 1 hr at 37°C, washed and later separated into three groups: one was

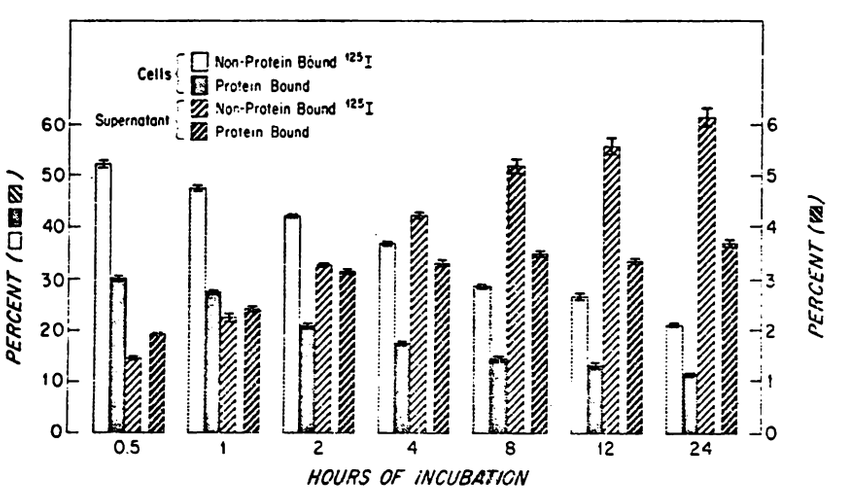

Figure 1. Experiment was carried out as described in *Materials and Methods:* 10^7 macrophages were planted for 24 hr, then cultured for 1 hr in media containing 17 μg of ^{125}I KLH (specific activity of 3 μCi/μg), washed and cultured for the times noted in the figure. Each value is the arithmetic mean of three dishes; standard error of the mean is indicated. The uptake of KLH after the 1-hr exposure at 37°C was 0.36% or 61.7 ng.

TABLE I

Release of protein-bound ^{125}I into culture supernatants[a]

No Change in Culture Medium				Culture Medium Changed		
Time of culture	CPM PB-^{125}I	Net release Time	CPM PB-^{125}I	Time of culture	CPM PB-^{125}I	Cumulative CPM PB-^{125}I
hr				*hr*		
0–0.5	4666			0–0.5	4471	
0–1.0	5703	0.5–1.0	1037	0.5–1.0	2176	6647
0–2.0	7586	1.0–2.0	1883	1.0–2.0	1710	8357
0–4.0	7544	2.0–4.0	0	2.0–4.0	1033	9390
0–8.0	7828	4.0–8.0	284	4.0–8.0	721	10111
0–12.0	7418	8.0–12.0	0	8.0–12.0	447	10558
0–24.0	8216	12.0–24.0	798	12.0–24.0	645	11203

[a] These are the same experiments as in Figure 2. The "No Change" column refers to dishes cultured for the entire span of time; the "Culture Medium Changed" column refers to a set of dishes cultured for 24 hr. but with changes in media as indicated. CPM, PB-^{125}I: Counts per minute of protein-bound ^{125}I in medium. Variations are indicated in Figure 2.

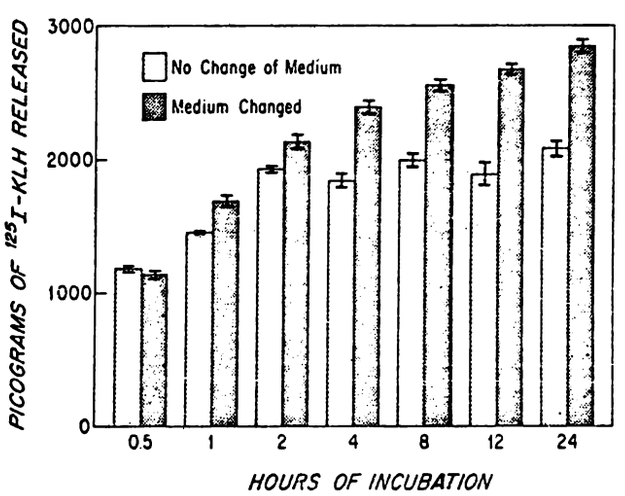

Figure 2. In the same experiment described in Figure 1, we included another set of dishes in which the culture medium was changed periodically and new medium was added. The changes in tissue culture took place at the indicated times. The graph shows the cumulative increase in protein-bound ^{125}I under both sets of conditions. Table I details this experiment.

left in the incubator for 4 hr; in a second, media were changed after the 1st and 2nd hr of culture; in a third group the dishes at 1 and 2 hr were taken out of the incubator, the media were aspirated with a Pasteur pipette but then placed back into the same dish. Hence, the second and third group were handled identically except that the fresh media were added in the second whereas the same media were added in the third group. There was an increased release of 20.8% of protein-bound ^{125}I (with a standard error of 1.4%) when the media were changed twice as compared to the group maintained in the incubator; the increase in the third group, however, was only 3.4% (with a standard error of 0.5%). Hence, the increase of the release of protein-bound ^{125}I was not as a result of the handling of the dishes during the change of media.

We next considered the effect of temperature in the release of protein-bound ^{125}I. Macrophages were exposed to ^{125}I KLH, washed, and cultured overnight. After 24 hr of culture, the media were removed, fresh culture media were added, and the culture continued for 8 more hours at 37°C, 22°C, or 4°C. The results are

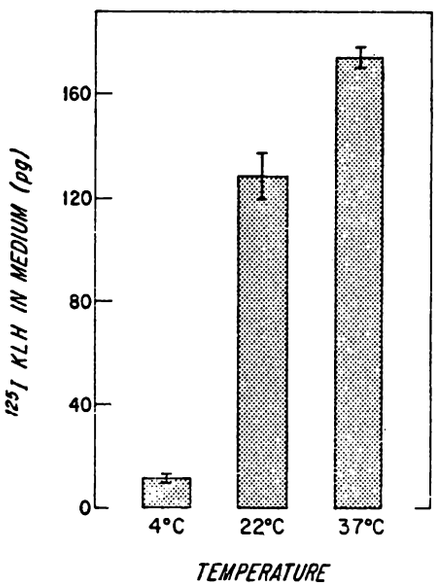

Figure 3. 10^7 macrophages were exposed for 1 hr to 18 μg of ^{125}I KLH (7.8 μCi/μg); washed, and cultured overnight; the medium was then changed, and the cells cultured 8 hr at the different times. Bars are arithmetic means of four dishes with standard error of the mean.

shown in Figure 3. There is a reduction of the release of protein-bound ^{125}I with a decrease in temperature—to 30% at 22°C, to 90% at 4°C.

The release of protein-bound ^{125}I as a function of the amounts of KLH bound initially to macrophages was investigated. A constant number of macrophages (10^7) were exposed to increasing amounts of ^{125}I KLH (1, 10, 100, and 500 μg) for 1 hr at 37°C, washed, and placed in culture for 4 hr. At this time the amounts of KLH bound to cells were 1.2, 12.7, 113.9, and 503.7 ng, respectively; under these conditions there was little reduction of the capacity of macrophages to take up KLH. The proportion of protein-bound ^{125}I released in 4 hr was similar for the four groups: 3.1% (40 pg), 2.8% (360 pg), 3.1% (3.6 ng), and 3.0% (14.96 ng), respectively.

The source of the released protein-bound ^{125}I. Several possibilities arise regarding the source of the released protein-bound ^{125}I into the media. A first one, raised in part by previous studies of Steinman and Cohn using a system similar to ours but with the protein horseradish peroxidase, is that the material may derive from protein loosely bound to the dish (3). A second possibility is that the material derives not from live cells but from cells that have died during the culture and that are partially or totally autolyzed. Experiments were set up to explore these two possibilities. First, ^{125}I KLH was added to a series of dishes containing a monolayer of macrophages or totally free of cells. Both sets of dishes were treated identically, i.e., washed after 1 hr exposure to ^{125}I KLH at 37°C and then placed in culture with media free of antigen for various periods of time. At the various times the culture supernatants were harvested and analyzed for ^{125}I radioactivity precipitable in 10% TCA. Figure 4 shows the results of six representative experiments comparing the amounts of macromolecular KLH in supernatants from empty dishes or dishes containing macrophages. The absolute radioactivity counts from the monolayer of macrophages have all been normalized to 100%. The results of Figure 4 are self-explanatory; there was some macromolecular ^{125}I released from dishes but this amount could not account for the release observed from dishes containing macrophages.

If the macromolecular KLH was released as a result of cell death and lysis, one would expect to find in the culture fluids chemical evidence from the lysed cell such as the cytoplasmic enzyme LDH. In a series of experiments supernatants from macrophages exposed to ^{125}I KLH were studied for their content of LDH and of macromolecular ^{125}I. Ten experiments are shown in Table II. There was no correlation between the release of protein-bound ^{125}I and the release of LDH; note two cultures that contained in the supernatant 2.0% and 2.2% of their total LDH but which only released 3.4% and 3.8% of protein-bound ^{125}I in contrast to one other which contained only 0.6% of LDH yet released the highest amount of protein-bound ^{125}I—7.1%. In a control experiment we lysed all macrophages previously pulsed with ^{125}I KLH by a brief exposure to hypotonic media (10 min) and then cultured in isotonic media for 4 hr. During this time the culture fluids contained 98% of the cellular LDH content and 21% of the ^{125}I as protein bound. Finally, the small amount of LDH released correlated well with the extent of cellular death as estimated by cytologic analysis; indeed as evidenced by trypan blue exclusion test no more than 1 to 2% of macrophages were dead during the 4-hr period of culture.

Hence, we concluded that the macromolecular ^{125}I present in the culture fluid was actually

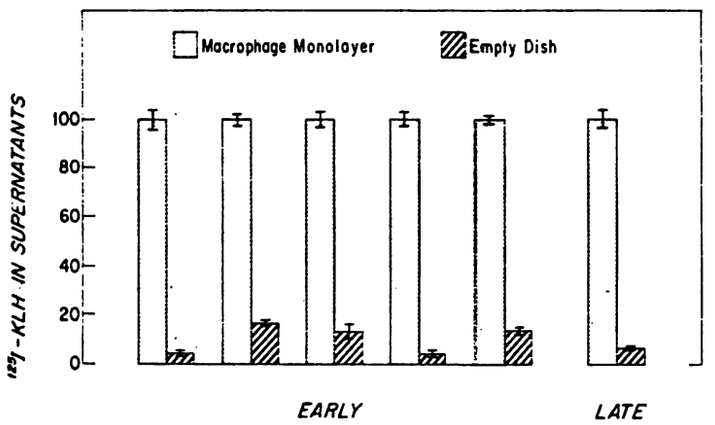

Figure 4. Empty dishes or dishes containing a monolayer of macrophages were exposed to 10 μg of ^{125}I KLH for 1 hr, washed, and then cultured. "Early" refers to cultures for 4 hr immediately following the pulse of ^{125}I KLH. "Late" refers to dishes cultured for 24 hr following the pulse, then washed, and cultured for 8 more hr. The cpm of dishes containing the monolayers of macrophages were taken as 100% value. Standard errors of the mean are included. Each experiment includes four dishes.

TABLE II

Relationship between LDH and protein-bound ^{125}I in supernatants[a]

Time of Incubation	LDH	Protein-Bound ^{125}I
	%	%
2	0.3	4.4
2	0.4	3.5
2	0.8	2.2
2	1.3	4.7
4	0.6	7.1
4	0.7	4.6
4	0.7	2.9
4	1.6	2.9
4	2.0	3.8
4	2.2	3.4

[a]Macrophages were exposed to media containing ^{125}I KLH for 1 hr, washed, and cultured 2 or 4 hr. The results represent the percentage of LDH or protein-bound ^{125}I in medium. 100% of LDH represents the amount obtained from cells lysed in 0.2% Triton X-100; 100% of ^{125}I represents the sum of all counts in macrophages and in supernatants. Each value represents the mean of two to four dishes with variations of no more than 15% over or under the mean. The interdependence of both parameters was assessed by Spearman's coefficient of rank correlation; no significant correlation was found ($r > 0.5$ $p < 0.6$). (The mean figure for total LDH activity = 687 ± 62 A.U. per dish.)

derived from live, active macrophages and not from protein loosely bound to dishes, nor from dead autolyzed cells.

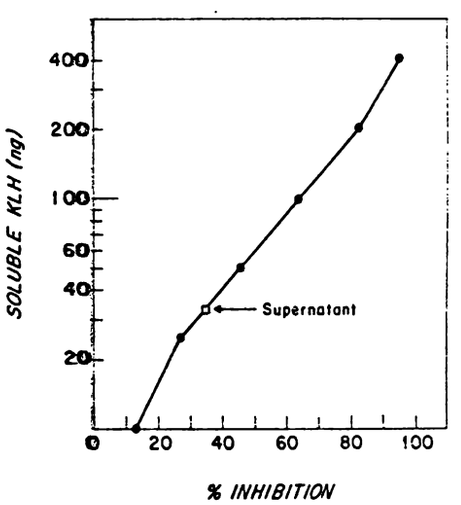

Figure 5. This graph depicts the degree of inhibition of an anti-KLH-^{125}I KLH reaction by increasing amounts of soluble KLH (curve with *closed circles*). The supernatant inhibited 35% of the reaction (*arrow, square*). This calculates on the standard curve to 33.5 ηg of KLH.

Immunochemical analysis of the protein-bound ^{125}I in culture fluids. The next problem that was investigated concerned the chemical nature of the released macromolecular ^{125}I and to this effect two sets of experiments were carried out. The first described herein studied whether the protein-bound ^{125}I was immunochemically related to the native ^{125}I KLH. The second experiment studied the molecular forms of the ^{125}I products by SDS acrylamide gel electrophoresis.

Aliquots of 24-hr culture supernatants from macrophages pulsed with ^{125}I KLH—dialyzed for 5 days against PBS in order to eliminate non-protein-bound ^{125}I—were added to a standard anti-KLH-KLH immune precipitation reaction or to an unrelated antigen-antibody complex as was described in the *Materials and Methods* section. Although 2.4% of the label was precipitated by the unrelated system, 20.6% of it coprecipitated in the anti-KLH-KLH reaction. In a different type of assay, macrophages were exposed to *non*-radioactive KLH for 1 hr at 37°C and then cultured for 4 hr. The supernatants were assayed for immunochemical KLH by their capacity to inhibit a standard anti-KLH-^{125}I KLH reaction. Note in Figure 5 the inhibition of a standard KLH-anti-KLH reaction by varying amounts of non-labeled KLH and the inhibition by the culture supernatant. The aliquot of culture supernatant inhibited 35% of the reaction which calculates to 33.5 ng of KLH. From previous experiments with ^{125}I, one expected the aliquot to contain 130 ng of ^{125}I protein bound. These calculations are explained in the text of Figure 5. Hence, we concluded from both results that about 20 to 25% of the protein-bound material released into the media was immunochemically identical to native KLH.

Since a great part of the released protein-bound ^{125}I will not bind to anti-KLH the possibility arises that it may not be related to the original native KLH that was bound to the cell but that it may represent cell proteins which have incorporated ^{125}I tyrosines or even some free ^{125}I through some oxidative process. Monolayers of macrophages (or empty dishes) were incubated with ^{125}I tyrosine for 4 hr; the concentrations of tyrosine, 2.7 ng, 27 ng, and 135 ng, were calculated to correspond 1-, 10-, and 50-fold to that present in the KLH bound to cells. (Under the usual experimental conditions, we estimated that cells contained 60 ng of KLH after 1 hr exposure; this amount of KLH contained 2.7 ng of tyrosine.) After this time, the culture fluid was studied for ^{125}I bound to protein. 0.82% and 0.84% of the total radioactivity in supernatants from macrophage monolayers and in culture media from empty dishes, respectively, was precipitable in 10% TCA. In another experiment the macrophages (or the empty dishes) were exposed to 0.006 or 0.060 μCi Na ^{125}I continuously from 4 to 24 hr and again the incorporation of the label into macromolecules in the culture fluid was studied. In these experiments no more than 0.6% of the ^{125}I from both sets of dishes was found in the TCA insoluble fraction. Hence, under our experimental setup we could not obtain evidence for incorporation of ^{125}I tyrosine or Na ^{125}I into a protein-bound fraction.

A different experimental setup was carried out with macrophages treated with puromycin in order to inhibit protein synthesis. Twenty-four-hour cultured macrophages were incubated for 8 hr in 1 ml of media containing 2 μg of puromycin per milliliter (18), then exposed to ^{125}I KLH for 1 hr, washed, and now cultured for 4 more hr. The uptake of KLH by normal and treated macrophages was identical; the amounts of protein-bound and non-protein-bound ^{125}I released in 4 hr, likewise, were identical in both cultures. The amounts of protein-bound ^{125}I was 3.0, 3.1, and 4.2% in the three control dishes, and 4.1, 4.1, and 4.6% in the puromycin-treated group. The amounts of non-protein-bound ^{125}I were 29.0%, 31.1%, and 30.9% in controls and 25.7%, 27.4%, and 27.9% in the puromycin-treated group. Hence, this result supported the previous experiments and strongly suggested that the ^{125}I protein-bound in the regular experiments was not the result of incorporation of labeled amino acids into newly synthesized protein.

We concluded that the protein-bound ^{125}I in supernatant was indeed KLH but in two forms: one which conserved its antigenic determinants, and a second which was unreactive, probably as a result of a change in the original conformation of the immunoreactive groups.

Effects of trypsin on the release of ^{125}I KLH. Most of protein-bound ^{125}I bound to macrophage surfaces can be released after trypsinization of the cells (6). We tested whether trypsin treatment would affect the release of protein-bound ^{125}I. Macrophages were exposed to ^{125}I KLH for 1 hr at 37°C at which time a set of dishes was trypsinized for 20 min, whereas the remaining was just exposed to media; both sets were placed in culture and the distribution of radioactivity studied at various time periods. In the 20 min of incubation, 22.6% of the ^{125}I KLH was removed by trypsin treatment. We assume that this amount, in great part, represents KLH present on the surface at the end of the 1-hr pulse. After culture, the release of protein-

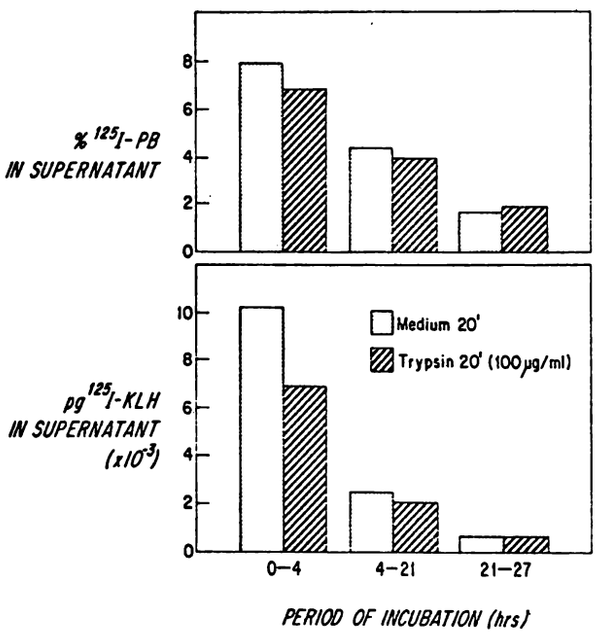

Figure 6. The graph depicts the release of ^{125}I KLH following trypsin treatment. In the top graph the values are expressed in absolute amounts; in the lower graph, in values relative to the total content of the cell.

bound ^{125}I was not affected by trypsinization, that is both controls and trypsinized cells released the same amount of protein-bound ^{125}I relative to their total content—about 2.8%. Figure 6 details these results.

Size of the released ^{125}I KLH (Figure 7). KLH is a large polymer of varying molecular sizes (up to 9 × 10^6 daltons), presumably made up of subunits of about 60,000 molecular weight (19). SDS polyacrylamide gels were made of the native KLH, of the KLH released from the cell during a 24-hr culture, and of the KLH remaining in the cell after this period of culture. The KLH remaining in the cell was heterogenous in size but formed mostly by large molecular size material. The KLH released into the media was also heterogenous but made of smaller molecular size (note small amount of material in the 0 to 20 fractions). In other experiments the culture supernatants obtained after various times of culture were studied. There was no significant difference among supernatants obtained from early, i.e., 0 to 4, 4 to 12, or late culture, 12 to 24 and 24 to 48 hr.

DISCUSSION

In these experiments we observed that a small amount of the antigen taken up by macrophages was released into the extracellular milieu in a partially degraded form. In trying to explain this phenomenon, we first set up a series of experiments for the purpose of establishing its veracity. Was the release of antigen a simple tissue culture artifact, or was it a true phenomenon associated with live, viable macrophages? Since the amounts of KLH released by macrophages were small, there was a possibility that such small amounts could be the result of the culture procedure. For example, KLH could be bound to the surface of the dish not covered by macrophages and released slowly thereafter; or it could be derived from cells that died during culture and which released their intracellular contents. Two sets of experiments were set up to analyze such alternatives. In one, we studied the release of KLH from empty dishes and found an amount too small to account for the results with live cells. In the second, we assumed that if KLH was released by cell lysis then a concomitant release of the cytoplasmic enzyme LDH should take place. No correlation could be established between both, which makes us conclude that the KLH release is selective in the sense that it doesn't correlate with the release of a cytoplasmic enzyme LDH (nor for this matter, with acid hydrolases; results to be published). These results, together with information obtained in the experiments involving the effect of temperature and of changes in media, make us conclude that the

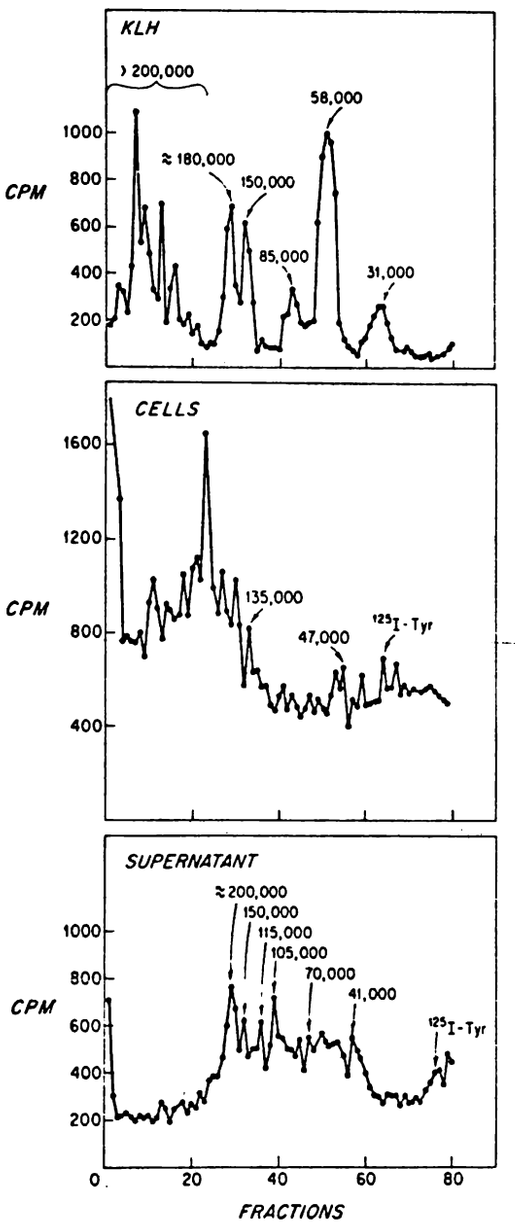

Figure 7. SDS polyacrylamide gel patterns of ^{125}I KLH and of cells and supernatant after pulsing with ^{125}I KLH. Culture medium was taken after 24 hr of culture.

KLH release is a true process associated with live, viable, and metabolically active macrophages.

Where does the released KLH derive from? The KLH could obviously come from two possible sources—the cell surface or the cell interior. A few years ago we reported that not all KLH bound initially to the surface suffered endocytosis and that few molecules remained at the surface for finite periods (6, 7). It is entirely possible that such molecules could be slowly detached from the cell surface and account for the present results. Since a great part of these residual surface molecules are removed by trypsin, we decided to study the release of KLH subsequent to treatment with this enzyme. Trypsin treatment did not affect the release of ^{125}I KLH, leading us to suspect strongly that the released molecules most likely derive from the cell interior. This suspicion is supported by the analytical study of the released KLH molecule which shows partial fragmentation of them. The above experimental results, however, do not firmly establish the intracellular origin of the released antigen. One could still postulate that the released KLH molecules were never interiorized but had remained on the surface, were partially fragmented from spill out of lysosomal enzymes, and moreover, were insensitive to removal by trypsin. Altogether, this explanation seems unlikely to us. Our working hypothesis is that the released KLH derives from the intracellular pool of partially degraded material. It is of interest to note that this is a relatively large pool of antigen which persists in the cell for long periods and perhaps in some way or form separated from rapid catabolism. The release of KLH from the intracellular pool to the exterior could be akin to a process of reversed endocytosis postulated by some to explain the release of lysosomal enzymes (20, 21).

The factors that regulate the KLH release are not clear. We reported one experiment (Fig. 2) where the release of KLH decreased with time in the presence of the culture media. In experiments to be reported, we have observed that the KLH release is also reduced if macrophages are allowed to pinocytose other materials. Further analysis of these types of experiments plus the study of macrophages at different stages of maturation or activation might give us some clue on the nature of the release process.

The phenomenon described herein may not be easy to study with all foreign proteins. For one, the degree of release seems to vary among different proteins. For example, in our experience we have seen it occur well with hemocyanins, aggregated albumins, or various hapten conjugates of bovine γ-globulin, but to a very small extent, if at all, with some synthetic

polymers made of D amino acids. It may be difficult to observe with proteins that are poorly taken up by macrophages or with proteins which bind with high avidity to the surface of culture vessels and are subsequently eluted off, obscuring any positive results. These may be the reasons for the difficulties of Steinman and Cohn to observe the release of horseradish peroxidase (3). Peroxidase is taken up about 50 times less than KLH, and furthermore, some of it binds nonspecifically to dishes.

The biologic significance of the phenomenon of antigen release needs to be established. Askonas and Jaroskova (9, 10) envisioned it as a mechanism for slow release of immunogenic material; Humphrey (22) speculated that if operative with nondigestible antigens it could serve to maintain tolerance; one of us (E.R.U.), with Cruchaud (11), envisioned it as a possible route by which large phagocytosed materials could be liberated for recognition by lymphocytes. Along these lines it is of interest to note that partially degraded antigens have been shown to be good inducers of delayed sensitivity reactions (23, 24). In summary, it is possible that this release may have different biologic significance depending upon the antigen in question. It is also possible that it may be totally irrelevant. However, now that we recognize it as a true biologic process one is in a better position for designing more meaningful experiments.

Acknowledgment. We appreciate the advice given to us by Prof. Manfred L. Karnovsky.

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11, pp. 4273–4277, November 1974

bitor of Cell Proliferation Released by Cultures of Macrophages

line incorporation/leukemia cells/concanavalin A/spleen cells)

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d by Baruj Benacerraf, August 16, 1974

Culture fluids from mouse peritoneal ls inhibited [³H]thymidine incorporation by, ration of, EL-4 leukemia cells, 3T3 cells, and mulated spleen lymphocytes. Inhibited EL-4 lls recovered their normal proliferative capacity ed and incubated in normal medium. The in- tivity resided in a low-molecular-weight sub- . could be absorbed by incubation with the . This substance was dialyzable and resistant digestion and phosphodiesterase treatment. nuclear phagocytes in the peritoneal exudate ae the source of the inhibitor. The inhibitory as found in the same amounts in exudates of e or mice injected with peptone or infected with *nocytogenes*; spleen cells adherent to plastic e inhibitor but in lesser amount. We suggest nhibitor may contribute to the deleterious d when various cells, including neoplastic ones, d in the presence of macrophages.

nclear phagocyte system comprises circulating and cells, monocytes and macrophages, respectively, from bone marrow precursors. They are endowed operty of phagocytosis and degradation of extra- ials (1). A wide variety of functions has been as- he mononuclear phagocyte—phagocytosis and of microorganisms, indeed an essential element in ense against infection, killing of tumor cells, inter- immunocompetent lymphocytes, elimination of d dead materials in inflammation, etc. Phago- ecomplish these diverse functions by two possible ot mutually exclusive: by direct contact with the t (as it necessarily happens in phagocytosis of d by the release of pharmacologically active ma- t mononuclear phagocytes can release biologically materials seems unquestionable at present, al- ost cases the chemistry of the putative substances, on, and the mechanisms of synthesis and release een explored. There are reports of activities in cultures that promote lymphocyte functions ulate growth and differentiation of stem cells (5), ultiplication (6), or kill cells or bacteria (7, 8). on process in macrophages has now been well d in the studies of Unkeless *et al.* (9), and Gordon

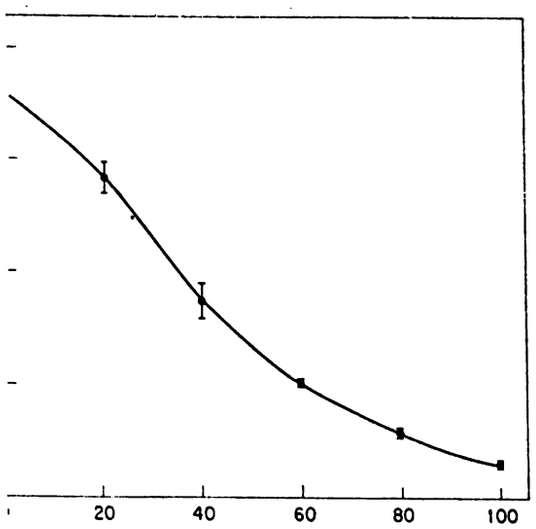
ttention in this report to an activity in cultures of s that stops the growth of various cells without ling them. This inhibitory activity is absorbed by cell, thus removing it from solution, and it is as- sociated with a dialyzable low molecular weight substance. The effect on cells is readily reversible upon removal of the inhibitor.

s: PEC, peritoneal exudate cells; i.p., intraperi- ymphocytes, thymic-derived lymphocytes.

MATERIALS AND METHODS

Culture of Peritoneal Exudate Cells (PEC). Cells were obtained aseptically from the peritoneal cavity of normal mice, of mice injected three days before with 1.5 ml of 10% proteose-peptone (Difco Laboratories, Detroit, Mich.) intraperitoneally (i.p.), or of mice injected with *Listeria monocytogenes* organisms i.p. [2×10^3 organisms 7 days before; the 50% lethal dose (LD_{50}) was 6×10^4]. In the latter two instances, the macrophages were presumed to be "activated" on the basis of their cytological characteristics. The mice were of the inbred C57Bl/6J strain (Jackson Laboratories, Bar Harbor, Me.), males, 10–15 weeks old. 10^7 freshly harvested PEC in 1.0 ml of culture medium were planted in 35 × 10-mm plastic dishes (Falcon Plastics, catalogue no. 3001) and incubated at 37° in 5% CO_2 in air incubator. The medium was RPMI-1640 (Associated Biomedics, Inc.) supplemented with 5% fetal calf serum, 2 mM glutamine, 50 units and 50 μg of penicillin and streptomycin, respectively, and 1% v/v of sodium bicarbonate solution (7.5% w/v, Microbiological Associates, catalogue no. 17-613). After 20 hr of incubation the cell monolayers were washed three times with medium, and, in most experiments, reincubated in 1 ml of culture medium for 48 more hours. More than 90% of the cells attached to the dishes were well-spread macrophages forming an interrupted monolayer. Fluids were obtained at various times after initiation of cultures, centrifuged at 500 × *g* for 10 min in order to remove detached cells, and filtered through sterile filters of 0.45 μm pore diameter (Swinnex-25, catalogue no. SXHA 02505, Millipore Corp.). We tested the capacity of the PEC culture fluids to influence the growth of various kinds of cells: EL-4 leukemia, spleen lymphocytes stimulated by mitogens, 3T3 fibroblasts, and polyoma-transformed 3T3 fibroblasts. These various cells were cultured in the presence of graded dilutions of the PEC culture fluids and examined for their incorporation of [³H]thymidine after several hours. (The medium was the same one described above, i.e., RPMI-1640 with 5% fetal calf serum and supplements.)

EL-4 leukemia cells of C57Bl/6J mice were maintained by *in vivo* i.p. passages. They were harvested from the mice and cultured at a density of 5×10^5 cells per ml of culture medium in 12 × 75-mm plastic tubes (Falcon Plastics, catalogue no. 2054). Four hours before the end of the incubation, 1 μCi of [³H]thymidine in a 50 μl volume was added to the culture (2 Ci/mmol, catalogue no. NET-027A, New England Nuclear



This graph shows the inhibition of [^{3}H]thymidine tion in EL-4 cells. EL-4 cells (5×10^5) were incubated ulture medium with graded concentrations of super- btained from 48-hr culture of PEC from mice injected cose-peptone. Here and in the following figures, error ate one standard error of the mean.

ton, Mass.). Trichloroacetic-insoluble fractions from ured cells were collected on glass fiber filters in a manifold (Millipore Co.) and radioactivity was l in a liquid scintillation spectrometer. Each experi- roup was done in triplicate.

cells from C57Bl/6J mice were cultured with con- A or *Escherichia coli* lipopolysaccharide by standard es.

roblasts and polyoma virus-transformed fibroblasts ained from Dr. Thomas Benjamin of our department.

ls. The important reagents were 3′:5′-cyclic phos- rase (P-0134, Sigma, St. Louis, Mo.), N^6,$O^{2'}$-di- denosine-3′:5′-cyclic monosphosphoric acid (D-0627, trypsin-TPCK (Worthington Biochemical Corp., ey), and trypsin inhibitor (T-9003, Sigma), concana- twice crystallized (79-001, Miles Laboratories, Ill.), (lipopolysaccharide from *E. coli* obtained from Difco ichigan).

RESULTS

tion of Cell Growth. The PEC culture fluid consistently the proliferation and growth of the various cells. A ny of the experiments were done with EL-4 leukemic which the inhibition of proliferation was striking. In iment shown in Fig. 1, the EL-4 cells were cultured in different amounts of PEC culture fluids. If the edium was made entirely of the PEC fluid, there was % inhibition of [^{3}H]thymidine incorporation; as little PEC culture fluid, the smallest amount used in the ex- , inhibited about 20%. Similar results were obtained ing EL-4 cells 24 or 48 hr in the PEC fluids. Fig. 2 he growth of EL-4 cells in PEC culture fluids and in nedium. The number of tumor cells cultured in PEC reduced to 52% and 31% of the normal values at 24 hr of incubation, respectively. The mitotic indexes at times were 2.3%, 2.3%, and 1.5% at 24, 48, and 72 culture in normal medium and 0.6%, 0.7%, and 0.9% ture in PEC fluids.

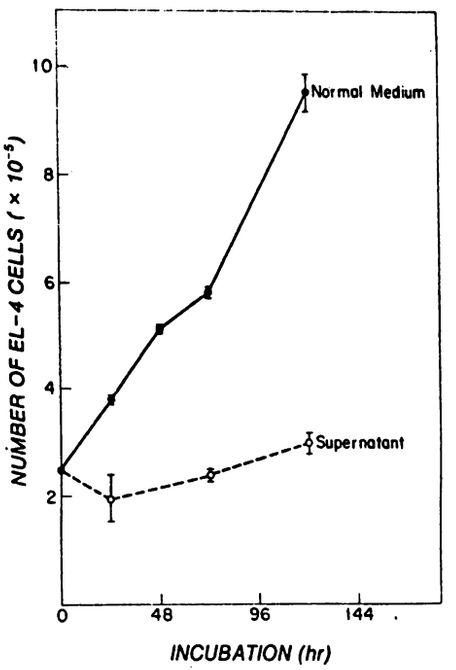


FIG. 2. This graph depicts the growth curves of EL-4 cells incubated in normal medium or supernatants from macrophages. The culture tubes containing 2.5×10^5 cells were incubated in normal medium or undiluted supernatants at 37°.

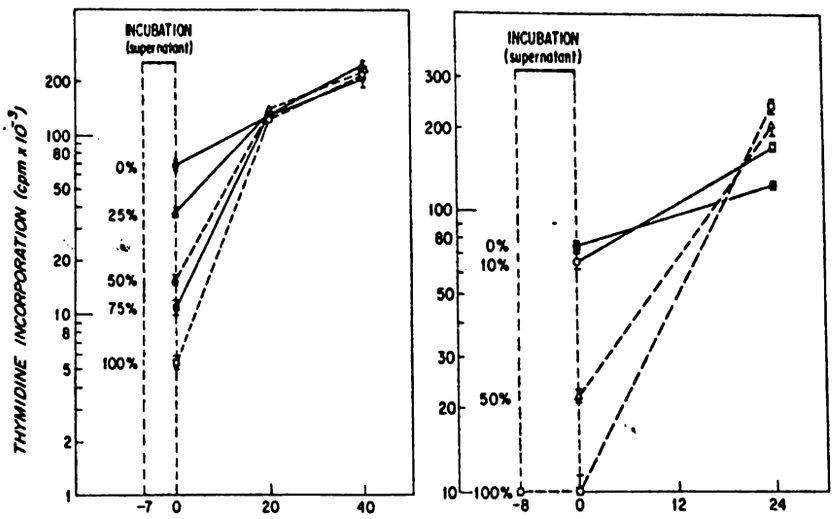
The inhibitory activity of the PEC fluids was also noted in cultures of normal spleen lymphocytes stimulated by addition of concanavalin A or *E. coli* lipopolysaccharide (Table 1). In one experiment normal and polyoma-transformed 3T3 cells 14 hr after being planted in a dish were inhibited 64% and 40%, respectively, in their incorporation of [^{3}H]thymidine. In unpublished experiments the PEC fluids markedly inhibited the production of antibody-forming cells in cultures of spleen cells with antigen (sheep erythrocytes) following the procedures of Mishell and Dutton (11).

Cytostatic Effects of PEC Culture Fluids. We investigated the behavior of EL-4 cells incubated with PEC fluid if later washed and incubated in normal medium (Figs. 3 and 4). In the two experiments in Fig. 3, EL-4 cells were cultured with various dilutions of PEC fluid for 7 or 8 hr, washed, and then incubated in normal medium for 19–48 hr, the last four in the presence of [^{3}H]thymidine. (The studies detailed previously indicated a marked inhibition of [^{3}H]thymidine incorporation by culturing from 8 to 24 hr; the period of 7–8 hr was selected

TABLE 1. *[^{3}H]Thymidine uptake by spleen cells in the presence of PEC fluids*

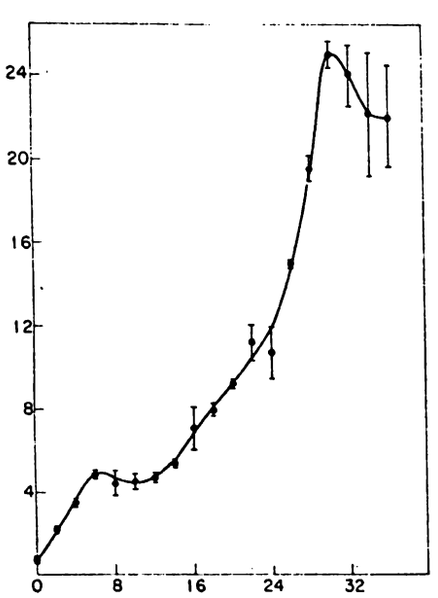
Addition to culture	cpm of [^{3}H]thymidine at 72 hr
None	7,580 ± 1,039
Concanavalin A, 1.5 μg	126,405 ± 10,350
Concanavalin A, 1.5 μg + PEC fluid, 100%	18,102 ± 957
Lipopolysaccharide, 5 μg	43,910 ± 3,573
Lipopolysaccharide, 5 μg + PEC fluid, 100%	6,359 ± 205

Spleen cells (1.5×10^5) were cultured for 72 hr; [^{3}H]thymidine was present for the last 12 hr of culture. Figures represent cpm in trichloroacetic acid-insoluble fraction (± one standard error of the mean). All results are means of triplicate cultures.



Two experiments demonstrating the restoration of [³H]thymidine incorporation by EL-4 cells after incubation in PEC res of 5 × 10⁵ cells were preincubated in normal medium or with different concentrations of supernatants from 48-hr culture n the cells were washed once and incubated in normal medium for the periods of time indicated.

l considerations.) There was a marked inhibition nidine incorporation during the first 8 hr in which lls were placed in PEC fluid, but the proliferative as regained after culture in normal medium but) fluids (data not shown). The kinetics of recon- the proliferative activity were studied in cultures ncubated at 2-hr periods with [³H]thymidine for . In Fig. 4 one can note a brief early rise in [³H]- incorporation followed by a lag period of about 8 steady increase in the incorporation of [³H]thymi- kinetics of incorporation of [³H]thymidine into shown in Fig. 4 are complicated, and it would be

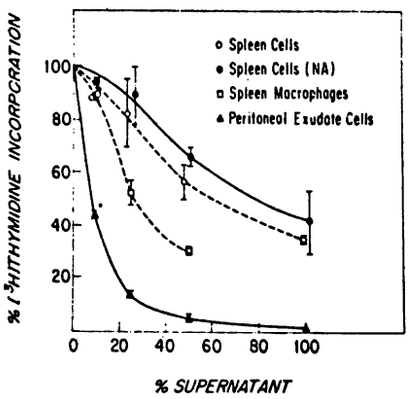


The figure shows the kinetics of [³H]thymidine in- of EL-4 cells after their release from inhibition. 5 × 10⁵ cells were incubated with supernatants of s for 20 hr, washed once, and incubated in normal he progression of nucleoside incorporation was as- y 2-hr pulses with 1 μCi of [³H]thymidine every 2 hr e cultures. The inhibition induced during the pre- with supernatants was 99%.

premature to try to interpret them. Suffice to say that removal of the inhibitor does allow the cells to regain their capacity to incorporate thymidine.

Cells Responsible for Inhibitory Activity. A most likely candidate for the inhibitory activity of the PEC fluid is the macrophage, since it is the most abundant cell in such fluids. Inhibitory activities, however, have been ascribed to activated thymic-derived (T) lymphocytes, also present to a small extent among the PEC (12). We tested for the possible contribution of the T cells by comparing the activity of fluids of normal PEC to PEC deprived of T lymphocytes by treatment with anti-θ serum and complement. Treatment with anti-θ and complement kills thymic-derived lymphocytes. The inhibitory activity of PEC treated with anti-θ was the same as that of untreated PEC, ruling out the role of T cells in the phenomenon. In this experiment fluid from untreated PEC inhibited the incorporation of [³H]thymidine by 92% (±0.7). Fluids from anti-θ-treated PEC inhibited 89.7% (±0.2%).

A further extension of this kind of experiment is shown in Fig. 5, which compares the inhibitory activity on EL-4 cells of culture fluids of PEC to those of spleen cells that adhere to dishes, of normal spleen cells, and of spleen cells deprived of macrophages, on growth of EL-4 cells. (All fluids came from 24-hr cultures of cells. Spleen cells were cultured, in suspension, on dishes at a density of 10^7 per ml. After culture, the suspension was centrifuged, and the fluids were obtained and tested. Nonadherent spleen cells were obtained after 1 hr of culture of 10^7 spleen cells in a 35 × 10-mm dish. This procedure was repeated three times in order to obtain a confluent monolayer of cells to be used as adherent cells.) The PEC cultures were strongly inhibitory, followed by the spleen adherent cells and normal spleen cells. The amounts of culture supernatants that produced 50% inhibition were 8%, 28%, 62%, and 72% for PEC, spleen adherent cells, normal spleen, and spleen cells depleted of adherent cells, respectively. We interpret this to indicate that the inhibitory activity is a property of cells adhering to dishes but more actively represented in peritoneal exudate than in normal spleen sus-



. This figure shows the release of inhibitory material PEC (▲), normal dish-adherent spleen cells (□), total lls (O), or spleen cells after removal of adherent cells (●). ls were obtained after 24-hr incubation with the cells. eriment was done with EL-4 cells incubated 8 hr in the

Since macrophages in the peritoneal cavity tend ıore mature and differentiated than those found in we have ascribed the inhibitory activity to mature ıages.

natant fluids from PEC of normal, peptone-injected, *ria*-infected mice inhibited [³H]thymidine incorpora- ɔ EL-4 cells to the same extent, indicating that the "activation" of macrophages did not influence the enon being described. The production of inhibitory from PEC of normal or peptone-injected mice was during the first 24 hr of culture, decaying slightly days of culture.

pts to Establish the Nature of the Inhibitory Activity. ibitory activity of PEC fluids on EL-4 cells was lost ·sis, suggesting that it was caused by a low-molecular- chemical. If the PEC fluid was dialyzed against an olume of medium, the inhibitory activity was dis- l equally on both sides of the membrane. (The ıd inhibited the incorporation of [³H]thymidine by ıd the dialysate inhibited 69.5%.) After extensive n on Amicon PM-10 and UM-2 filters, the inhibitory · was found in the filtered fraction, indicating a ma- ·less than 1400 daltons.

inhibitory activity was resistant to tryptic digestion /ml of trypsin for 1 hr) and was stable to freezing and ɛ (five times); we have routinely maintained the cul- ıids at −20° without loss of activity. The possibility e inhibitory activity was cyclic AMP secreted by the as considered. The PEC culture fluids, treated with hosphodiesterase (100 μg/ml with 0.014 M $MgSO_4$,) were as inhibitory as normal fluids.

rption of Inhibitory Activity to Tumor Cells. Can the ory activity of PEC fluids be absorbed by the tumor)ne milliliter aliquots of PEC fluids were incubated ⁄ EL-4 cells for 1 hr at 4°, the cells were removed by ıgation, and the fluid was diluted and tested for its y to inhibit EL-4 growth. Fig. 6 shows that a sig- l percent of activity was indeed removed by incubation L-4 cells. Not shown in Fig. 6 is the observation that tion of PEC fluid with EL-4 *at 37°* resulted in a very degree of absorption. We interpret this to mean that

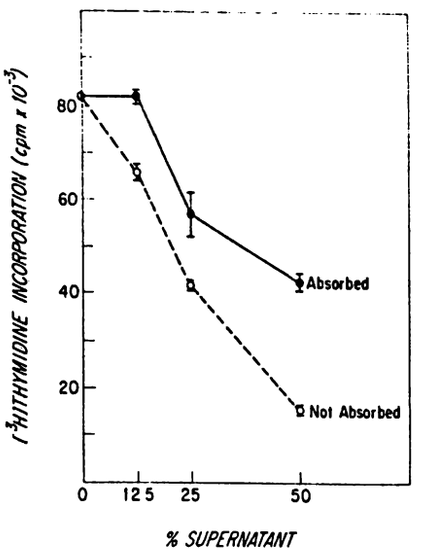


FIG. 6. This graph shows the absorption of the inhibitory activity from supernatants of macrophages. Culture fluids from 48-hr cultures of macrophages were incubated with EL-4 cells at 4° at a density of 10^8 cells per ml during 1 hr. Graded dilutions of absorbed supernatants were compared with equivalent amounts of nontreated supernatants in their ability to inhibit [³H]thymidine incorporation by 5×10^5 EL-4 cells for 8 hr of incubation.

the PEC fluid activity can indeed be absorbed to cells but that the binding is weak at 37°.

DISCUSSION

We have consistently found a cytostatic activity in cultures of lymphoid cells rich in mononuclear phagocytes. The culture fluids inhibited both the incorporation of radioactive thymidine into, as well as the proliferation of, various cells. The activity responsible for the inhibitory effect was dialyzable and absorbable by incubation with EL-4 cells, suggesting that it resides in a small molecule capable of interacting with some cell constituent. It is possible that this molecule may block at the cell membrane the uptake and transport of nucleosides or nutrients; alternatively, since it is smaller than 1400 daltons, the possibility exists that it readily enters the cell to block in some way, directly or indirectly, the biosynthesis of DNA. Clearly, these points need investigation. Two further points need clarification: One, we have not answered conclusively the question of whether different kinds of cells react differently to the inhibitor, since only limited experiments were performed with nonlymphoid cells—the point which is clear is that lymphoid cells are very sensitive to the inhibitory effect. Secondly, all evidence indicates that the mononuclear phagocyte is the main source of the inhibitor, but we cannot exclude the participation of some other lymphoid cells besides macrophages. In one experiment (Fig. 5) cultures of spleen cells presumably depleted of macrophages inhibited, albeit at high concentration. It is difficult to eliminate all macrophages by their adherence to surfaces, so the possibility remains that the effect seen with spleen was caused by some remaining phagocytes.

The biological activities found in cultures of macrophages fall into two groups: one showing stimulation; the other, inhibition. Among the first are those described in the report of Hoffman and Dutton (4) showing that 24-hr culture fluids of murine macrophages allow lymphocytes to respond immunologically to sheep erythrocytes. The activity was absorbable

ırocytes and hence was directed against the anti-
lhan at the lymphocytes. Gery *et al.* found that
ıdherent cells stimulated by endotoxin enhanced
: of thymocytes to lectins (2, 3). Chervenick and
)tained evidence for a factor in culture of mono-
stimulated granulocyte colony formation (5).
ory activities from macrophages fall into two
hose associated with a lytic effect (7) and those
vith inhibition of growth (6, 13). Kramer and
nd a "toxin" in cultures of "immune macro-
l active against many kinds of cells. This toxin
generated under conditions where antigen was
macrophage cultures (7). Bast *et al.* have recently
or that lyses bacteria in cultures of macrophages
; immune processes (8). An activity similar, if
l, to the one reported here was found by Wald-
ottlieb (6) using rat macrophages. It should be
t in the past many investigators have found in-
lymphocyte function by addition of excessive
nacrophages to the cultures (reviewed in ref. 14).
e of different activities in cultures of mononuclear
may not be surprising. It is possible that the
may release various kinds of biological activities
:e with their maturational stage and/or response
stimuli (microorganisms, lymphocyte products,
xperiments suggest some quantitative differences
)itory activity of spleen macrophages and peri-
but no differences among the latter cells regard-
her they were "activated" or not.) In favor of
: of different biological activities in macrophage
ds are recent observations we have made when
)ody formation *in vitro*. In dilutions of 25–100%,
hage culture fluids consistently inhibited anti-
ion; but when further diluted to 10%, some cul-
:hibited stimulatory effects.

amental question concerns the relevance of the
ıbstance secreted by the macrophage in culture
inhibitory phenomena associated *in vivo* with
activation or seen *in vitro* when macrophages are
other cells (15). Although we are impressed by
enon that we have studied because of its repro-
ıd its relatively strong activity, we are still skepti-
biological role, knowing that it is a small molecule
ıably diffuses readily—unless one considers its
role in circumstances where macrophages agglomerate around target cells, as in granulomas.

Note Added in Proof. Experiments made after the manuscript was submitted have disclosed the presence in the peritoneal cell culture fluid of a nondialyzable material which enhances the proliferation of thymocytes or spleen cells in response to lectins. The nondialyzable material does not enhance proliferation of EL-4 cells. The stimulatory activity for thymocytes or spleen cells is partially or completely masked by the inhibitor described herein and can be revealed following extensive dialysis. The stimulatory activity is probably similar to that described by Gery *et al.* (2, 3), and discussed above.

This study was supported by National Institutes of Health Grants NCI-14723 and AI-10091. J.C. is supported by an International Fellowship Program of the National Institutes of Health. E.R.U. is a recipient of a Research Career Award from the National Institutes of Health.

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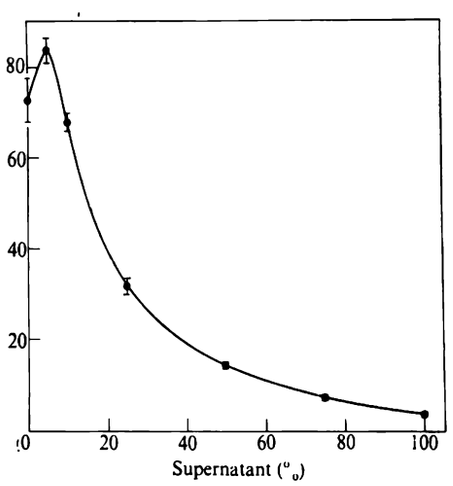
(Reprinted from Nature, Vol. 253, No. 5490, pp. 359–361, January 31, 1975)

logical activities regulating iferation found in cultures neal exudate cells

ound two distinct biological activities in cultures al exudate cells rich in macrophages. One con- inhibitory effect on cell proliferation, described caused by a low molecular weight product[1]; the stimulatory effect brought about by a non- material. Both activities were found in the same id; and different kinds of cells exhibited different s to them. Our results offer a possible explana- ie discrepancies in the literature concerning the effects of macrophages or their products on

tured the peritoneal exudate from A/St or mice injected 3 d previously, intraperitoneally, nl of a 10% (v/v) solution of proteose peptone he cells were plated at a density of 10^7 or 2×10^6 l on 35 mm plastic dishes (Falcon) in RPMI-1640 edium supplemented with 5% foetal calf serum. a, the medium was collected; the cell monolayer



Incorporation of ^{3}H-thymidine by EL-4 leukaemia ured in the presence of different amounts of culture m peritoneal exudate cells. Cells were cultured at a f 5×10^5 per ml in plastic tubes for 8 h, the last 4 h resence of ^{3}H-thymidine (New England Nuclear Ci mmol^{-1}). Each result is the mean of three culture rackets refer to standard error of the mean. In this nt, the EL-4 cells (a tumour line from C57BL/6 ere cultured in the presence of fluids from A/St entical results have been obtained if the cells are tured in fluids from C57BL/6 peritoneal cells.

ed, fresh medium was added, and the culture for 48 h. The activities of the culture fluids during the first 24 h or from the 24–72-h period d. Most cells forming the exudate were typical ges as judged morphologically; during the first 0% lymphocytes were present with the macro- at most were eliminated by the washing at 24 h. the incorporation of ^{3}H-thymidine by EL-4 cells, P815 mastocytomas cells, thymocytes and s cultured in the presence of various amounts of n macrophage cultures. Results have been con- more than twenty different experiments. The lts shown in Figs 1–3 were obtained with a fluid lture of 10^7 cells per ml.

ration of ^{3}H-thymidine into EL-4 leukaemia cells or 8 h in the peritoneal cell supernanant was inhibited markedly (Fig. 1). This inhibition correlated with that of cell mitotic activity and with the increase of cell density with time of culture[1]. The culture fluid, if dialysed extensively against normal culture medium, lost all inhibitory activity. EL-4 cells cultured in dialysed culture supernatants incorporated the same amount of thymidine as cells culured in normal medium. Another cell line, the mouse mastocytoma P815 used extensively in current *in vitro* studies of cell-mediated immunity, behaved as the EL-4 line, and its incorporation was inhibited markedly by the supernatants (data not shown).

We examined for the amounts of inhibitor extracted by freeze-thawing the peritoneal exudate cells or released into the medium after culture for 24 h. The amounts of material (v/v) required to reduce by 50% the incorporation of ^{3}H-thymidine by EL-4 cells were determined. The amounts were: 63% from cells lysed immediately after plating; 83% from cells lysed 24 h after plating, and 22% from the fluids after culture for 24 h. Thus, the inhibitory activity found in culture fluids was generated during the culture and did not result from lysed or dead cells. The chemical nature of this material is not known. In our previous experiment, we indicated that the material was resistant to tryptic digestion, to phosphodiesterase treatment, and to boiling and freeze-thawing[1].

The spontaneous incorporation of ^{3}H-thymidine into spleen cells cultured with the untreated supernatant was inhibited slightly. When these cells were cultured with dialysed supernatants, c.p.m. were the same as controls in the range of doses used, 10–50% (v/v). (In other experiments, a 75% concentration of the dialysed peritoneal cell

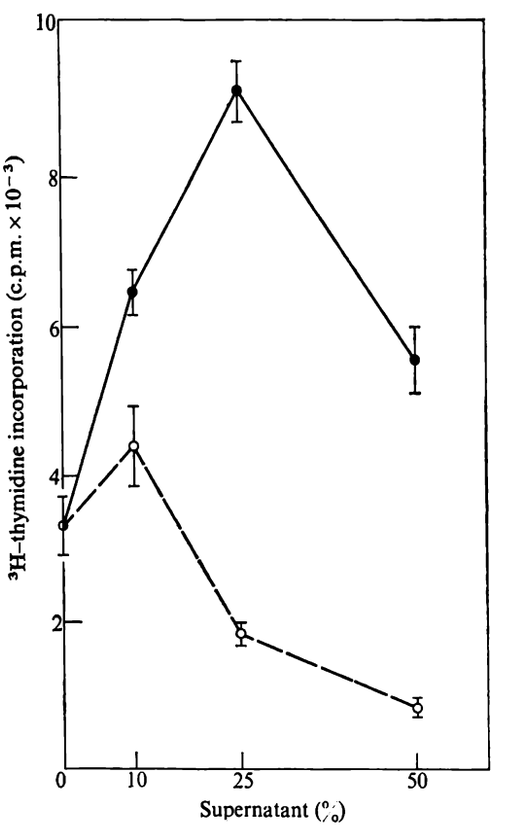


Fig. 2 Spleen cells from A/St mice were cultured in the presence of various amounts of the supernatants and PHA. Time of culture was 72 h, the last 12 h in the presence of ^{3}H-thymidine. Cultured fluids were dialysed against ten times the volume of normal medium for 72 h, changing the dialysate on four occasions. ●, Dialysed supernatant; ○, untreated supernatant.

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SECRETION BY MACROPHAGES OF TWO MOLECULES MODULATING CELL PROLIFERATION

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It is well accepted that macrophages play an important role in immune induction as well as in cell-mediated immune reactions. In immune induction, macrophages have been thought to act as an antigen focusing cell favoring T and B cell interactions (1). In cell-mediated immunities, phagocytes form the major component of the inflammatory reaction - their concentration and activation appear to curb the growth of bacteria, viruses or tumor cells. The question has arisen whether phagocytes may carry out some of their diverse roles by secreting molecules that moderate the activities of surrounding cells. The purpose of this paper is to report on two contrasting biological activities found in cultures of peritoneal exudate cells rich in macrophages. Both activities are found in cultures of macrophages markedly depleted of T lymphocytes by treatment with anti-θ antibodies and complement. The fluids are generated by cultures of peritoneal macrophages in regular minimal essential medium with 5% fetal calf sera. Figure 1 depicts the general procedure.

Inhibition of Cell Proliferation

Culture fluids from peritoneal exudate cells rich in macrophages markedly inhibit the spontaneous proliferation of EL-4 leukemia cells, P815 mastocytoma and 3T3 transformed or non-transformed fibroblasts; also the cultures inhibit the proliferation of spleen lymphocytes induced by Concanavalin A or bacterial endotoxin as well as antibody formation in vitro by Mishell-Dutton method. Most of these results have just been published in an article in Proc. Natl. Acad. Science (2). Figures 2 and 3 are examples of the effects of the culture fluids on EL-4 cells. The following results have been obtained:

1. The material responsible for the inhibition is dialyzable (Fig. 2). By the use of filters of differing sizes, the material is thought to have a molecular size of 1400 daltons or less.

This work was supported by National Institutes of Health Grant AI 10091 and NCI Grant 14723.

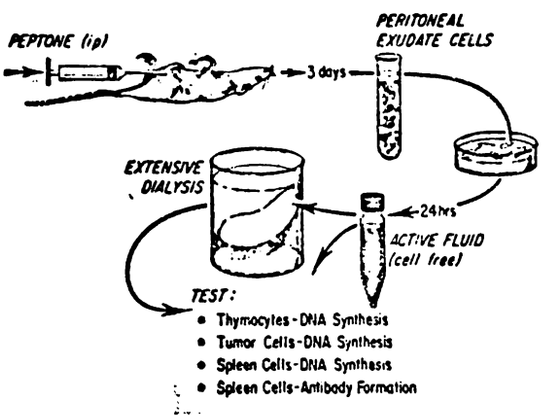


Fig. 1 The graph depicts the procedure used in most experiments. Mice are injected i.p. with peptone and their peritoneal cells harvested and cultured. The culture fluids are tested prior to or after dialysis.

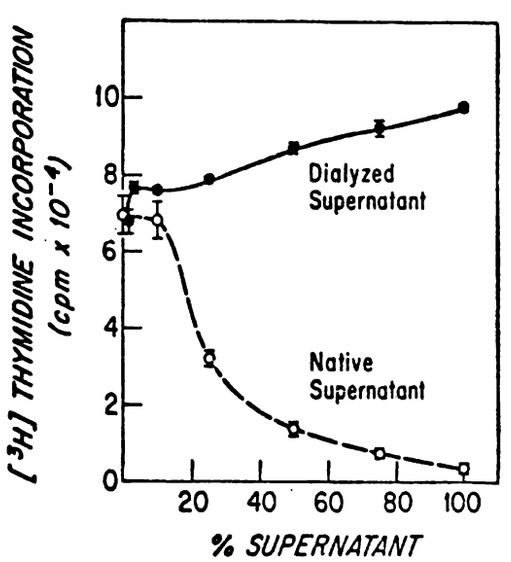


Fig. 2 EL-4 leukemia cells were incubated with macrophage culture fluid for 8 hrs, the last 4 in the presence of tritium labeled thymidine. Culture conditions: 5×10^5 EL-4 cells, 1 ml or RPMI 1640 medium with 5% fetal calf serum. % supernatant refers to concentration (v/v) of undialyzed or dialyzed macrophage fluid.

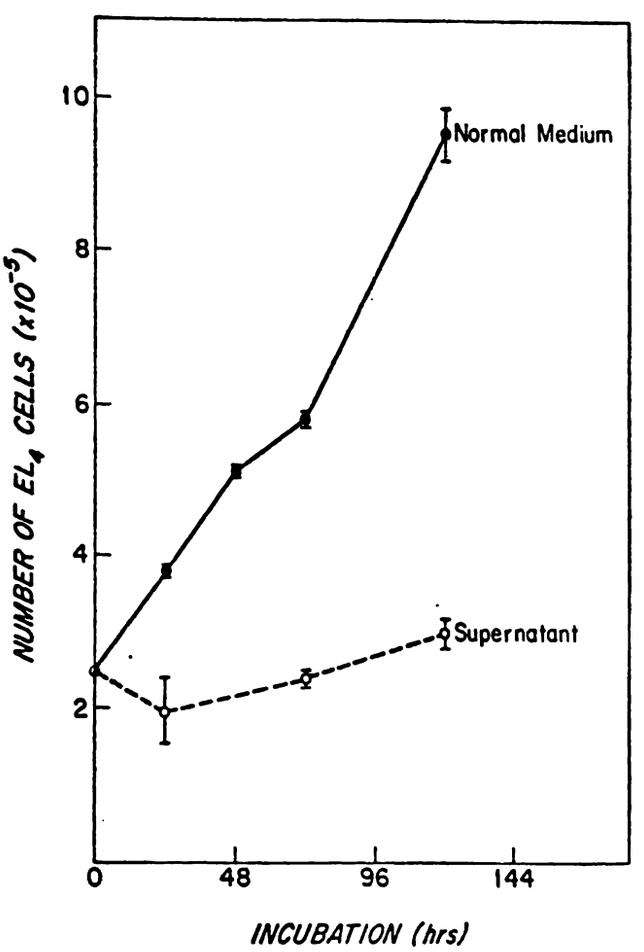


Fig. 3 In this experiment, the EL-4 leukemia cells were grown for the indicated times in normal medium or in the macrophage culture fluids.

2. The inhibitory material resists tryptic digestion and treatment with phosphodiesterase; it resists boiling and freeze-thawing.

3. The effects on cells is cytostatic. Cells cultured in the inhibitor will recover their normal growth activity if cultured in fresh medium.

4. The inhibitor is synthesized by the macrophages and is not simply released from cells dying in the culture. The experiment in Figure 4 substantiates this point (3).

5. The inhibitor is synthesized in equal amounts by peritoneal macrophages from unstimulated, peptone stimulated, or Listeria monocytogenes infected mice. Spleen macrophages synthesize it in small amounts.

6. The inhibitor acts on differing strains of mice and also acts on cells from other species. Murine inhibitor can act on human lymphocytes and vice versa.

7. The inhibitor can be absorbed by the target cells and removed from solution.

8. Inhibiting activity has not been found in cultures of fibroblasts or EL-4 leukemia cells. Spleen "non-adherent" cells secrete it in small amounts probably because of a small number of residual macrophages.

Stimulation of Lymphocyte Proliferation

The experiments so far have been done only with peritoneal exudate cells activated by peptone. One experiment has employed cultures of human spleen macrophages. In most experiments, the effects of the stimulatory molecule are best seen following removal of the inhibitory molecule. The following results have been obtained:

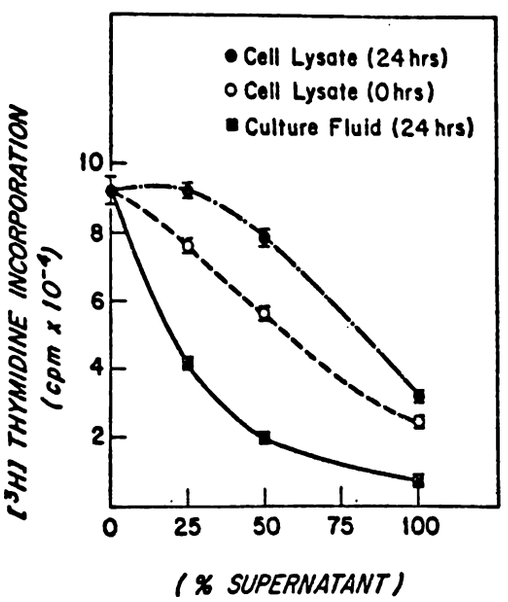
1. The cultures stimulate proliferation of thymocytes. This proliferation is seen best after removal of the inhibitor by dialysis (Fig. 5). The stimulatory molecule enhances the PHA response of thymocytes (3).

2. The stimulatory activity allows spleen cells from nude mice in vitro to make an antibody response both of the IgM and IgG class.

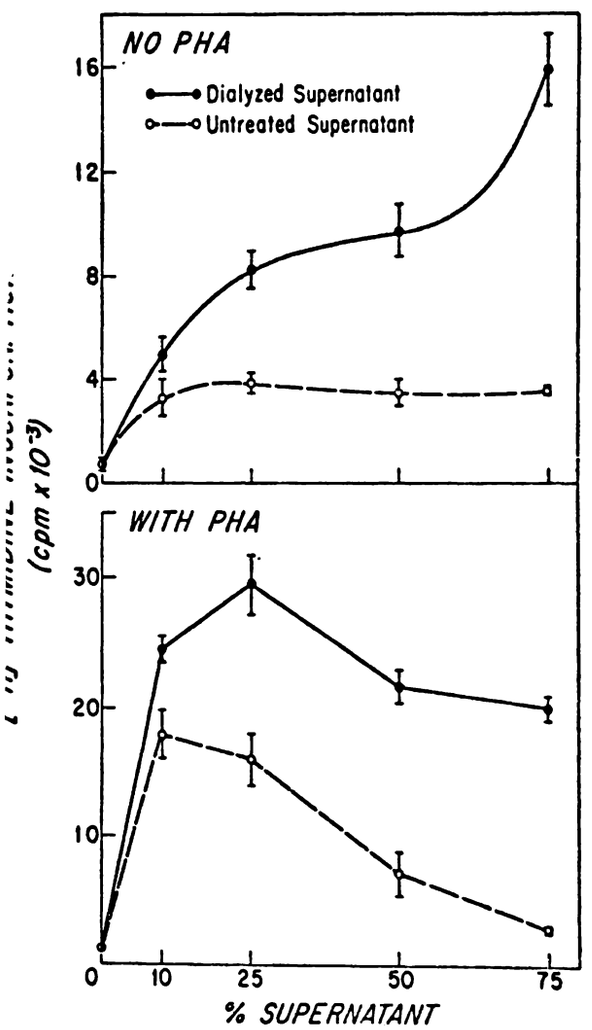
3. The stimulatory molecule enhances the response of hapten carrier primed cells. Indeed, spleen cells from mice primed to Fluorescein hemocyanin (FITC-KLH) will develop 3 to 4 times more PFC, both of IgM and IgG class. This response to FITC on a heterologous carrier is also enhanced but much less.

4. Finally, anti-θ-treated spleen cells from FITC-KLH primed mice will make a peritoneal anti-FITC response when cultured in the macrophage culture fluids.

5. The stimulatory substance crosses strains and species.



This figure shows that the inhibitor is synthesized and released by the macrophage monolayer in culture. Macrophages were planted and immediately lysed by freeze-thawing. Another set was cultured 24 hrs at which time the fluids were obtained as well as a lysate from the cells. Each was tested for inhibitor on EL-4 cells, as in Figure 2. Inhibitor is formed in the initial cell lysate but it is released in larger amount after 24 hrs of culture.



Thymocytes were incubated with dialyzed or untreated cultures from 72 hrs, with or without PHA, the last 12 hrs in the presence of titrated thymidine. Culture conditions: 5×10^6 thymocytes in 1 ml of RPMI 1640 medium with 5% fetal calf serum; PHA: 1 µg per ml of PHA-P from Difco Labs.

e stimulatory substance can be generated from cul-
peritoneal exudate cells depleted of T cells and
toneal cells obtained from x-irradiated mice. It
een obtained from cultures of nonadherent spleen

mary, the stimulatory molecule appears to act on
d B cells. Indeed, it stimulates proliferation of
s and enhances the response of carrier primed T
t also allows B cell preparations to make a signi-
sponse probably as a result of proliferation and
iation. Unfortunately, we have no idea at present
quirement for its production by macrophages, that is
ether its production reflects, as we suspect, the
activation of the macrophage. Similar stimulatory
s were previously reported by Gery et al. (4),
(5) and Wood (6).
mary it would appear that phagocytes can secrete at
kinds of molecules that have important modulatory
n surrounding cells. The stimulatory molecule could
mportant role in responses of lymphocytes and may be
le for some of the helper effects previously report-
role of the small molecular weight inhibitor is not
nce it should readily diffuse in tissue fluids. It
at the end result inhibition or stimulation depends
al factors: number of macrophages, their state of
n, their relationship to the target cells (for
if forming a granuloma), the nature of the target
c. The presence of these potentially important
s again focus on the macrophages as an important
ry cell in immunity.

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.uation of the role of macrophages ı immune induction[1,2]

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ɜed for cellular cooperative ıns involving macrophages, ɜrived T lymphocytes, and g B lymphocytes has been ɔut in a multiplicity of sys- ˈing the past several years This paper reviews some of ɔtions of macrophages in induction and points out he problems and difficulties, ıs possible areas for future on.

ɜ the extensive progress in mmunology during the past he role of macrophages has ı well defined. The great ity of inductive events, our ed information of the nature ıs lymphocyte functions, and esponse of lymphocytes to has not allowed us to estab- a definite way the precise the mononuclear phagocyte. ɔlem with research on macro- ıas been to determine which many systems—in vivo or —where it has been analyzed ɗied is relevent to normal responses. The multiplicity ns has led to contradictory ɜ are not suggesting that one .her system is right or wrong y wish to emphasize that ɜell that carries out diverse s such as uptake and deg- ı of macromolecules, secre- ɗ the like, one would expect ı results that depend in part ɔarticular conditions and cir- ces of a given assay or test Thus, the roles of macro- n destroying (16) or retaining (21), in secreting modulating ɜes (9), or in producing super c complexes (6, 10) have been ɗ. In the context of the ex- ıt where they were tested, such functions of macrophages are entirely correct. However, now is the time when one can place the functions of the macrophage in a better perspective and decide which experimental results fit better in the context of an in vivo situation; indeed, it is only now when we have reached a better understanding of some basic phenomena in immune recognition and induction.

PARTICIPATION OF MACROPHAGES IN IMMUNE INDUCTION

There are three points in immune induction that are well grounded on hard experimental evidence, which one should keep in mind when interpreting the function of macrophages. The first point concerns the specificity of the immune response. This is a sole function of committed lymphocytes with specific recognition units on their surface. Indeed, prior to antigen stimulation, lymphocytes are present with specific receptors on their membranes. The antigen receptors are immunoglobulin, at least in the B class of lymphocyte, and are presumably restricted in their specificity to one antigenic determinant (15, 22). It is clear that the responses do not develop in the absence of these lymphocytes despite the presence of NORMAL macrophages capable of interacting with antigen (14). This is well evident in immune tolerance where normal macrophages are found with functional absence of the specific T or B lymphocytes, or in the suicide kind of experiment devised by Humphrey and Keller (11) and Ada and Byrt (1) where highly radioactive antigen kills or inactivates the clone lymphocyte, leaving a population of cells incapable of responding when transplanted to a host with normal functioning macrophages. Hence, we conclude that macrophages do not play an instructional role but one related directly or indirectly to the uptake and handling of the antigen. In contrast to lymphocytes, macrophages can interact with many antigens, albeit to variable extent. The degree to which an antigen binds to macrophages depends in part on the degree of aggregation or polymerization of the molecule in question and whether it is complexed to specific antibody. Macrophages have surface receptors for Ig and C3.

The second point is that the uptake or trapping of antigen in lymphoid organs favors immunogenicity. This point has been extensively analyzed and was just recently reviewed (18). There is a strict correlation between the degree of immunogenicity of an antigen molecule and the extent to which it abandons the extracellular fluid spaces and is trapped in lymphoid organs by macrophages. The classical example is that of highly polymerized proteins versus their monomeric counterparts: the former are highly immunogenic while the latter tend to become tolerant. As was stated in a recent report, "diffusion alone cannot govern the en-

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[1] From a Symposium, *Function of Macrophages*, presented by the American Association of Immunologists at the 58th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 11, 1974.

[2] Supported by National Institutes of Health Grants AI 10091 and N.C.I. 14723.

[3] Recipient of a Research Career Award from the National Institutes of Health.

[4] Supported by an International Fellowship Program from the National Institutes of Health.

Abbreviation: DNP, dinitrophenyl.

antigen with lymphocytes; words, antigen does not gas but in a more com-23).

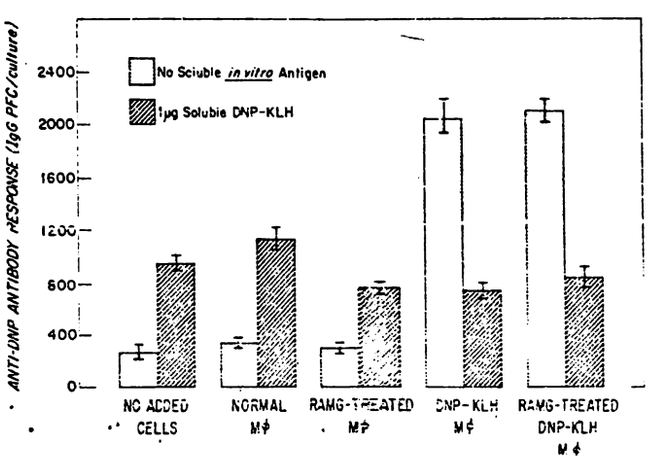
d point is that an antigen and to a macrophage is munogenic. More than igens have now been ex- in vivo or in vitro sys- arious investigators (re- ef 18). In the system first tchison (14), Unanue and 0), and Gallily and Feld- nacrophages are isolated peritoneal cavity of mice ed to antigen for a brief time. The macrophages d and then assayed for nicity in several ways— cells can be transferred al syngeneic mice or to- h immune lymphocytes n cells from previously mice) into X-irradiated he macrophages can be immune lymphocytes and in vitro culture. In both e antibody response is d several days later. Every ed was found to be highly nic when bound to macro- omparisons have been g in vivo systems between nicity of an antigen ad- in live macrophages and antigen administered in m. It was found that the munogenicity of macro- id antigens versus soluble s higher with proteins that up poorly by macrophages those that were trapped e macrophages. Thus, for bumins or immunoglobu- n are poorly taken up by macrophages—induced g response if adminis- ophage-bound relative to mocyanin, a large protein dly taken up by tissue es, does not appear as nic when given in live es. In the latter example, ng the antigen already the phagocyte does not immunogenicity since a of the soluble antigen also lymphoid macrophages. e way to circumvent these to compare the immuno- a macrophage-bound anti- soluble in in vitro systems formation. Using totally in vitro systems, Katz and Unanue found that on a molar basis, dinitrophenylated hemocyanins bound exclusively to macrophages were more immunogenic (about 1,000-fold) than when given in soluble form (13). In no instance has a macrophage-bound antigen induced tolerance.

A very important observation is that the addition of soluble antigen can abrogate the high immunogenicity of macrophage-bound antigens. A representative experiment from the paper of Katz and Unanue is shown in Fig. 1. This means to us that the immune system operates best under conditions where the antigen is concentrated by fixed cells *and in the absence of free diffusing molecules*.

In the assays described before, macrophage-bound antigen did not bypass the requirement for a thymic helper cell. Most of the antigens tested were thymic-dependent antigens that required T lymphocytes. Thus, thymectomized mice unresponsive to soluble hemocyanin, a T-dependent antigen (19), did not respond immunologically even if the hemocyanin was administered bound to live macrophages. This point was made very clear using Mishell-Dutton in vitro systems and mice primed to the hapten dinitrophenyl (DNP) bound to different carrier proteins. Thus, spleen lymphocytes from mice primed to DNP-hemocyanin (containing DNP-primed B cells and hemocyanin-primed T cells) were stimulated by the addition to the culture of macrophages containing DNP-hemocyanin but not by macrophage-bound DNP-bovine gamma globulin. The reverse was also true using lymphocytes primed to DNP-bovine gamma globulin. Thus, macrophages served as an excellent substrate to bring about T–B cell interaction but were insufficient under those conditions employed above to stimulate the isolated B lymphocyte.

There are obvious problems in trying to explain the points analyzed and discussed above. One substantial problem is that macrophages are well known for destroying antigen and that immune recognition usually precedes *extensive* catabolism (17). Indeed, it is known that antigen needs to retain both "helper" and "carrier" determinants for inducing collaboration between T and B lymphocytes (reviewed in ref 12). With globular proteins, many of which are highly immunogenic in macrophages, the response is directed to conformational determinants of the antigen (17). On one hand, we are stating that the lymphocytes interact best with macrophage-bound antigens, a cell that catabolizes antigen; and on the other, we state that the lympho-

Figure 1. The deleterious effects of soluble antigen in the immune response to DNP-hemocyanin (DNP-KLH). Dinitrophenyl-KLH-primed spleen cells were cultured with and without DNP-KLH either alone or together with normal macrophages (Mø), macrophages exposed briefly to DNP-KLH (DNP-KLH Mø), or macrophages pulsed briefly with DNP-KLH in the presence of anti-immunoglobulin antibody (RAMG-treated DNP-KLH Mø). The graph depicts the secondary IgG plaque-forming cell response 4 days after initiation of the culture. Results are geometric means of three cultures; bars represent the ranges of standard error. Note in the fourth and fifth group the excellent response to DNP-KLH bound to macrophages and its reduction by the addition of soluble DNP-KLH. (Taken from the paper of Katz and Unanue (13), Figure 6).



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can be explained if we
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NOGENIC MOLECULES
N MACROPHAGES

art of the work done in our
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with attempts to identify
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ɔf antigen bound to macro-
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above. We now envision two
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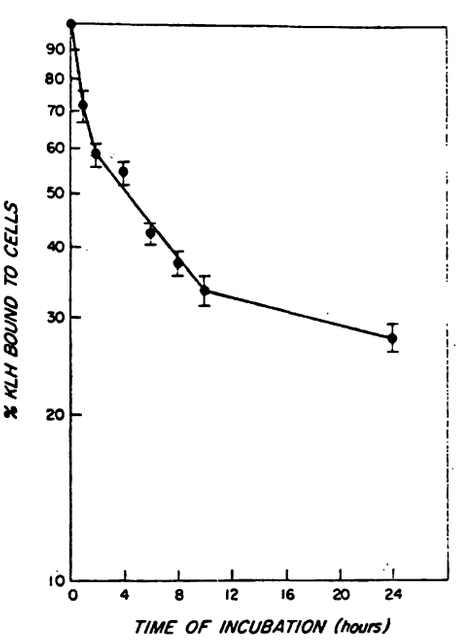
is degraded by lysosomal

Figure 2. Surface replica of macrophages pulsed at 4 C with keyhole limpet hemocyanin for 30 min. Hemocyanin is distributed throughout the cell surface with a tendency to form small clusters.

digestion. Figure 3 shows the fate of macrophage-bound hemocyanin. In this experiment, the peritoneal macrophages were first planted on dishes and cultured for 18–25 hr; at that time, one eliminated loosely adherent cells and was left with an interrupted monolayer of typical—healthy-looking—macrophages. These cells were then pulsed briefly with hemocyanin labeled with ^{125}I, washed, and placed back into culture. There was an exponential loss of material from the cell. The lost material was being released as degraded products into the culture fluids. Note that there was heterogeneity in the rates of catabolism—an early phase of rapid degradation having half-life varying among experiments from 0.5 to 2 hr and a slow catabolic phase with half-lives of 8 to 22 hr.

Figure 3. Elimination of labeled hemocyanin from murine macrophages. Macrophages were planted in culture and exposed to ^{125}I-labeled hemocyanin for 30 min at 4 C; the cells were washed and cultured in medium free of antigen. The amount of trichloroacetic material remaining in the cells was determined at the different times. Each point is the average of two to three dishes; the standard error of the means is shown by the bars.



One point to observe is that there was a substantial amount of intracellular protein still in the cell for fairly long periods of time. The reasons behind the heterogeneous intracellular handling of hemocyanin are not clear—we have reason to believe it is not caused by heterogeneity of macrophage, but this point has not been strictly ruled out. It is certainly not caused by exhaustion of lysosomal enzymes with time of culture. Macrophages pulsed for a second time with the same antigen but radiolabeled with a different isotope of I, degrade this second pulse of antigen at the same rates as the first pulse. Our inclination is that the heterogeneity in catabolism might reflect differences of lysosomal-pinosome interaction.

Not all the antigen initially bound to the surface membrane was rapidly

1. With some protein anti-
molecules remained asso-
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presented to the lympho-
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aterials, such as red cells
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it ranged from 1% to 10%
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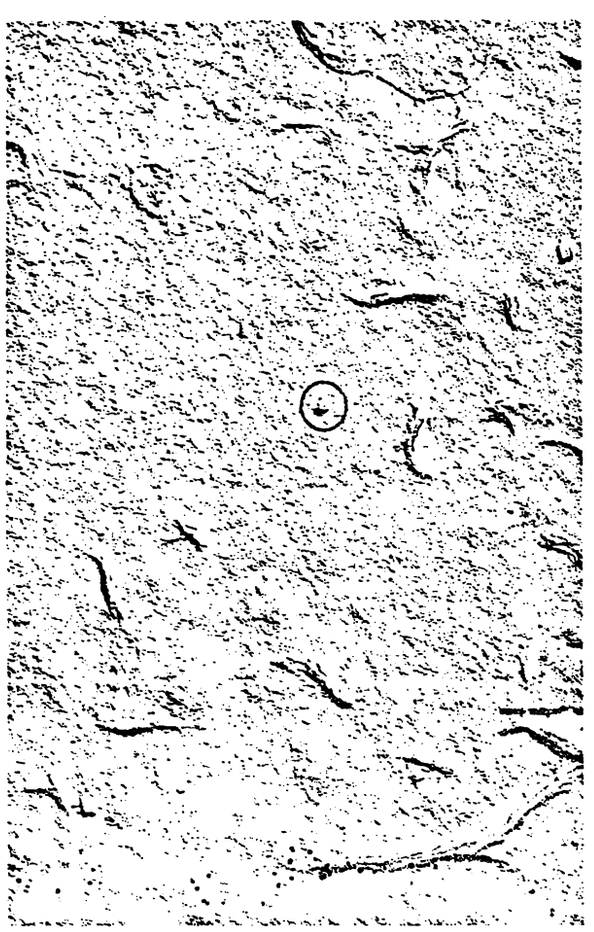
ended to dissociate faster.

The antigen remaining on the surface was certainly immunogenic and accessible to the lymphocytes. Indeed, its removal by trypsinization abrogated to a significant degree the immunogenicity of some macrophage-bound protein antigens (21). The reasons for the persistence of antigen on the surface were not clear. As we analyzed before, size of the antigen determined to some degree the extent and life of the persistent antigen. A small protein rapidly dissociated, while larger molecules were interiorized in bulk. With a given antigen like hemocyanin, the retained surface antigen might reflect the functional mosaicism of the macrophage membrane. Perhaps some of the "receptors" for these antigens on the macrophage membrane were somewhat slower in pinocytosis. The persistent molecules might simply represent a heterogenicity in functional membrane receptor sites.

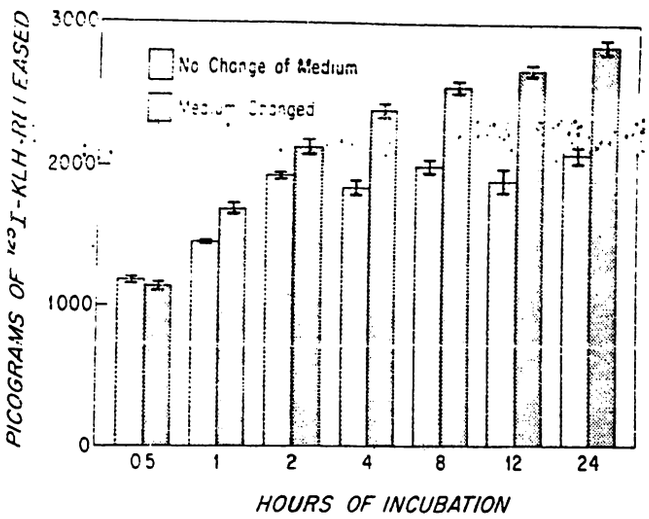
The second way by which antigens can be presented to lymphocytes is by being released from the interior of the cell following their interiorization. If one pulses macrophages planted in culture with radio-labeled antigens, after a short period washes the excess antigen, and then places the cells in medium free of antigen, one can consistently note the appearance of protein-bound radioactivity in the culture supernatant, a phenomenon first reported by Askonas and Jaroskova (2). During the past year, we have been studying this phenomenon in depth for, if indeed true, it could represent another pathway by which antigens are presented to the lymphocytes in the milieu of the macrophage (3). The antigens that we have employed are hemocyanin or rabbit IgG. Figure 5 represents the release of macromolecular hemocyanin into the culture supernatant at various times and under two different conditions. In one set of dishes the cultures were sampled from 0.5 hr to 24 hr; the other set, the medium was removed (and radioactivity counted), fresh medium was added, and the cultures allowed to proceed. A great part of the hemocyanin that is released is already present within the first 2 hr and after this time the release decreases markedly. However, in the cultures where fresh medium is added, release continues, indicating that the composition of the medium does influence the extent of release. The nature of this last phenomenon is not clear.

me experiment as in Fig. 2. In this instance, the macrophages were cultured for
ig the pulse at 4 C with hemocyanin. The majority of molecules have been cleared
face. In this field, one isolated molecule can be identified.



It was obvious that the release of antigen from macrophages did not represent material that came from dead, autolyzed cells—the culture conditions were excellent as judged by the minimal release of the cytoplasmic enzyme lactic dehydrogenase. Moreover, other possible artifacts of culture-like attachment—and subsequent detachment of the radio-labeled protein—to the plastic dish were also ruled out (3).

It was important to determine the source of the release material. There are two experimental results that strongly suggest that it derives from intracellular vesicles. First, macrophages pulsed with hemocyanin, then trypsinized to remove surface molecules, did release ^{125}I material (Fig. 6). Second, the molecular size of the released antigen was smaller than the native molecules. Indeed, only about one-third to one-fourth of released hemocyanin precipitates with anti-



ine macrophages were harvested from the peritoneal cavity, planted in culture posed for 1 hr to medium containing ^{125}I-labeled hemocyanin. The dishes ight times and then cultured in media free of antigen for the times indicated. dishes the medium was changed at the different times, fresh medium was culture continued (medium changed). In a second group of cultures the medium ed from the initiation of culture, the cultures were terminated at the designated amounts of hemocyanin released in the medium were determined (no change of bars represent the cumulative increase of hemocyanin released into culture. released were about 3% of the hemocyanin taken up by the macrophages. Calderon and Unanue (3), Figure 2.)

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Our tendency is to believe
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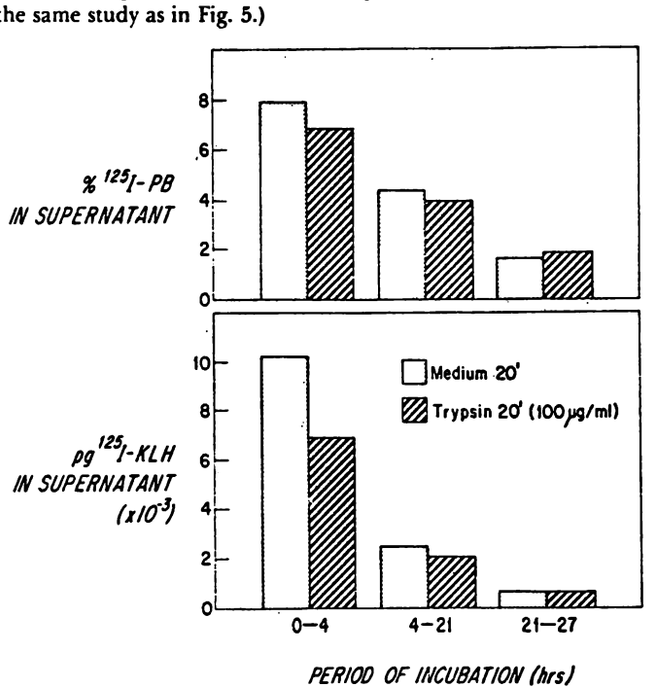
In summary, there are many undecided points concerning macrophages and handling of immunogenic molecules: the exact immunogenic moiety; its mode of contact with lymphocytes; the regulation of the two pathways described above; their relative importance with different antigens; and so on. The point that has to be made is that intracellular degradation is not 100% perfect, leaving room *always* for finite number of molecules that escape complete or total degradation and which could be sufficient to stimulate lymphocytes. Basically, macrophages operate by removing the extracellular antigen and by creating in tissue critical points where antigen molecules remain accessible for relatively long periods. Obviously, the process of antigen presentation to the lymphocyte is deeply associated with the process of degradation and elimination.

CONCLUSION

It seems that the immune response has been built to operate under conditions where antigen is concentrated and focused by mononuclear phagocytes. Indeed, one should look at the macrophage–lymphocyte–antigen

Figure 6. Effects of trypsinization on the release of hemocyanin from macrophages. Macrophages were pulsed with hemocyanin at 37 C for 1 hr, washed, and cultured; the amounts of hemocyanin released into the culture fluids were determined in the time periods shown (0–4 hr, 4–21 hr, or 21–27 hr). Prior to cultures one set of dishes was trypsinized. (Trypsin removed about 22% of the hemocyanin bound, presumably representing molecules still bound to the surface.) The upper panel represents the amount of hemocyanin released, expressed as a percentage of the content of the cell at the start of the culture. The percentage of hemocyanin released is the same whether the macrophage has been trypsinized or not. Presumably, the trypsinized macrophage has no hemocyanin on its surface, suggesting that the released products should derive from the cell interior. The lower panel shows the results expressed in absolute amounts of hemocyanin. (Taken from the same study as in Fig. 5.)



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cannot explain everything, such as, for example, macrophage–T lymphocyte interaction where T cells apparently need the macrophage for induction. One then presumes there is something *special* about the macrophage presentation of antigen. This special effect could be related to some membrane-to-membrane interaction that stimulates the lymphocyte, as is suggested by the recent work of Rosenthal and co-workers discussed in this symposium. The possibility exists that macrophages may release modulatory substances as that described by Gery and Waksman (9), Further work along these lines is needed. The second point concerns the relationship of macrophage-bound antigen to stimulation of the B lymphocyte in absence of a T lymphocyte. This point needs close reappraisal—the kind of experiments that Marc Feldmann has done (5), where a putative antigen–T cell antigen bound to macrophages stimulates B cells, needs urgent extension and/or confirmation.

The third point concerns the immunosuppressive role of macrophages and its relationship, if any, to positive inductive events. It is well known in various in vitro systems that excess macrophages are deleterious (reviewed in ref 18). Macrophages have some inhibitory effect to growth of cells, including tumors; whether this presumed immunosuppressive effect influences immune induction at certain stages and under special circumstances needs to be established. The final point concerns the anatomical relationship between macrophage-bound antigens and the lymphocytes —one very early stage in immune induction is the marked increase in lymphocytes of the deep cortex, what Eugene Lance has termed the "lymphocyte trap" (7). Frost and Lance have speculated on some good, although indirect, evidence that any agent that is readily phagocytosed triggers the lymphocyte trap; they have raised the question that in some way macrophages may alter the traffic of lymphocytes. We find this to be an interesting speculation that is worth our attention.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, July 1, 1975,
Vol. 142, No. 1, pp. 151-164]
Printed in U.S.A.

THE MODULATION OF LYMPHOCYTE FUNCTIONS BY MOLECULES SECRETED BY MACROPHAGES

I. Description and Partial Biochemical Analysis*

BY JESUS CALDERON, JEANNE-MARIE KIELY, JANET L. LEFKO, AND EMIL R. UNANUE‡

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Mononuclear phagocytes are thought to play a regulatory role in immune induction, although there is no general consensus on their exact mode of action. Phagocytes are capable of multiple functions—uptake, endocytosis, catabolism and retention of foreign molecules, activation by extracellular events, secretion of enzymes and other regulatory molecules, etc. As a result of these diverse functions, it would not be surprising for phagocytes to influence the immune process at different stages of its development and evolution. One of the explanations for the role of macrophages in inductive events is that it takes up and presents antigen molecules in an appropriate manner, serving as an antigen-focusing cell for T-B cell interactions (1–3). This is the explanation that appeals more to us based on the superior immunogenicity of macrophage-associated antigen and its counteraction by free soluble materials (4, 5). However, other experiments strongly suggest that phagocytes contribute in some other way to inductive events apart from simple presentation of antigen: (*a*) in the guinea pig, macrophage associated antigen stimulates T-cell proliferation best when the macrophage and the T-cell share their histocompatibility background—this indicates that in this system T-lymphocyte proliferation requires some other unknown factor besides antigen presentation (6, 7); (*b*) antigens bound to macrophages stimulated by adjuvants are more immunogenic than bound to unstimulated macrophages despite the fact that antigen handling is identical in both cells (8); (*c*) viability of lymphocytes in cultures is increased by the presence of macrophages (9); and (*d*) factors that enhance or support lymphocyte function have been found in macrophage cultures (10–15). Of particular interest is the observation of Gery, Gershon, and Waksman reported in this journal (10, 11) of an activity in cultures of human macrophages stimulated by endotoxin that enhanced the response of thymocytes to lectins. We have recently confirmed and extended this observation using murine macrophages stimulated by peptone (16). Upon removal from the cultures of a small molecular weight inhibitor of DNA and protein synthesis (17), an activity was found that

*This study was supported by National Institutes of Health grant AI 10091 and National Cancer Institute grant 14723.

‡Recipient of a Research Career Award from the National Institutes of Health.

stimulated proliferation of spleen and thymocytes without the need of addition of lectins. This paper reports on this phenomenon. We have confirmed that culture fluids from macrophages contain activities that stimulate lymphocyte proliferation and influence B-cell differentiation. These activities are only secreted by macrophages and not by unstimulated lymphocytes, are not restricted in action to cells of the same species, and are in molecules that do not contain antigens of the *H-2* complex.

Materials and Methods

Culture Fluids. Peritoneal exudate cells (PEC)[1] were harvested from mice injected 3 days previously with 1.5 ml of 10% proteose peptone solution (Difco Laboratories, Detroit, Mich.), intraperitoneally. The mice were killed and their peritoneal cells harvested after an injection of 3 ml of minimal essential medium (MEM, Microbiological Associates, Bethesda, Md., catalogue no. 12-683) containing 10 U of heparin per ml and 5% vol/vol heat-inactivated fetal calf serum (Associated Biomedics, Inc., Buffalo, N. Y.). The cells were centrifuged (500 *g*, 10 min), washed with same medium (not containing heparin), and suspended at a density of 10^7 per ml in RPMI 1640 (Associated Biomedics) with 5% fetal calf serum, 0.5 mM Na pyruvate, 2 mM L-glutamine, 1% Na bicarbonate, 50 U of penicillin, and 50 μg of streptomycin. (In some experiments fetal calf serum was omitted and replaced by 200 μg per ml of lactalbumin hydrolyzate [Mann Research Laboratory, New York].) The cells were planted on 35 × 10 mm dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at 37°C in 5% CO_2 in air incubator for 2 h, washed vigorously three times, and incubated for 24–72 h. Culture fluids were harvested, centrifuged, and dialyzed 72 h against 1640 medium, changing the dialysate three to four times. This procedure is necessary for removing the low molecular weight inhibitor of DNA and protein synthesis (17).

In one experiment the PEC monolayer after the first 2 h of culture was treated with anti-θ serum and guinea pig serum, as a complement source, in order to kill and eliminate any possible dish-adherent T cells. The anti-θ serum, raised in AKR mice by immunization with CBA thymocytes, had been previously standardized. The PEC monolayer was incubated in 1 ml of MEM containing 10% anti-θ serum or normal mouse serum at 4°C for 30 min; then the cells were washed three times, incubated at 37°C for 45 min in medium containing 20% guinea pig serum, as a complement source, washed again, and allowed to culture for 24 h.

Culture fluids were also obtained from 3T3 cells obtained from Dr. Thomas Benjamin of our department and from a line of normal mouse embryo cells.

Mice. Most experiments were done in A/St mice from West Seneca Laboratories, Buffalo, N. Y., of either sex, of 8–10 wk of age at the time of initiation of the experiments. Nu/nu athymic mice, obtained from the colony of Dr. Gabriel Michael, University of Cincinnati Medical School, were used at 10–12 wk of age. In occasional experiments the donors of PEC were Swiss outbred mice obtained from Charles River Laboratories, Boston, Mass.

Culture of Thymocytes and Spleen Cells. Thymocytes and spleen cells were harvested by conventional procedures and cultured at a density of 5×10^6 or 10^6 per ml, respectively, in 1640 medium, as described above, with 5% fetal calf serum. The cells were cultured in 12 × 75 mm tubes (Falcon Plastics, catalogue no. 2054) for 72 h in 37°C incubated with 5% CO_2 in air. 12–15 hours before termination, the cultures received 1 μCi of [^{3}H]thymidine (2 Ci/mmol from New England Nuclear Company, Boston, Mass.). Trichloracetic acid insoluble material from cultured cells was collected on glass fiber filters in a sampling manifold. Radioactivity was measured in a liquid scintillation spectrometer. The basis of the experiments consisted of evaluating the incorporation of [^{3}H]thymidine of the cells cultured in medium containing various amounts of PEC culture fluids. The time of culture was always 72 h—pilot experiments indicated this to be the optimal time to see effects. All results are means of triplicate cultures expressed as counts per minute (cpm) plus or minus the value of one standard error of the mean. In some experiments we express the ratio of cpm of experimental

[1] *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; F, fluorescein; KLH, keyhole limpet hemocyanin; PEC peritoneal exudate cells; PFC, plaque-forming cells; PHA, phytohemagglutinin; RGG, rabbit gamma globulin.

tubes over cpm of control tubes not containing the PEC fluid-E/C value. In many experiments phytohemagglutinin (PHA) (Difco Laboratories) was also added to the cultures.

Antibody Response In Vitro. The methodology of Mishell and Dutton (18) was applied for the study of the response of fluorescein (F) as a hapten. This compound behaves as a typical hapten, the immune response depending on T-cell cooperation via an appropriate carrier protein (J.-M. Kiely and E. R. Unanue, unpublished). We followed the exact methodology reported by Mishell and Dutton employing a cell density of 10^7 per ml. In all experiments we report the total plaque-forming cells (PFC) per culture at the fourth day, which was the peak time of the response. Each result represents the arithmetic mean of three culture dishes.

The PFC response to F was assayed by conventional plaque technique using F conjugated to sheep red blood cells (SRBC). The technique of conjugation of SRBC to F was as follows: a solution of 0.5 mg per ml (in bicarbonate buffer, 0.125 M, pH 9.2) of fluorescein isothiocyanate was freshly prepared, filtered to eliminate small undissolved particles, and slowly added to an equal volume of 20% SRBC (also in bicarbonate buffer). The suspension was stirred with a magnet for 40 min at ambient temperature. The SRBC were then washed four times with 0.5% ClNa solution and taken to a 7% concentration in MEM. Direct and indirect PFC were assayed by conventional procedures. A polyvalent rabbit antimouse immunoglobulin (Ig) was used as a facilitating antisera. In control experiments it was determined that anti-F PFC were readily blocked by addition of 10^{-6} M to 10^{-8} M free F into the medium.

All experiments employed spleen cells from A/St mice immunized with F-keyhole limpet hemocyanin (KLH) in alum. Two groups of mice were used: (*a*) mice primed with KLH in alum (i.p. with 50 to 100 μg) once to twice about 3–4 mo previously; these mice usually responded with about 300 to 400 direct and indirect PFC with background number of PFC never exceeding 150; and (*b*) mice primed as above but boosted 1–4 wk before the experiment with 50 μg of F-KLH in alum; these cells responded with about 1,000 to 3,000 direct and indirect PFC.

Fluorescein was conjugated to KLH or rabbit gamma globulin (RGG) by standard procedures. KLH was prepared by ultracentrifugation of the hemolymph of giant keyhole limpets. Rabbit IgG was obtained from Pentex Biochemical, Kankakee, Ill. Ratio of F:P were 9.0 for 100,000 mol wt of KLH and 2.5 for RGG.

Physicochemical Analysis. PEC from CD-1 mice injected with peptone were placed in culture with 1640 medium containing lactalbumin hydrolyzate for 24 h at which time the fluids were harvested and concentrated about 10-fold by ultrafiltration. 2 ml of the concentrated fluid were passed through a Sephadex G200 column (Pharmacia Fine Chemicals, Piscataway, N.J.), 39 cm long and 3 cm in diameter and eluted with 0.15 M phosphate-buffered saline, pH 7.2, at a flow rate of 4.5 ml per hour. Eluted fractions, each of 32 ml, were dialyzed 48 h against distilled water, lyophilized, and resuspended to one-tenth of the original volume with 1640 medium and tested. The fractions containing the stimulatory activity were pooled, passed through a Sephadex G100 column under the same conditions as described above, and the eluted fractions tested. Each of the columns had been calibrated with known protein markers. The molecular weight of the stimulatory molecule was calculated from a chart where the Kav values for markers were plotted against the logarithm of the molecular weight.

In one experiment culture fluids were passed through a Sepharose 4B column which had anti-*H-2* antibodies bound covalently. Anti-$H\text{-}2^a$ antibodies were prepared in C57BL/6 mice by repeated immunization with A/St spleen cells. The antiserum had been standardized and found to be cytolytic to A/St spleen cells at dilutions of 1 × 160. A globulin-rich fraction of the anti-*H-2* serum and of normal mouse serum was obtained by ammonium sulphate precipitation (at 40% concentration); 10 mg of the globulin was bound to 20 ml of Sepharose 4B by cyanogen bromide as per details given by March, et al. (19). Control experiments with radioiodinated globulin confirmed that 32% of it was bound to the column. 8 ml of macrophage culture fluid was passed through each column.

Miscellaneous Manipulations. In one experiment PEC fluids were incubated with a known batch of diisopropylfluorophosphate (DFP) (from Aldrich Chemical Co., Milwaukee, Wis.) 10^{-4} or 10^{-5} M, for 1 h at 37°C, then dialyzed and tested.

An active fraction eluted from G100 column was treated with various enzymes: trypsin (up to 2 mg per ml), papain (70 μg per ml), pronase (50 μg per ml), carboxypeptidase A (70 μg per ml), ribonuclease (50 μg per ml), chymotrypsin (2 U per ml), pepsin (82 μg per ml) and neuraminidase 50 μg per ml), for 4 h at 37°C under appropriate conditions. All proteolytic enzymes, obtained from Worth-

ington Biochemical Company, Freehold, N. J., were tested on a hemoglobin substrate and found to be active.

Results

Effect of Macrophage Culture Fluids on Thymocyte and Spleen Cell Proliferation. Most of the experiments were made with culture fluids obtained from PEC of A/St mice harvested 3 days after injection of 1.5 ml of 10% proteose peptone (Difco Laboratories). In Table I we present the result of 19 different

TABLE I

Thymocyte Response to A/St PEC Culture Fluids

Culture fluid no.	Background	Amount of PEC supernate		
		10%	25%	50%
	cpm			
147	678 ± 115	4,957 ± 608	8,294 ± 699	9,719 ± 1,008
154	314 ± 41	13,138 ± 819	14,580 ± 1,564	—
157	1,082 ± 50	22,628 ± 5,032	12,255 ± 1,753	18,922 ± 2,265
158	1,199 ± 136	1,624 ± 109	—	2,717 ± 175
160	445 ± 86	2,591 ± 202	—	9,890 ± 669
161	586 ± 77	910 ± 124	—	876 ± 164
171	571 ± 10	761 ± 86	—	1,801 ± 262
172	468 ± 39	3,123 ± 1,314	—	7,716 ± 221
174	785 ± 128	10,522 ± 1,648	20,827 ± 1,057	27,747 ± 1,919
175	615 ± 77	7,060 ± 935	17,040 ± 1,309	31,057 ± 1,827
176	801 ± 58	3,331 ± 157	7,448 ± 206	15,911 ± 1,130
176-A	736 ± 48	1,419 ± 160	2,840 ± 235	5,112 ± 371
189	425 ± 22	647 ± 54	621 ± 48	629 ± 48
190	425 ± 22	628 ± 59	774 ± 65	1,120 ± 130
191	961 ± 47	—	3,400 ± 450	6,115 ± 625
192	961 ± 47	—	1,493 ± 272	2,216 ± 186
196	425 ± 22	3,870 ± 200	6,571 ± 256	—
197	836 ± 176	—	6,629 ± 251	12,912 ± 1,623
200	402 ± 82	644 ± 120	998 ± 109	2,124 ± 135

Thymic cells were cultured for 72 hs with different amounts of macrophage culture fluid or in regular medium. Values represent cpm of [^{3}H]thymidine in trichloracetic-insoluble material.

culture supernates tested on thymocytes. Of the 19 supernates, 15 stimulated significantly the incorporation of thymidine into DNA, while four had minimal, if any, effect. The degree of stimulation varied among the 15 stimulatory fluids—indeed, six of these produced stimulation of over 10,000 cpm over background incorporation usually not exceeding about 600 cpm, while the remaining produced stimulations ranging from about 2,000 cpm to about 9,000 cpm.

Several experiments clearly indicated that the stimulatory activity of PEC fluid was not restricted in its action to thymocytes of the same strain. For example, PEC fluids generated in cells of A/St (an *H-2*^{a}) mice stimulated proliferation of syngeneic thymocytes or thymocytes from BALB/c (*H-2*^{d}) or A.By (*H-2*^{b}) to the same extent.

The response of spleen cells to macrophage culture fluid was of small magnitude, not exceeding in nine different culture fluids tested an E/C value of three (see also Reference 16). Culture fluids that produced good stimulation of thymocytes only induced meager stimulation of spleen cells. One such culture fluid, for example, resulted in an E/C value on thymocytes of 30 while only 1.9 on spleen cells.

The In Vitro *Antibody Response to F in the Presence of Macrophage Culture Fluids.* We set up cultures of spleen cells from mice immune to F-KLH without the addition of antigen, adding F-KLH or F-RGG, and with or without different concentrations of macrophage culture fluids. In most experiments the macrophage culture fluids were obtained from peptone-stimulated mice—of nine such culture fluids tested, seven displayed good stimulatory activity. In the experiment shown in Fig. 1, spleen cells were cultured at three concentrations of the two antigens as well as in three different concentrations of macrophage supernates (10, 25, and 50% vol/vol). The spleen cells were obtained from mice primed 3 mo previously with F-KLH in alum. At a concentration of 0.1 μg of F-KLH the PFC response was markedly augmented proportional to the dose of macrophage supernate added to the culture. Of interest are the results using a heterologous carrier, F-RGG. PFC to F-RGG in the absence of macrophage supernate were not over the background level. In the presence of macrophage supernates, however, the cells responded with 530 direct PFC and 840 indirect PFC. That is to say the macrophage supernate had stimulated the cells for a

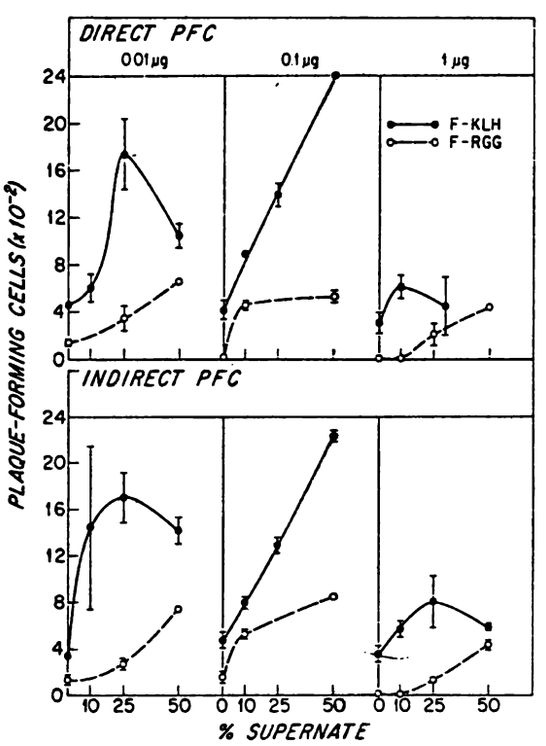

FIG. 1. The PFC response of F-KLH-primed spleen cells to the F-KLH or F-RGG at three concentrations of each in the presence of different doses of macrophage culture fluids. Bars represent value of one standard error of the mean.

response similar to that present under conditions of complete stimulation with F-KLH. (Cell viability was not determined. However, in other experiments, after 4 days of culture, viability was about 30% regardless of whether the cultures contained macrophage supernatants and/or antigens.)

In the experiment of Fig. 1 we did not include the effects of macrophage cultures in the absence of antigen. This point was studied in the experiment of Fig. 2 which also evaluated macrophage supernates obtained from various strains

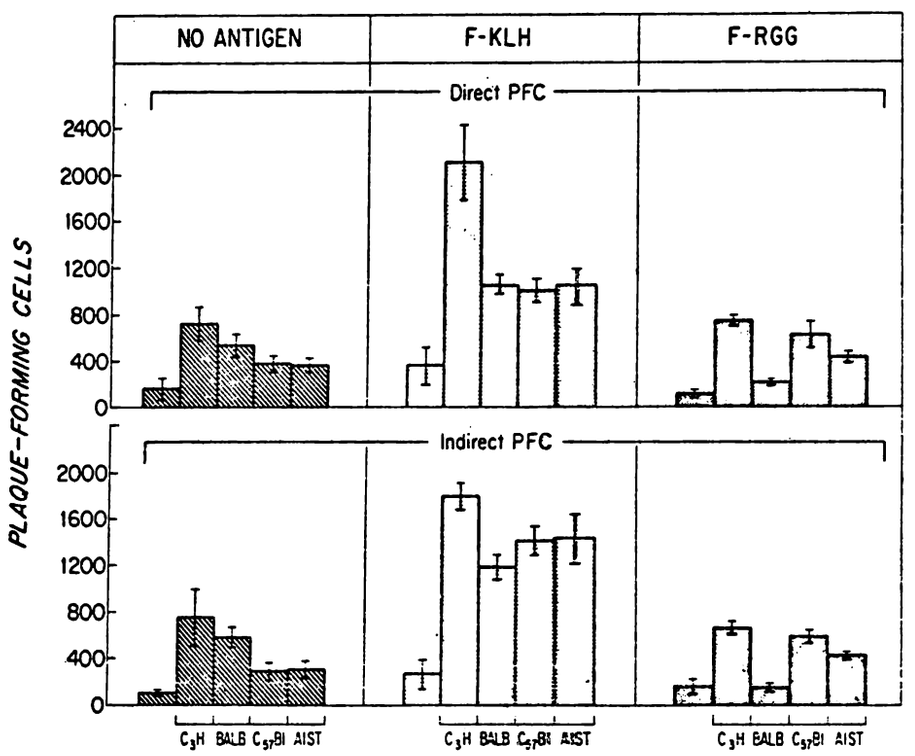

FIG. 2. The PFC response to F induced by macrophage supernatants from various strains of mice differing in their *H-2* haplotype. F-KLH-primed A/St spleen cells were incubated 4 days in the presence or absence of hapten conjugates on homologous (KLH) or heterologous carrier (RGG).

of mice differing in their *H-2* haplotype from the A/St spleen cells used in cultures. Despite some variations, all the macrophage culture fluids irrespective of the donor strain increased the response to F. In this experiment of Fig. 2 the response to F-KLH was of the order of 367 direct and 287 indirect PFC; addition of F-KLH and the macrophage supernate—for example, from C3H mice at a 50% concentration—increased it to 2,133 direct and 1,817 indirect PFC; the culture of spleen cells with the macrophage culture fluids in the absence of any antigen increased the response to 730 direct and 773 indirect PFC, which was of the same order of magnitude as cultures with F-RGG plus the macrophage supernate (760 direct and 660 indirect PFC). In other words, the culture fluids from macrophages had produced a stimulation of the B cells without the need for addition of antigen to the culture. Likewise, as in the experiment of Fig. 1, the stimulation of carrier T-cell function was quite evident.

The Use of Anti-θ-Treated Spleen Cells. In the experiment shown in Fig. 3 we examined the PFC response of spleen cells depleted of T cells by treatment with anti-θ and complement. Untreated spleen cells made a modest number of PFC upon challenging with F-KLH; similar results were obtained by culturing the cells in the presence of macrophage culture fluids without addition of F-KLH; as before, a marked enhancement was observed in cells cultured in the presence of

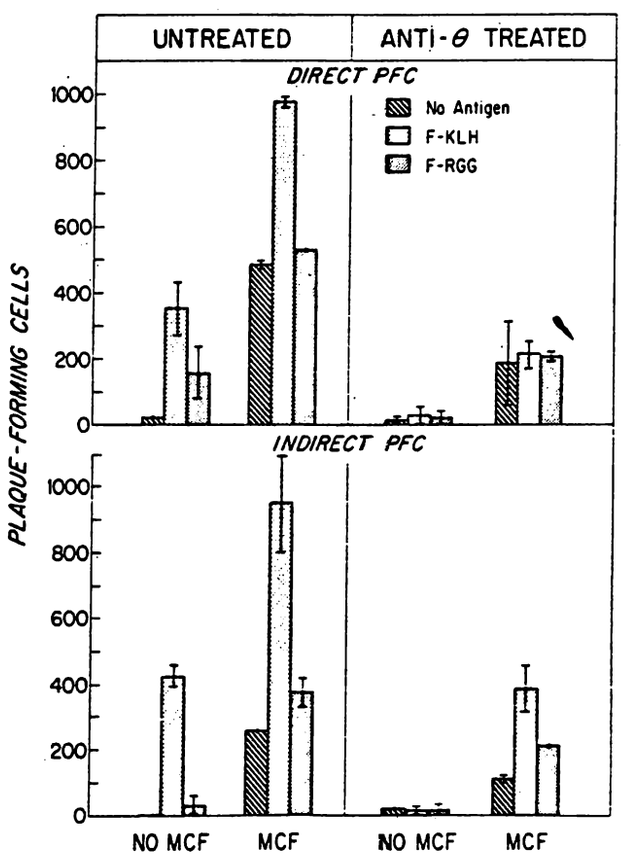

FIG. 3. The PFC response of spleen cells treated with anti-θ and complement to macrophage culture fluids (MCF). Normal or treated F-KLH-primed spleen lymphocytes were incubated with or without antigen as in the previous figure in the presence or absence of macrophage supernatants.

both macrophage culture fluids and F-KLH. Anti-θ-treated spleen cells did not respond as expected. Culture of such cells in macrophage fluids, however, produced a response to about 200 direct and indirect PFC. Not shown in Fig. 3 is the additional control that brings out an interesting relationship. We added to the anti-θ-treated spleen cells (10^7) an equal number of spleen cells from a KLH-immune mouse given 750 R of whole-body X-irradiation just before harvesting. Such cells provided helper T-cell activity producing now strong responses upon challenged with F-KLH of 3,010 direct and 4,560 indirect PFC, respectively. This strong anti-F response was not enhanced any further by culturing the cells with macrophage culture fluids and F-KLH (i.e., 2,740 direct and 4,500 indirect PFC). The experiment suggested that in order to see the effects

of the macrophage culture fluids in enhancing helper activity it was best to use culture conditions of limited T-cell function.

Effects of Macrophage Supernates on Cultures of Nude Mice Spleen Cells. Spleen cells from nude athymic mice developed no indirect PFC in culture and a small direct PFC response (Fig. 4). The addition of culture supernates from macrophages plus SRBC increased the direct PFC response and allowed for an indirect PFC response. Note that the increase in PFC necessitates the addition of both SRBC plus macrophage supernate; the latter by itself only produced a marginal increase in PFC.

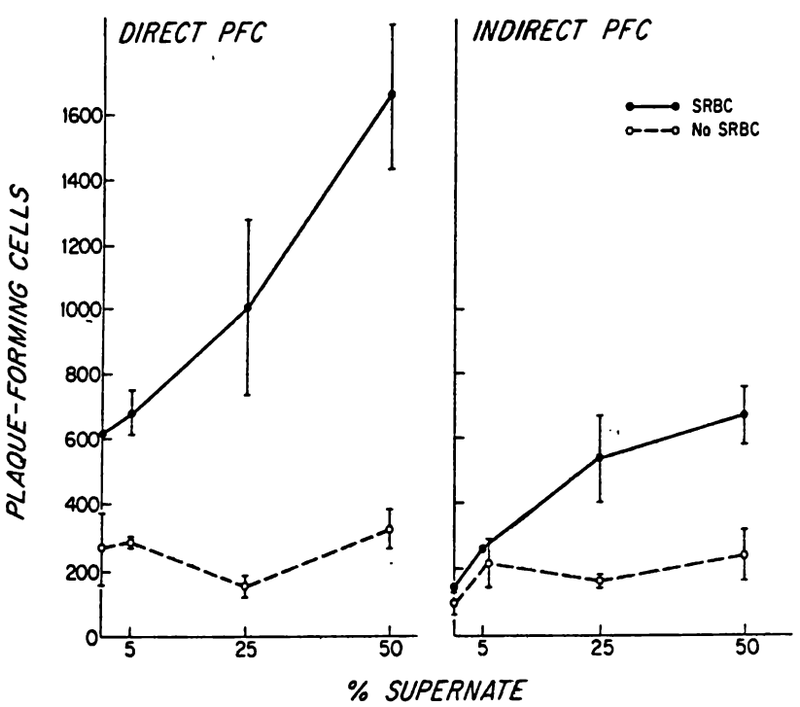

FIG. 4. The PFC response of nude mouse spleen cells to SRBC in the presence of macrophage supernates.

The Cell Source of the Stimulatory Activity. It is possible that other cells in the PEC besides the macrophages could be the source of the stimulatory material. The best candidate could be a T lymphocyte adherent to the dish and not eliminated by the handling and washing procedure. To eliminate such putative T cells, the PEC were treated with anti-θ antibodies in the presence of fresh guinea pig serum. Culture fluids were generated from these anti-θ-treated PEC, from cells treated with normal mouse serum and complement, and from untreated cells. It was observed that PEC treated with anti-θ produced the same degree of stimulation as PEC treated with normal mouse serum. This was tested on thymocyte proliferation and on the PFC response to F with identical results. In agreement with this observation, it was found that PEC obtained from X-irradiated mice generated in culture good stimulatory activity. Such PEC were made entirely of macrophages. Culture fluids from 3T3 cells, mouse embryo cells, or unstimulated spleen lymphocytes did not contain stimulatory activity.

The experiment in Fig. 5 shows similar increases of PFC without the need of addition of antigen in the presence of PEC fluids generated from either untreated PEC or anti-θ-treated PEC. The experiment of Fig. 5 also stresses the important relationship between the stimulatory activity from macrophages and the T-cell cooperative activity. The spleen cells used for this experiment were harvested from recently boosted mice. These cells, upon challenge with F-KLH, exhibited

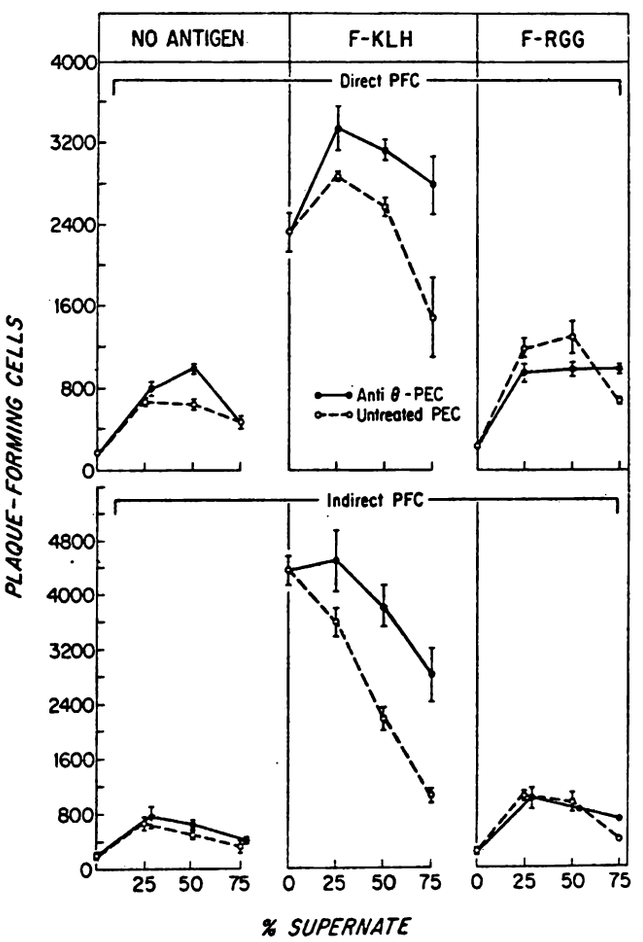

FIG. 5. Assessment of PFC on spleen cells incubated with supernates from peritoneal exudate cells treated with anti-θ or normal serum and complement.

a much larger number of PFC than those shown in the previous series depicted in Figs. 1–3. In the presence of such larger responses, the addition of macrophage supernates results only in a slight enhancement of the F-KLH response (central panels); furthermore, at higher concentrations, the supernates clearly had an inhibitory effect, the response being lowered up to one-half or one-third of its original level.

Attempts to Establish the Nature of the Stimulatory Molecule. The nature of the stimulatory factor is not known. The factor can be generated in cultures free of fetal calf serum, is resistant to freezing and thawing, and sensitive to boiling.

Because macrophages can secrete a plasminogen activator-like material (20),

we examined whether the stimulatory material was sensitive to DFP. There was no loss of activity by treatment with DFP (i.e., 25 to 50% concentrations of untreated supernates stimulated thymocyte incorporation of thymidine to 2,910 cpm and 12,722 cpm, respectively; figures for DFP-treated supernates [at 10^{-4} M] were 3,367 cpm and 10,341 cpm; background incorporation was 642 cpm).

Culture fluids generated from A/St PEC were found not to lose any of their thymocyte-stimulating activity by passing through an anti-*H-2*^{a} Sepharose 4B column (i.e., untreated PEC fluid: stimulation of 3,917 cpm ± 423; PEC fluid after passing through normal mouse serum column: 4,923 ± 392; PEC fluid passed through anti-*H-2*^{a} column: 6,087 ± 698; all fluids were tested at three concentrations, but the indicated results are at 50%.

The size of the active fraction was investigated using gel filtration (Fig. 6). The active moiety eluted from a G100 column in a position lighter than trypsin and heavier than ribonuclease. Activity stimulating thymocyte proliferation was only found in fractions ranging from 15,000 to 21,000 daltons (Fig. 6), in general agreement with data obtained by Gery and Handschumacher (21). Limited studies have been done, so far, assaying on PFC responses. It is clear, however, that a pool of fraction active in thymocyte proliferation likewise resulted in PFC cell stimulation. The active fraction eluted from a G100 was treated with several enzymes and analyzed for stimulation of thymocyte proliferation. The stimulatory material was resistant to trypsin, ribonuclease, carboxypeptidase A, papain, and neuraminidase. It was reduced by 41% by treatment with pronase and totally destroyed by chymotrypsin and pepsin.

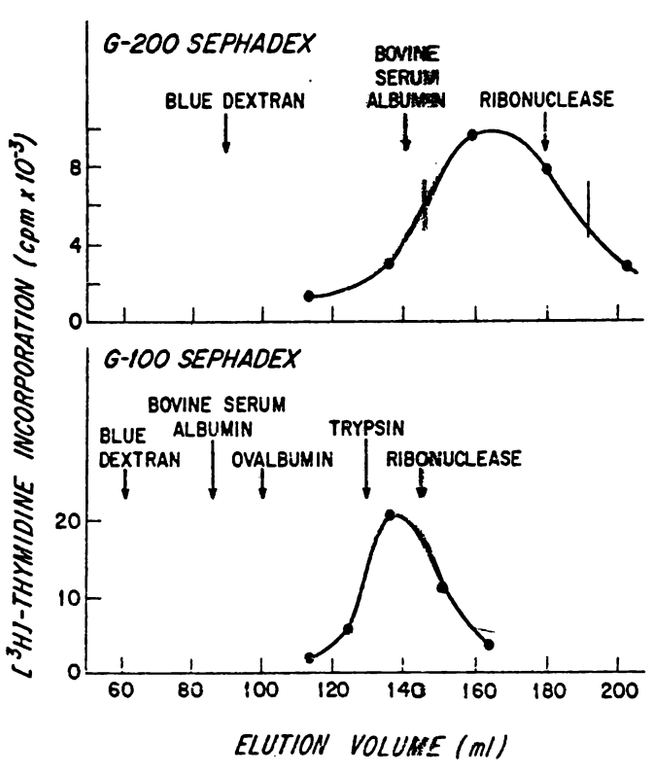

FIG. 6. The graph shows the fractionation of macrophage culture fluids in Sephadex G200 or G100. The figure shows only those fractions with cpm over background.

Discussion

Cultures of peritoneal cells rich in macrophages contained activities that stimulated DNA synthesis in thymocytes and, to lesser extent, in spleen lymphocytes. The stimulation of thymocytes did not require the addition of lectins to the culture, as was first reported in the original papers of Gery, et al. (10, 11). In agreement with them, a marked potentiation of proliferation was observed by addition of PHA (16). The culture fluids also contained an activity that influenced B-cell differentiation. Whether both activities—thymocyte proliferation and B-cell stimulation—reside in the same molecule, as suggested by the early fractionation experiments, needs to be verified using more purified materials. (In the context of this discussion, both activities are considered as a single entity.) An important requirement for detecting the stimulatory molecule was to eliminate by dialysis a small molecular weight inhibitor of DNA and protein synthesis (17). This inhibitor of less than 1,000 daltons stopped cell division and protein synthesis of a number of cells in a reversible manner. As with the stimulatory molecule, it was found associated with macrophages in cultures. The exact relationship of these contrasting molecules clearly needs to be established.

Several points concerning the stimulatory molecule have been reasonably well documented. All evidence points to the macrophage as the cell source. Indeed, cultures of macrophages free of lymphocytes, in particular those of the T class, generated active fluids. In contrast, cultures of unstimulated lymphocytes or of other cell lines failed to produce the activity. A second point of interest is the relationship between the biosynthesis and/or secretion with the activity of the macrophage. Indeed, in experiments to be reported, we have found that phagocytosis enhanced the amount of active material in culture by several fold. Further studies are in progress attempting to dissect the relationship between membrane binding, interiorization, and/or cellular activation by various materials with the actual biosynthesis and secretion of the stimulatory molecule.

The stimulatory molecule has a size ranging between 15,000 to 21,000 daltons and lacks *H-2* determinants. Hence, by both these criteria, this molecule can be separated by the thymic factors which are of larger size and which contain some antigens coded by *H-2* complex, therefore, binding to anti-*H-2* antisera (22–29). It is possible, however, that crude, unfractionated preparations of thymic factors could contain this macrophage molecule, since, under the culture condition where they are generated, macrophages have not been excluded. The stimulatory molecule was heat labile, resistant to trypsin treatment, and partially affected by pronase. This suggests that it is a protein with a low content of basic residues not available to tryptic digestion. The fact that it is resistant to DFP rules out that it is a serine esterase such as the plasminogen activator-like molecule secreted also by macrophages.

In order to obtain a better perspective of the functional significance of this stimulatory molecule from macrophages, certain points need to be clearly evaluated: (*a*) The stimulatory molecule clearly exerted an effect on thymocytes and on T helper cells; the observation that anti-θ-treated spleen cells or nude mice spleen cells responded can be interpreted to mean that the stimulatory molecule also acted directly on B cells. Alternatively, the above results may be

explained by a primary effect on some low level of residual T cell or of stem cells. Hence, it is possible that the macrophage stimulatory molecule may act directly on B cells or via T-cell stimulation or under both conditions. (*b*) The stimulation of hapten-primed cells without antigens raises the possibility that macrophage stimulatory molecule by itself—directly or indirectly—could allow for expansion and differentiation of any B-cell clone. However, the need for antigen in the experiments with nude mice spleen cells contrasts with the apparent lack of requirement in the F-KLH system. Either lymphocytes differ in the response to the molecule—the primed population having a lower threshold for response—or some sequestered antigen, present in the F-KLH-primed cells, is being exposed as a result of disruption of the spleen and serves as an added stimulus to the macophage molecule. (*c*) The fact that macrophages are made to secrete more of the stimulus as a result of uptake and phagocytosis of particles clearly indicates that the process of synthesis and/or secretion is under some form of modulation by extracellular materials that interact with the macrophage. Investigation of the factors that modulate the synthesis and secretion of this molecule may give us some clue as to its possible intervention in immune process—indeed, we are thinking along the lines that lymphocyte products or a number of adjuvants (most of which are known to be taken up by phagocyte) could have some of their effects via the stimulation of the macrophage. And (*d*) the relationship between the stimulation of macrophages and the T-helper function needs to be closely evaluated—the results suggest a clear synergism of both when T-cell helper function is less apparent (Figs. 1 and 2) and an antagonistic effect under optimal T-cell helper function (Fig. 5). Perhaps the B cell is turned off after a certain level of stimulation or perhaps the antagonism reflects competition for a membrane receptor of two activities.

It is our hypothesis that the macrophage plays a very early regulatory role in immune induction. Clearly, the regulatory role of phagocytes could be accomplished not only as a result of antigen uptake, degradation, and focusing, but also as a result of secretion of the kind of molecule described herein.

Summary

Culture fluids of peritoneal exudate cells rich in macrophages stimulated DNA synthesis of thymocytes and, to lesser extent, of spleen cells. We also investigated the effects of culture fluids from macrophages on the in vitro response to a hapten-carrier protein (fluorescein-hemocyanin) using spleen cells from immune mice. Macrophage culture fluids contained an activity that increased the plaque-forming cell response of both IgG and IgM class. This increase was observed in the absence of any added hapten protein to the culture. The helper function of T lymphocytes (as evidenced by challenging with the hapten on the homologous carrier) was also increased by the macrophage culture fluid. However, this enhancement was best observed in conditions of relatively low T-cell activity. Also, the macrophage fluid allowed spleen cells of nude athymic mice to make a plaque-forming cell response to sheep red blood cells of both the IgM and IgG class. The macrophage was the cell source of the stimulatory molecule since it was generated only in cultures of macrophages devoid of

significant number of lymphocytes. Stimulatory activity was not found in cultures of lymphocytes, mouse embryo cells, or 3T3 cells. The thymocyte stimulatory molecule did not contain *H-2* antigens, was resistant to diisopropylfluorophosphate treatment, eluted from Sephadex with a size ranging from 15,000 to 21,000 daltons, and was sensitive to chymotrypsin and pepsin.

We are grateful to Doctors Baruj Benacerraf, Martin E. Dorf, and David H. Katz for their comments and advice.

Received for publication 27 February 1975.

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THE MODULATION OF LYMPHOCYTE FUNCTIONS BY MOLECULES SECRETED BY MACROPHAGES*

II. Conditions Leading to Increased Secretion

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Culture fluids rich in mononuclear phagocytes have a powerful effect on various in vitro reactions of lymphocytes (1–9). The culture fluids contain a mitogen for thymocytes and mature lymphocytes (1–4, 8). Also found are active principles which differentiate memory B cells into antibody-secreting cells and which increase helper activities of T lymphocytes (2, 3). In a previous report we obtained from experiments in which lymphocytes were depleted from the cell preparations, strong evidence that the cellular source of the active component(s) was the macrophage (3). The activity secreted into the medium, however, was variable from experiment to experiment, perhaps related to the state of macrophage activation. In this paper we present results of experiments in which we studied the relation between macrophage activation and/or stimulation with the secretion of the various activities. We have found two ways in which to generate high levels of lymphostimulatory activities in macrophage cultures: one is to add a series of materials that are readily taken up by the macrophages; the second is to add a small number of activated T cells to the macrophage-rich cultures. Other experiments indicate that there is an inverse relationship between macrophage activation and the secretion of the lymphostimulatory activities.

Materials and Methods

Culture Fluids. Peritoneal exudate cells (PEC)[1] were harvested from mice, washed once, and cultured at an initial density of 4×10^6 cells per ml, usually, in 1640 medium containing 5% fetal calf serum (vol/vol) (3) in 35 × 10-mm dishes (Falcon Plastics, Division of Bioquest, Oxnard, Calif.). In all experiments the macrophages were planted for an initial period of 2 h, after which the dishes were shaken hard to remove loosely adherent cells. The dishes containing the adherent cells were washed and then cultured for various times, usually for 24–48 h. The PEC were obtained from A/St mice (West Seneca Laboratories, Buffalo, New York), about 8–12 wk of age, of either sex. The A/St mice were either untreated or had been injected intraperitoneally (i.p.) with one of the following: 1.5 ml of a 10% proteose peptone solution (Difco Laboratories, Detroit, Mich.); 1 ml of thioglycollate broth (Difco Laboratories, Catalog no. 0236-01); or 100 μg of *Escherichia coli* lipopolysaccharide (LPS) (Difco Laboratories, Westphal preparation). The doses used and time of

* This work was supported by grants AI 10091 and NCI 14723 from the National Institutes of Health and from a grant from The National Council for Tobacco Research – U. S. A., Inc. Dr. Unanue is the recipient of a Research Career Development Award. Dr. Calderon's present address is: Instituto Politecnico Nacional, Mexico 14, D. F.

[1] *Abbreviations used in this paper:* F, fluorescein; F.KLH, fluorescein-conjugated hemocyanin; F.RGG, fluorescein-conjugated rabbit IgG; PEC, peritoneal exudate cells; PEL, peritoneal exudate lymphocytes; PFC, plaque-forming cells; LPS, *E. coli* lipopolysaccharide.

harvesting, 3-days postinjection, gave rise to populations of PEC containing mostly macrophages activated by morphological criteria (i.e., large degree of spreading, abundant lysosomes, and pinocytic vesicles). A series of experiments was done using PEC from *Listeria monocytogenes*-infected mice. Mice received an i.p. dose of 2×10^3 organisms (LD 50, 2×10^5), 7 days later 1.5 ml of 10% peptone i.p. 3 days later, the mice were sacrificed and the PEC harvested (10 days after infection). These exudates were sterile and contained abundant activated macrophages. All culture fluids were dialyzed against culture medium for 72 h before testing.

Assay for Lymphostimulatory Activities. Two assays were done (3). In all experiments culture fluids were tested for mitogenic activity on thymocytes. Thymocytes (5×10^6) were cultured in 1 ml of 1640 medium in 12 × 75-mm tubes for 72 h at 37°C in a 5% CO_2 in-air incubator (3). The cultures received 1 μCi of [^{3}H]thymidine about 12 h before harvesting. Incorporation of labeled ^{3}H into acid insoluble precipitate was measured by standard methods. The background counts per minute (cpm) of thymocytes ranged from 400 to 1,000 cpm, usually about 500. The macrophage culture fluids were mixed (vol/vol) with normal medium and assayed for their mitogenic principle on thymocytes as above. Frequently, phytohemagglutinin (PHA) was added to the cultures together with macrophage culture fluids (1).

The second assay used in most but not all experiments was to test for antibody responses, in vitro, using the method of Mishell and Dutton (10). Spleen cells from mice immunized to fluorescein (F)-conjugated hemocyanin (KLH) were cultured with 0.1 μg of F.KLH, 0.1 μg of F-conjugated rabbit IgG (RGG), or without antigen for 4 days in the presence or absence of the macrophage culture fluids. Anti-F-secreting cells (plaque-forming cells, PFC) were detected by a plaque method using F-conjugated red cells. Two effects of macrophage culture fluids were usually noted. One was an increase of PFC to F, in the absence of antigen challenge, an effect considered to be a B-cell differentiating activity (3). The second effect was a higher increase in PFC in the presence of F.KLH which we attribute to a combined effect of the active principle(s) on both B and T cells. Full details of methods and of our interpretation of the effects of macrophage fluids were outlined in our previous paper (3).

Separation of T Cells. T lymphocytes from PEC (peritoneal exudate lymphocytes, PEL) or from spleen were isolated by the method of Julius et al. (11). We first planted cells in culture dishes for 1 h (10^7 per ml) and passed the nonadherent population through nylon wool columns. The preparations consisted of about 98% T cells and were essentially devoid of macrophages and B cells. Only a very occasional macrophage was found upon 2 or 3 days of culture of the T-cell preparations. The T cells were cultured in 1640 medium, as described, usually with the addition of 2-mercaptoethanol (10^{-4} M). Survival of cells in culture for the first 48 h was excellent (approximately 75%).

Challenge of Macrophages. We tested the effects of various materials on the production of the lymphostimulatory activities: *(a)* sheep red cells incubated with a subagglutinating dose of rabbit IgG antibody; dose of red cells to PEC was 10:1; *(b) Listeria monocytogenes* vaccine, 50 bacteria per PEC; *(c)* latex beads of 1.09 μm diameter (Dow Chemical Company, Midland, Mich.), 50 latex beads per macrophage; *(d)* LPS, 50 μg per ml; and *(e)* beryllium sulfate ($BeSO_4$), 0.1 μg per ml.

Miscellaneous. In one experiment PEC were treated with anti-θ antibodies and complement in order to kill any T cells adhering to dishes (3). The anti-θ antibodies were from the same batch used previously (3). The source of complement was guinea pig serum.

Results

Effect of Various Stimuli on the Production of Lymphostimulatory Activities. PEC culture fluids stimulated DNA synthesis of thymocytes and an increased PFC response in immunized spleen cells. The activity of PEC cultures under basal conditions was variable and usually tended to be low (3). However, the addition to the macrophage culture of various agents, such as bacteria, antigen-antibody complexes, latex particles, etc., led to an abrupt but usually temporary increase in the activities. In the experiments shown in Figs. 1 and 2, peptone-induced PEC were planted in culture and challenged for 24 h with antibody-coated sheep red cells or *Listeria* vaccine. Fig. 1 shows that culture

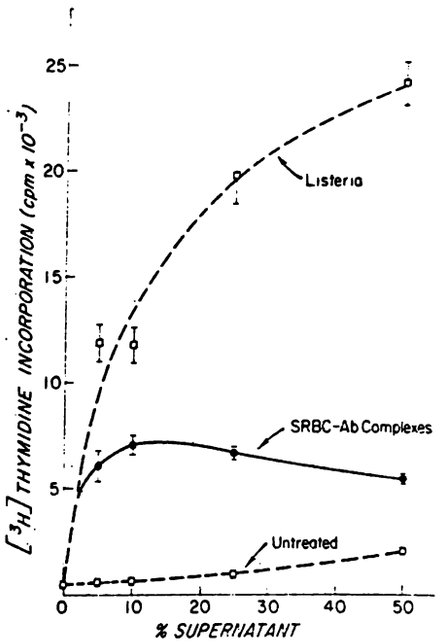

FIG. 1. The figure indicates the incorporation of radiolabeled thymidine by thymocytes cultured in PEC culture fluids after exposure to antibody-coated red cells or to *Listeria* vaccine. The PEC culture fluid was obtained during the first 24 h of culture.

fluids from treated PEC contained a higher amount of the mitogenic factor compared to cultures from untreated macrophages. The incorporation of [^{3}H]thymidine was increased about fivefold by fluids from PEC challenged with opsonized sheep cells and about 20-fold by fluids from macrophages challenged with *Listeria* organisms.

The effects of the culture fluids on antibody formation are depicted in Fig. 2. Spleen cells from mice immunized to F.KLH were cultured for 4 days with F.KLH, F.RGG, or without antigen in the presence or absence of the macrophage culture fluids. The following points can be made from this experiment: *(a)* as before (3), the macrophage fluids stimulated the development of PFC in the absence of antigen challenge (left-hand box, no antigen), but fluids from macrophages exposed to *Listeria* and antibody-coated red cells had higher activity than those from untreated macrophages; *(b)* all fluids increased the response to F.KLH but more markedly the fluids from stimulated PEC (middle box); and *(c)* the response to F.RGG (last vertical box) was comparable to that seen with lymphocytes cultured without antigen.

Not shown in Figs. 1 and 2 are control results testing culture fluids in which the dead *Listera* organsisms were incubated for 24 h with medium. Such culture fluids did not affect the lymphocytes' responses.

We explored the above phenomenon further in the experiment shown in Fig. 3 and Table I. Peptone-induced PEC were exposed to various agents and cultured for a period of 3 days, harvesting the medium every 24 h. There were increased amounts of mitogenic activity in cultures of PEC challenged with *Listeria* organisms, the increased activity decreasing after 48 h of culture. Latex beads were as stimulatory, the activity decaying also at 72 h. Antibody-coated sheep

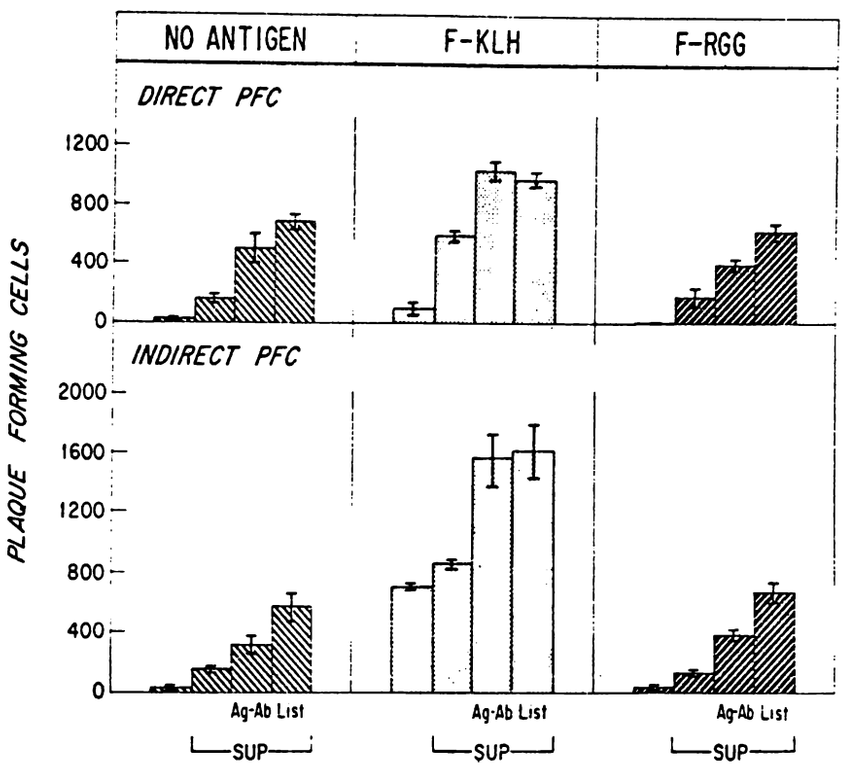

FIG. 2. The same PEC culture fluids of the experiment of Fig. 1 were tested on antibody formation in vitro. Ab-Ag refers to those PEC fluids obtained after uptake of antibody-coated red cells; list refers to fluids from PEC exposed to *Listeria*. The first vertical column in each box are cultures devoid of any PEC culture fluids. All cultures were tested at 25% concentration of PEC fluids.

TABLE I

Effects of Different Macrophage Culture Fluids on the PFC Response

Addition to spleen cultures	No antigen		F.KLH (0.1 μg)	
	IgM PFC	IgG PFC	IgM PFC	IgG PFC
No addition	60 ± 0	53 ± 11	200 ± 40	203 ± 25
25% PEC fluids				
Untreated PEC	260 ± 40	293 ± 110	887 ± 75	1,180 ± 111
After latex	707 ± 70	527 ± 527	2,280 ± 485	2,093 ± 411
After *Listeria*	680 ± 69	527 ± 50	2,047 ± 121	2,633 ± 325
After SRBC-antibody	220 ± 53	200 ± 60	1,160 ± 69	1,373 ± 219
After Be	147 ± 31	160 ± 60	1,247 ± 133	1,740 ± 201
After LPS	300 ± 52	320 ± 35	1,440 ± 260	1,753 ± 500

IgM PFC refer to direct PFC; IgG are PFC detected with the use of facilitating antiserum. Each result is the mean PFC from two to three dishes (± standard error of the mean). Spleen cells were obtained from mice primed with F.KLH in Freund's adjuvant. The PEC culture fluids were obtained during the first 24-h period of culture. All PEC were from peptone-injected mice.

red cells were much less stimulatory. $BeSO_4$, a salt that stimulates macrophage activity in vitro (and which is also a potent adjuvant [12]), produced a slight increase in activity. Comparable results were obtained in antibody formation in vitro. We show in Table I only the results of fluids from the first 24 h of culture.

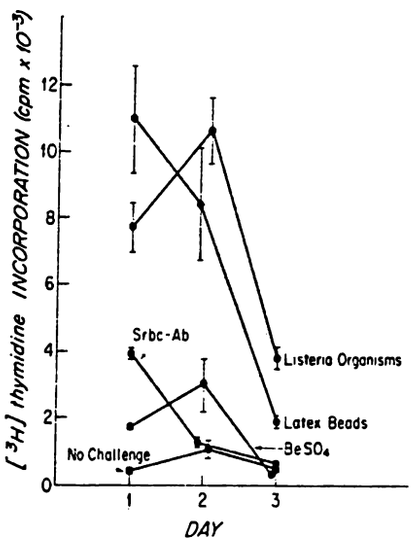

FIG. 3. PEC culture fluids were tested at a 25% concentration on thymocyte DNA synthesis. The explanation of the experiment is in the text.

The effects of LPS on the secretion were next examined. Peptone-induced PEC were exposed to LPS for 24 h. The culture fluids obtained during 24-h periods of culture were tested on the thymocyte DNA synthesis assay. A representative experiment is shown in Fig. 4. Exposure of PEC to LPS generated a marked increase in activity (1) during the first 48 h, which decreased by 72 h (data not shown). The dose of LPS was critical (not shown in Fig. 4). In all experiments stimulation required 50 μg of LPS per ml of culture and was not seen with 20 μg or less. Incubation of spleen lymphocytes with LPS did not result in fluids with mitogenic activity.

Lymphostimulatory Activities in Cultures of Activated Macrophages. PEC from mice injected with various materials known to generate morphologically activated macrophages were tested for the secretion of lymphostimulatory molecules. PEC were harvested from mice injected 3 days previously with LPS i.p. The macrophages exhibited clear morphological signs of activation but, as shown in Fig. 4, were not actively secreting the mitogenic principal. Furthermore, in several other experiments such in vivo LPS-stimulated macrophages responded very weakly, if at all, to challenge with latex beads, despite extensive phagocytosis. Thus, direct addition of LPS to peptone macrophages in vitro (data of Fig. 4) or to normal PEC (data not shown) did stimulate increased secretion. However, it appeared that once a macrophage reached a state of "activation" by LPS, it became refractory to secretion of the lymphostimulatory activities.

Thioglycollate-stimulated PEC had very little basal activity. Furthermore, in three experiments, these macrophages did not increase their production after phagocytosis of latex beads.

In contrast to the results with LPS or thioglycollate-activated PEC, striking effects were seen in cultures of PEC from *Listeria*-infected mice (Table II). In this culture note that the response of thymocytes to a 25% vol/vol concentration of culture fluids was of 75,409 cpm compared to a background of 583 cpm. The results of this experiment are not unique, in six different culture fluids the

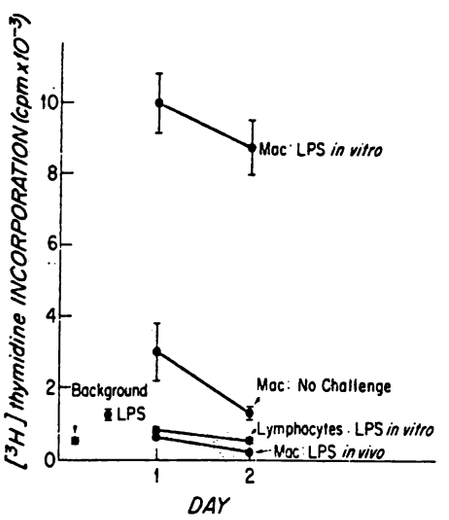

FIG. 4. The same setup as of Fig. 3 also testing the fluids at 25% concentration on thymocyte DNA synthesis. Mac: LPS in vitro, fluids from PEC exposed in culture to LPS; Mac: No challenge, cultures from unstimulated PEC; Lymphocytes: LPS in vitro, fluids from lymphocytes cultured in LPS; Mac: LPS in vivo, fluids from PEC obtained from mice injected with LPS in vivo.

TABLE II
Mitogenic Activity of PEC from Listeria-Infected Mice

Time of culture	cpm incorporation			
	5	10	25	50
	% PEC fluids			
Day 1	15,440 ± 1,350	39,117 ± 1,256	75,409 ± 3,653	92,203 ± 1,644
Day 2	2,294 ± 179	5,219 ± 443	9,062 ± 1,340	11,313 ± 809
Day 3	1,671 ± 17	2,736 ± 100	5,851 ± 780	6,513 ± 147
Day 4	1,259 ± 101	2,453 ± 226	4,282 ± 136	6,074 ± 493

Background cpm was 583 ± 103. Each result is the average of three dishes. The PEC culture fluids were obtained every 24 h for 4 days (first vertical column) and assayed at different concentrations on thymocytes.

range of stimulation at 25% vol/vol concentration ranged from about 20,000 to 75,000 cpm. At the time of harvest, the exudates were sterile and made up of highly activated macrophages.

The Effects of Activated T Cells on Macrophage Cultures. The high activity found in PEC from *Listeria*-infected mice contrasted with the meager activity found in PEC activated by LPS or thioglycollate. One possible explanation was the presence of some activated T cells in *Listeria* PEC which could contribute and/or influence the secretion of active molecules. This explanation was found to be true. Figs. 5 and 6 show two key representative experiments. In the experiment shown in Fig. 5 PEC from *Listeria*-infected or normal mice were cultured for 1 or 2 days. The PEC from *Listeria*-infected mice generated highly active mitogenic factor, but the activity decayed during the 24- to 48-h period of culture (Day 2). At the end of 24 h, when the cultures were no longer actively secreting the principles, we added to one set of dishes (or to dishes containing the nonstimulated PEC) a small amount, 3×10^6, of T lymphocytes from the

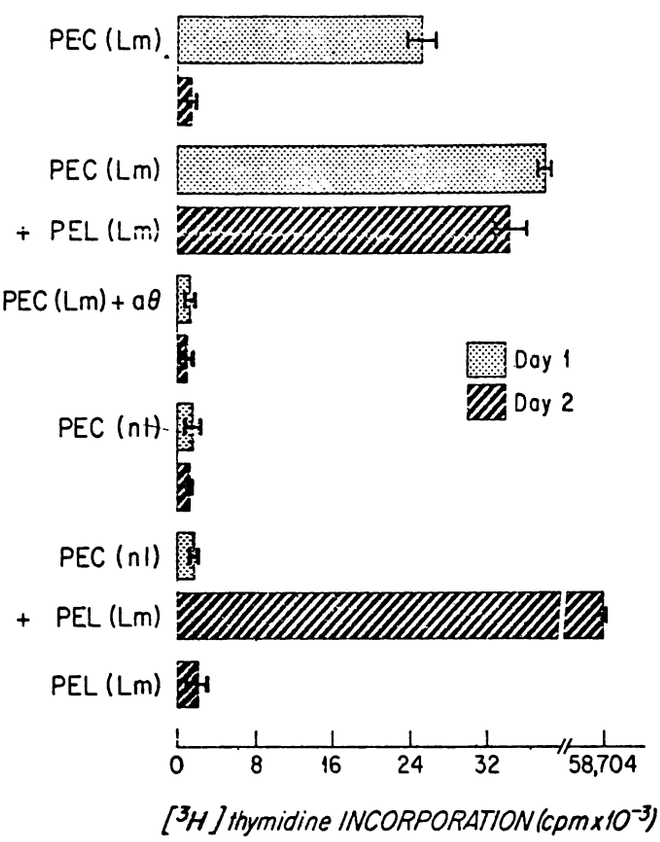

FIG. 5. PEC from *Listeria*-infected (PEC Lm) or normal (PEC Nl) mice were cultured for 1 and 2 days. At the 2nd day peritoneal exudate lymphocytes from *Listeria*-infected mice (PEL Lm) were added to some of the cultures. PEC (Lm) and anti-θ refers to cultures treated with anti-θ and complement. All cultures were tested at 25% concentration on thymocytes.

itoneal cavity (PEL) of the infected mice. This resulted in the secretion of ikingly high amounts of mitogenic factor. By themselves cultures of T lym-ocytes (in the presence or absence of 10^{-4} M mercaptoethanol) did not gener- active principle. Another point to note in the experiment of Fig. 5 is that atment of the PEC from *Listeria*-infected mice with anti-θ and complement olished the high production of mitogenic factor seen during the first 24 h of tures.

The experiment of Fig. 6 extends the previous results, showing that the dition of activated lymphocytes from *Listeria* exudates, but not from normal udates, to 48-h culture of *Listeria* PEC resulted again in an increased secre- n of the mitogenic principle. Also note that the PEC challenged at 48 h with tibody-coated red cells responded very well, secreting large amounts of mate- l.

Cytological examinations of cultures from normal or *Listeria*-activated PEC which activated T cells were added showed lymphocytes in between the acrophages and frequent ones attached to them. The number of lymphocytes th ameboid features was high, implying active motility of the activated T ls.

One point concerning the lymphostimulatory activities of PEC from *Listeria*-

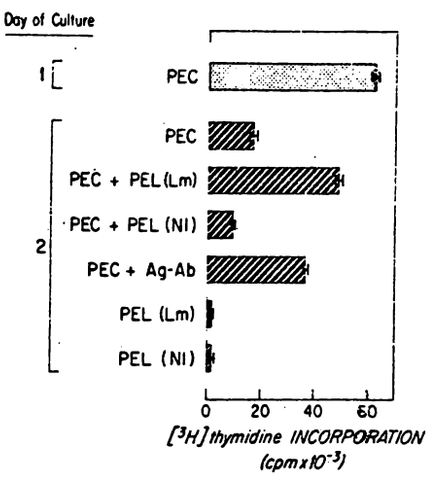

FIG. 6. The experiment has the same basic protocol of Fig. 5 using PEC from *Listeria*-infected mice. At Day 2 the PEC from *Listeria*-infected mice received peritoneal lymphocytes from *Listeria*-infected (PEL Lm) or normal (PEL Nl) mice or antibody-coated sheep red cells (Ag-Ab). Cultures of lymphocytes by themselves are included. All culture fluids were tested on thymocytes at 25% concentration.

infected mice is worth emphasizing. In most experiments with PEC, normal or stimulated, the mitogenic activity and the B-cell differentiating activities have usually run in parallel, but notable exceptions have occurred. We tested five culture fluids from PEC of *Listeria*-infected mice in both assays (i.e., thymocyte DNA synthesis and antibody formation in vitro). All had strong mitogenic activity. In two culture fluids the B-cell activity was negligible; in another the B-cell activity was maintained high during the 24–48-h period of culture, while the mitogenic factor markedly decreased; in the remaining two, both activities ran parallel.

The relationship between lymphocytes and macrophages in the production of the lymphostimulatory activities was studied using antigen-primed T cells. Mice immunized a month previously with F.KLH were boosted with 50 μg in complete Freund's adjuvant i.p., and a week later the T cells were isolated from the spleen. The T cells were added to cultures of PEC and the culture fluids harvested after 24 h of culture and tested for thymocyte activity. Table III shows the results of one such experiment. Cultures of T cells by themselves did not generate significant activity. The addition of activated T cells to the macrophages increased markedly the production of the factor(s). In experiments not shown, the optimal ratio of T cells to macrophages has been in the order to 5–10. The presence of antigen increased the activity of the T cells, although this was not essential in all experiments. In the experiment of Table III (or in those of Figs. 5 and 6), significant activity was found just by addition of the T cells to the macrophages. However, in other experiments using F.KLH, addition of the antigen was necessary. Note in Table III that cultures of normal lymphocytes, macrophages, and antigen also contained some activity. The requirement for antigen may be a reflection of the state of activation of the T cell.

In one further experiment, we explored whether the F.KLH-activated T lymphocytes interacted best with the normal PEC or PEC activated by thiogly-

TABLE III

Effect of Immune T Lymphocytes

Composition of cultures			Incorporation of [^{3}H]thymidine	
PEC	Lymphocytes	F.KLH	No PHA	PHA
+	–	–	1,734 ± 187	7,534 ± 972
+	Immune: 4 × 10^6	–	9,701 ± 2,106	20,746 ± 2,671
+	Immune: 4 × 10^6	+	15,717 ± 605	29,321 ± 3,123
–	Immune: 4 × 10^6	–	681 ± 57	1,645 ± 21
–	Immune: 4 × 10^6	+	936 ± 47	4,053 ± 774
+	Immune: 8 × 10^6	–	10,029 ± 1,087	23,959 ± 1,914
+	Immune: 8 × 10^6	+	31,098 ± 2,004	37,339 ± 2,328
–	Immune: 8 × 10^6	–	516 ± 165	1,532 ± 114
–	Immune: 8 × 10^6	+	498 ± 91	1,922 ± 110
+	Normal: 4 × 10^6	–	3,705 ± 282	11,224 ± 8,647
+	Normal: 4 × 10^6	+	5,455 ± 880	16,470 ± 115

The incorporation of [^{3}H]thymidine by thymocytes cultured in the absence or presence of PHA was 08 ± 60 and 1,567 ± 79 cpm, respectively. Not included in this experiment are controls of cultures of normal lymphocytes; these have never generated active materials in subsequent experiments. The culture fluids were tested at 25% concentration.

collate, peptone, LPS, or *Listeria*-infected mice. The PEC from *Listeria*-infected mice were tested after 48 h of culture, by which time there was no secretion of the mitogenic principle. Along the lines seen before, a comparable response was obtained from PEC of normal, peptone, or *Listeria*-infected mice, but little from PEC after injection of thioglycollate or LPS.

Discussion

The present series of experiments outlined some of the conditions for secretion of the lymphocyte stimulatory factor(s) by macrophages. In our previous study we had shown, in agreement with others, that culture fluids rich in macrophages exerted quite powerful action on lymphocytes in culture. The culture fluids contained a mitogenic principle for thymocytes and to lesser extent for B and T lymphocytes. Besides the thymocyte mitogenic principle, macrophage culture fluids, under appropriate conditions, increased the helper activity of primed T lymphocytes and differentiated primed B cells to active antibody-secreting cells. Whether or not several molecules were responsible for these different effects is not clear to us at present. The fact that on occasion the mitogenic and B-cell differentiating activities were not found in the same culture fluid suggests that there may indeed be at least two different molecules. In our preliminary biochemical studies, both activities were found on a single fraction of about 20,000 daltons (3). However, other subsequent examinations have shown heterogeneity in size of the mitogenic principle, suggesting various molecules or a tendency for a single molecule to polymerize or dissociate; in some instances some dissociation of both activities was noted.

In this study we outlined two conditions that led to increased secretion of the

active products. One was the challenge of macrophages by various agents. Clearly, latex beads, endotoxin, sheep cell-antibody complexes, *Listeria* organisms, even Be salt produced an increased secretion of the activities. The increased activity was seen during 1 or 2 days of culture and then declined. The increased secretion was consistent but with some degree of variation from experiment to experiment. This phenomenon is consistent with several observations made on secretion of enzymes and other molecules by macrophages. Plasminogen activator-like molecules (13), elastase (14), collagenase (15), complement factors (16), pyrogen (17), and some lysosomal enzymes (18, 19) are secreted best upon phagocytic challenge. However, our observations differ from some of the published reports in two important aspects: *(a)* while an undigestible particle like latex stimulated some enzyme secretion continuously (13–15), the secretion of the lymphostimulatory activities was usually short lived; *(b)* while phagocytic stimuli best worked on "primed" or activated macrophages (20), this was not the case with the secretion of the activities described herein; it was clear from the experiments with LPS and thioglycollate that no direct relationship was found between morphological appearance and the secretion of lymphostimulatory material. The behavior of these PEC contrasted with *Listeria*-activated PEC, which were capable of responding to external stimuli but not as well as nonstimulated macrophages (Fig. 5).

A second condition for the secretion of the lymphostimulatory molecules was the presence of activated T cells in the culture together with the macrophages. Clearly, in all instances, the activated T-cell-macrophage culture did not necessitate the addition of antigen to secrete the factor (experiments of Figs. 5 and 6 and Table III), although antigen challenge increased the production (Table III). At this point, one ought to question what cell in the T-cell-macrophage mixtures secretes the factor—the macrophage, the activated T cell, or both. We know from experiments reported previously (3) that macrophages secrete the activities under basal conditions. Furthermore, the secretion as shown herein increases following phagocytosis. In contrast, cultures of activated T cells do not contain the active principle. We are inclined to ascribe the increased secretion in the activated T-cell-macrophage mixture to the macrophage affected in whatever form, by the activated T cell. However, it would not surprise us if the converse also is true and that the T cells upon interaction with the macrophage could be made to secrete the activities. Further experiments are in progress attempting to dissect this phenomenon.

We consider it likely that secretion of the active lymphostimulatory moieties may play some role in immune induction. We envision three conditions where this could occur: *(a)* after the uptake of a large bolus of antigen by macrophages of lymphoid tissue; *(b)* subsequent to interaction of the phagocytes with adjuvants (such as LPS, Be); or *(c)* after the generation of highly activated T lymphocytes. These conditions, however, are restricted by the "activity" of the phagocytes inasmuch as highly stimulated cells appear to become refractory. The observation that the activity or maturation state of the macrophage conditions the extent of secretion of some molecules but not others indicates a rather sophisticated regulation of its secretory process which may explain the protean role of macrophages in immunity and in inflammation.

Summary

Cultures of peritoneal exudate cells rich in macrophages were studied for the ecretion of lymphostimulatory molecules. Two conditions produced increased ecretion: *(a)* addition to the cultures of various agents that readily interacted ith macrophages, such as latex particles, antibody-coated red cells, endotoxin, *isteria* organisms, or Be salt; or *(b)* addition of activated lymphocytes.

In the first case the increased activity was found during the first 24 or 48 h fter uptake of the stimuli. Increased activity was found in normal or peptone-imulated macrophages but not in macrophages after injection of endotoxin or ioglycollate.

The addition of T lymphocytes from *Listeria*-infected mice to macrophage ltures increased greatly the activities. This increase was also produced by ldition of antigen-primed T cells together with antigen. The lymphocytes by emselves did not secrete active factors.

The lymphostimulatory activities were tested on thymocyte DNA synthesis nd on antibody formation in vitro. The latter assay was done on spleen cells om immunized mice where one striking effect was the stimulation of differen-ation to antibody-secreting cells. Some dissociation of both activities (thymo-te DNA synthesis and B-cell differentiation) was observed with selected lture fluids.

e thank Miss Janet Lefko, who provided excellent technical assistance in some of the experi-ents, and Dr. B. Benacerraf and Dr. D. Katz for their comments and advice on the manuscript.

eceived for publication 13 February 1976.

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Regulation of Immunity and Inflammation by Mediators From Macrophages

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Mononuclear phagocytes secrete a number of materials into the extracellular environment. The materials secreted by phagocytes can be grouped into three categories: a) enzymes affecting extracellular proteins (collagenase, elastase, lysosomal proteases, plasminogen activators), b) materials involved in defense processes (complement proteins, interferons, lysozyme), and c) factors regulating activities of surrounding cells. The latter include lymphostimulatory molecules, a colony-stimulating factor, and inhibitors of cell growth. The conditions for secretion of the materials depend on the activity of the phagocytes. The lymphostimulatory molecules secreted by macrophages exert various effects: 1) an increase in DNA synthesis of lymphocytes, 2) a maturation of early thymocytes to mature T cells, and 3) the differentiation of some B cells to antibody-secreting cells. The mitogenic principle has been partially isolated as a protein of 15,000 to 20,000 daltons. The secretion of lymphostimulatory molecules is increased following uptake of various materials by macrophages or by addition of activated T cells to macrophage cultures. (Am J Pathol 85:465-478, 1976)

THE MONONUCLEAR PHAGOCYTES are cardinal cellular elements in host resistance and in inflammation. The monocyte-macrophage system of cells is actively involved in phagocytosis and intracellular digestion. These cells participate in an essential way in the elimination of various kinds of microorganisms as well as in the elimination of debris, dead cells, and tissue during the cleaning of wounds. Phagocytes have a fundamental role in specific immunity through an intimate functional association that has developed between them and the lymphocyte, principally those of the thymic class. Phagocytes participate in early inductive events favoring in some way or another the early interaction of T and B lymphocytes with antigen. In cellular immunity the phagocytes are the main effector cells, the lymphocytes representing the specific recognition limb of the reaction.

The precise ways by which phagocytes carry out their many functions are not clear. In this paper, we consider the secretory function and raise the point, also discussed by others,[1,2] of its potential biologic importance. It is clear that the mononuclear phagocytes release a number of active

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Supported by grants from the National Institutes of Health and The Council for Tobacco Research—USA, Inc.

Presented at the Sixtieth Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, Calif., April 11, 1976.

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products into the extracellular environment in a truly secretory process. Through this secretory function, which can be extensive, the phagocytes can modulate the activity of surrounding cells and/or tissues. As a result of the intensive work in several laboratories during the past few years, we have substantial information on the various secreted materials and, more importantly, on the conditions for increased secretion. The secretory function of mononuclear phagocytes has been reviewed recently in detail.[3] In this article, we summarize the most significant features of this function and highlight the secretion of lymphostimulatory molecules, which has been the subject of our own research interest.[4-7]

Products Secreted by Phagocytes

Table 1 summarizes the various products found in cultures of phagocytes. Among the secreted products are enzymes including lysosomal enzymes and others like lysozyme, plasminogen activator, collagenase, etc.; also secreted are complement proteins, interferons, and various biologically active materials. Among the biologically active products are included the colony-stimulating factors, the lymphostimulatory factors, and various inhibitory or cytolytic molecules. As noted in Table 1, the secreted products can be grouped into three interrelated categories: a) enzymes that affect extracellular proteins, b) products involved in host defense, and c) products affecting surrounding cells. All of these secretory products have been identified in culture fluids from phagocytes obtained either from peripheral blood as monocytes or from the peritoneal cavity, usually of mice or guinea pigs, as macrophages. A brief comment on the various secretory products follows. (See Unanue [3] for a comprehensive reference list on this subject.)

Part of the content of lysosomal enzymes is released into the medium during the interaction of phagocytes with various materials.[1,8-12] Release takes place during phagocytosis of zymosan or polystyrene particles, bacteria or bacterial cell walls, antibody-antibody complexes, etc. The release of lysosomal enzyme has also been studied in fibroblasts[13] and neutrophils (reviewed by Becker and Henson [14]). In the neutrophil the release takes place, as in the macrophage, during phagocytosis or as a result of contact with surfaces coated with antigen-antibody complexes. In the former case, part of the release occurs when the lysosome fuses with an endocytic vesicle that has not yet closed, maintaining a communication via a stoma to the outside. In the latter case, the release is attributed to migration of the lysosomal vesicles to the stimulated surface membranes in a process resembling endocytosis, but in reverse.

In both cases, the release of lysosomal enzymes is selective—a cyto-

Table 1—Products Secreted by Phagocytes

Enzymes affecting extracellular proteins
Lysosomal enzymes
Plasminogen activator
Collagenase
Elastase
Products involved in host defense
Lysozyme
Complement proteins
Interferon
Microbicidal products
Products modulating cells
Colony-stimulating factors
Lymphostimulatory proteins
Low-molecular-weight inhibitors

plasmic enzyme like lactate dehydrogenase is not secreted or released unless the cell is killed. The amount of lysosomal enzymes released upon brief contact with a large phagocytic stimuli is usually about 10 to 25% of the total enzyme contents, that is, only a relatively small portion. This release usually takes place in a question of minutes. There are two important features concerning lysosomal enzyme release from macrophages. First, under certain circumstances, in contrast to the neutrophil, it can be quite extensive. This point became evident through the work of Davies *et al.*, in which macrophages challenged with streptococcal cell wall preparations become "activated," releasing, after a few days, up to 80% of their content of lysosomes.[11] Thus, an activated macrophage phagocytizing large, partially insoluble materials releases, continuously, amounts of enzymes which could affect the structure of extracellular proteins. Davies *et al.* hypothesized that such extensive release could account for the inflammatory reaction seen when the streptococcal cell walls are injected into animals.

A second important feature of lysosomal enzyme release in macrophages is that it can be stimulated by products from activated lymphocytes.[15] Thus, the process is stimulated not only by contact of the macrophage membrane with an insoluble particle but also by interaction with soluble active materials.

Besides lysosomal enzymes, phagocytes secrete a number of other enzymes which include lysozyme,[16-20] plasminogen activators,[21,22] collagenase,[23,24] and elastase.[25]

Lysozyme, a protein of about 14,000 daltons, is found in abundance in body fluids. It acts on the peptidoglycan of certain bacterial cell walls, specifically hydrolyzing the one to four glycosidic linkages between acetylmuramic acid and acetylglucosamine. Plasminogen activators con-

vert plasminogen to plasmin, resulting in fibrinolytic activity. Macrophages secrete two molecular species of 48,000 and 28,000 daltons.[21] The macrophage collagenase is a metalloprotein of the same specificity as other collagenases, acting at neutral pH by splitting tropocollagen into fragments of one-fourth to three-fourths of the original chain length.[24] The elastase from macrophages is a serine protease which degrades insoluble elastin. It has properties different from pancreatic and granulocyte elastase.[25]

The secretion of these four enzymes differs somewhat from that of lysosomal enzymes. Secretion of lysosomal enzyme is usually limited to part of their content; usually the majority of the enzymes remain inside the cell to function in intracellular digestion. This is not the case for the others which are synthesized for export and are not retained by the cell. However, within this group, the characteristics of secretion differ quite markedly, a clear indication of the complexity of the secretory process. Gordon *et al.* found that macrophages secreted, continuously, about 80 to 90% of their content of lysozyme regardless of whether or not the cells were stimulated to phagocytize.[19] Activated macrophages secreted larger amounts of lysozyme.[18] In contrast, the other three enzymes were secreted mostly following phagocytosis. Two other important points need to be highlighted: a) the extent of secretion of plasminogen activator following phagocytosis depended upon the previous "activity" of the macrophage.[22] For example, macrophages previously activated by endotoxin secreted much larger amounts of the enzymes upon subsequent challenge than nonactivated cells. b) The nature of the phagocytic stimuli conditioned the time course of release; while digestible antigen–antibody complexes triggered a burst of secretion, indigestible latex beads stimulated persistent secretion for days and days of culture.[22]

It is well established that phagocytes make some of the proteins of the complement system.[26-30] Phagocytes synthesize and release C2, C4, C3, and most likely C5. The release of C4 and C2 increases following phagocytosis of bacteria but not of latex particles.[29]

Also secreted in macrophage cultures are interferons [31-33] and a pyrogenic material [34-36] as well as a colony-stimulating factor. The colony-stimulating factor is necessary for the formation of hematopoietic colonies from stem cells.[37-41] Exposure of macrophages to endotoxin [40] or to synthetic polynucleotides results in increase in colony-stimulating factor.[42]

The Lymphostimulatory Molecules

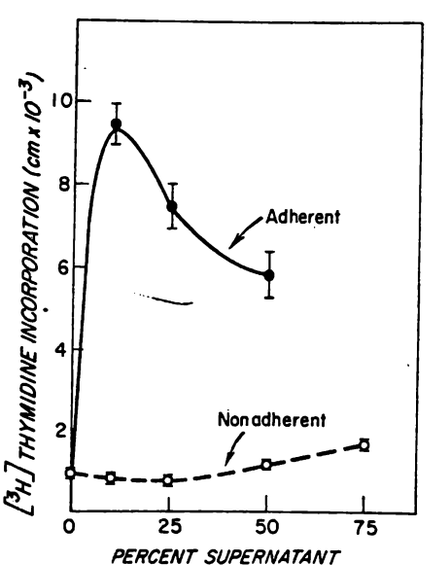
Our purpose here is to summarize the main results of our own research in this field.[4-7] Others have also reported on similar results,[43-49] but these

will be referred to only in passing. Most of the observations reported here have been published or are in press.

We have found that medium in which macrophages have been cultured for 6 to 24 hours can exert marked biologic effects on lymphocytes *in vitro*. The lymphostimulatory medium is first dialyzed to remove low-molecular-weight components which inhibit cell division (described later in this section).[6] The effects of the conditioned medium on the proliferation or differentiation of lymphocytes can then be assayed.

Several biologic activities can be identified in macrophage culture medium. One consists of a mitogenic principle which stimulates thymocytes preferentially; peripheral B and T lymphocytes show rather limited enhancement of ^{3}H-thymidine incorporation (Text-figure 1). Gery *et al.*[43,44] and others have described a lymphocyte-activating factor (LAF) which allows thymocytes to respond to lectins such as phytohemagglutinin (PHA) which normally stimulates only mature T cells. It is plausible that our mitogenic principle is similar to LAF, although we find that the macrophage culture medium not only enhances the response to thymocytes to con A and PHA but is strongly mitogenic by itself.

The effects of macrophage secretions on thymocyte maturation have been studied by one of us (DIB).[7] Immature thymocytes cultured with samples of the conditioned medium for 24 to 48 hours change their content of H-2 antigens from a low level to the high level characteristic of the cortisone-resistant, mature thymocyte. At the same time, the thymocyte responds much more effectively in a mixed lymphocyte reaction (MLR). This mature phenotype is stable, since the MLR and response to



TEXT-FIGURE 1—Medium obtain from 24-hour cultures of dish-adherent (i.e., macrophage-rich) and nonadherent (i.e., lymphocyte-rich) spleen cells was used to culture thymocytes. Thymocytes were cultured for 3 days at various concentrations of the conditioned medium.

lectins have been assayed up to 4 days after the macrophage-conditioned medium has been removed and replaced with fresh culture medium.

The increase in amount of H-2 and sensitivity to the MLR reflects true maturation of the immature thymocyte. That it is not the result of differential cell growth of the mature thymocytes already present in the thymus is demonstrated by assaying the conditioned culture medium on immature thymocytes isolated on an albumin gradient. The immature thymocytes are operationally defined as that fraction showing no significant PHA responsiveness and severely limited ability to respond in the MLR or absorb anti-H-2 antibodies.

Furthermore, inhibition of 99% of stimulated cell division by mitomycin does not appreciably affect the H-2 increase. This rules out the possibility that a few contaminating mature thymocytes could be dividing preferentially in response to the macrophage-conditioned medium. It also clearly shows that cell division is not required in this differentiation assay. It, therefore, appears that maturation follows *activation* of the thymocyte by a macrophage factor. Cell division also ensues but is not required for differentiation. This is supported by our kinetic studies which show that H-2 transition preceeds blastogenesis in these cultures. The molecule responsible for the maturation has not been identified yet.

Another biologic activity found in macrophage cultures is a B-cell maturation principle. B cells from mice primed to hapten-proteins incubated with the macrophage-conditioned medium differentiate to antibody-secreting cells. The differentiation is best seen with hapten-primed cells and is poorly seen in unprimed cells (Table 2). For differentiation to occur, the cells must be incubated in macrophage culture medium but do not require the addition of antigen to the culture.

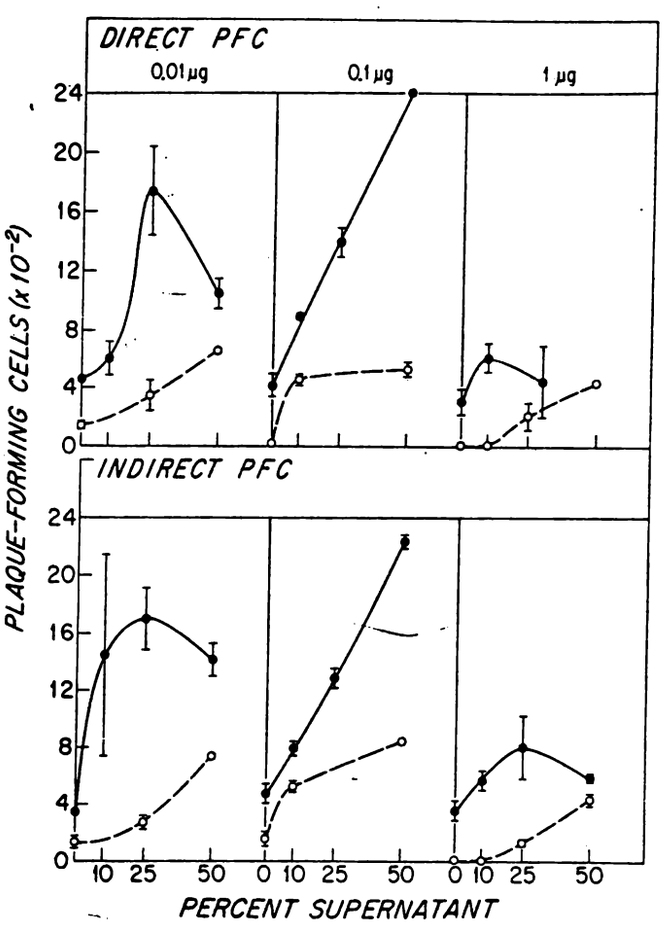
Finally, there is a marked increase in the production of antihapten-

Table 2—Differentiation of B Cells by Macrophage Culture Fluids

Method of priming	Plaque-forming cells		
	No MCF	25% MCF	50% MCF
F-KLH in complete Freund's adjuvant			
IgM	60	867	1,467
IgG	73	1,930	3,560
F-KLH in alum			
IgM	20	653	753
IgG	30	607	1,100

Spleen cells of mice immunized 30 to 45 days before, as described, were placed in culture in Mishell-Dutton type conditions for 4 days, at which time the number of antibody-forming (plaque) cells to F were determined. Cells were cultured in regular medium or in medium containing 25% and 50% macrophage culture fluids. Values are mean number of plaque-forming cells from triplicate cultures secreting IgM or IgG antibodies. Results similar to this were obtained in cultures devoid of T cells. The experiment is interpreted to indicate the capacity of the macrophage culture fluid to differentiate memory B cells.[60]

specific, antibody-secreting cells by culturing lymphocytes from hapten-protein–immunized mice in macrophage-conditioned medium. We interpret this effect as probably reflecting a combination of both principles (Text-figure 2). We have tested this effect by culturing spleen cells from mice immunized with hapten fluorescein (F) conjugated to the strong immunogenic protein hemocyanin (KLH). In such a system, production of antibody-forming cells to F depends upon collaboration of carrier-specific T cells with the hapten-specific B cells. Thus, addition to the culture of F-KLH triggers antibody formation; but this is not the case with F bound to an unrelated carrier protein-like rabbit IgG (RGG). Culture of F-KLH–primed spleen cells in macrophage fluids results in an increase in antibody-forming cells regardless of whether F-RGG is added, a reflection of the B-cell differentiating activity described above. Furthermore, addition of F-KLH together with the macrophage principles now produces a spectacular rise in antibody-secreting cells (Text-figure 2), a



TEXT-FIGURE 2— Spleen cells from mice immunized with F-KLH were cultured for 4 days with different amounts of F-KLH (*solid circles*) or F-RGG (*open circles*) in the presence or absence of macrophage culture fluids. Note that at the optimal antigen dose (0.1 µg) there is an increase in the response to both F-KLH and F-RGG in the presence of macrophage fluids.

summation of the helper T-cell activity plus the action of the macrophage principles.

It is interesting to note that there appears to be a fine balance between the stimulation produced by the macrophage principle and the T-cell helper stimulation. In our hands, the best stimulation by macrophage fluids in such a hapten–protein system was noted in cultures from mice primed for longer periods of time before culture—these cultures by themselves gave weak responses which were readily augmented by addition of the antigen plus the macrophage principle. In contrast, spleen cells from recently primed mice, which, by themselves, gave strong responses *in vitro*, behaved somewhat differently. In these instances, the degree of added stimulation by the macrophage principle in cultures challenged with the antigen F-KLH was slight. Furthermore, the dose was critical in that suppression of response could be seen with relatively low doses of the macrophage factor. We have interpreted this to mean a competition of two active moieties—that from the macrophage factor and that from the T cell—for the B cell.

Biochemical studies on the macrophage culture fluids [4,51] indicated that the mitogenic principle was a protein sensitive to chymotrypsin and papain but resistant to trypsin or to DFP. The principle passed through immunoabsorbant columns of anti-H-2 antibodies. In our first studies, fractionation on Sephadex columns indicates all activities to be in one peak corresponding to a molecular size of about 15,000 to 20,000 daltons. Subsequent studies, however, have disclosed heterogeneity with additional activities in fractions of higher molecular weights. Further studies are in progress.

The mitogenic protein found in macrophage cultures can act on B and T cells from different strains of mice and can even cross species. Factor produced from human monocytes can act on murine lymphocytes.[15] It should be noted that strains of mice differ in the extent of response to the mitogenic principle, some responding consistently at a high level, while others only at a low level. These differences are unrelated to the H-2 haplotype of the strain. C57BL/6 (an $H\text{-}2^b$ mouse) thymocytes respond poorly to macrophage culture fluids from macrophages of the same strain or from others; A/St (an $H\text{-}2^a$) or BALB/c (an $H\text{-}2^d$) respond strongly to the factor from C57BL/6 macrophages or from syngeneic macrophages. The lymphocytes from C57BL/6 mice also responded poorly to other mitogens.

The conditions for secretion of the active principles have been under investigation. The basal production of the active principles varies greatly from preparation to preparation of macrophages, but the production changes upon stimulation. There are two situations that lead to an in-

creased secretion: a) addition to the cultures of various soluble or particulate materials that interact with the macrophages or b) addition of activated T lymphocytes.[5]

We have found that addition of antibody-coated red cells, latex particles, endotoxin, beryllium salts, or bacteria to macrophage cultures produced a five- to tenfold increase in secretion of the active principles. The production of material was rapid, reaching maximum usually at 24 hours and then declining to basal levels by 72 hours, irrespective of the nature of the stimuli. This increase was seen for all biologic activities.

A second manipulation that causes a very marked increase in the production of active principles is the addition of activated T lymphocytes to the macrophage cultures. We came across this finding when studying the activities of peritoneal exudate cells from mice infected with *Listeria monocytogenes*. These exudates produced a marked amount of active materials. In this experiment the mice were infected intraperitoneally with a sublethal dose of *Listeria* organisms, the exudates were harvested 10 days later at a time when the peritoneal cavity was clean of bacteria. Because *Listeria* immunity produces marked activation of T cells, the exudates were depleted of any possible adherent T cells by treatment with anti-θ antibodies in a cytolytic system. The very high production of active material was totally abolished by this treatment. In other experiments the T lymphocytes from the peritoneal cavity were isolated and added to normal macrophages or culture alone. While the T lymphocytes by themselves did not produce any activity, mixing them with macrophages generated very potent principles. In other experiments we have been able to show that the addition to macrophages of purified T lymphocytes from mice immunized to KLH resulted in a marked increase in activity. In these instances, however, addition of the antigen was required. The optimal T cell/macrophage ratio was about 5:1 to 10:1. We do not know which cell makes the active principles in these situations. The data is consistent with the macrophage, but this point must be further explored. In all these experiments, cultures of isolated T lymphocytes with or without antigen did not produce any activity. These experiments clearly indicate the fascinating interrelationships between the T lymphocyte and the macrophages in that only when the two were mixed did synthesis of the factors take place.

We have asked the question whether activated macrophages secrete more of the lymphostimulatory principles than the normal, nonactivated counterparts. The secretion of enzyme discussed before was markedly augmented by activation of the cells—macrophages harvested from mice injected with endotoxin or thioglycollate broth, for example, secreted

much larger amounts of lysosomal enzymes, lysozyme, plasminogen activators, collagenase, or elastase. To our surprise, activated macrophages (by endotoxin or thioglycollate) secreted very *small* amounts of the active principle under basal conditions or following phagocytosis. Thus, for secretion of the lymphostimulatory molecules, the state of macrophage activation is critical in that it appears that, after a certain state of activation, the secretion is stopped, in contrast to what happens with secretion of enzymes.

Table 3 is a summary of the above-mentioned points.

Inhibitors in Macrophage Fluids

Macrophage fluids have been found to contain low-molecular-weight inhibitors of DNA and protein synthesis. We found, in agreement with others,[52-56] an inhibition of tritiated thymidine incorporation when lymphocytes were cultured with undialyzed macrophage-conditioned media. Later, it was found that the growth of a thymic leukemia, the EL-4 line (assayed by cell counts and mitotic indices), was markedly inhibited by the macrophage culture fluids. The inhibitor of EL-4 growth was dialyzable where it could be recovered.[6] Opitz *et al.* described the presence of thymidine in macrophage fluids and interpreted some of the "inhibitory" effects reported in the literature as resulting from competition between the released thymidine and the tritiated thymidine used for assaying the DNA synthesis.[55,56] Biochemical analysis of our culture fluids has confirmed the presence of a rather large amount of a thymidine-like material.[57] Indeed, a culture of 10^7 macrophages secreted 1 μg/ml nucleotide/24 hours of culture. The effects of thymidine extend beyond a simple competition for the radioactive thymidine used in the assay for

Table 3—Summary of Lymphostimulatory Molecules

Activities
Mitogenic principle for thymocytes and, to lesser extent, for B and T lymphocytes
Rapid maturation of immature thymocytes into mature, competent thymocytes
Differentiation of memory B lymphocytes to antibody-secreting cells
Increase helper activity in hapten-protein assays
Properties of mitogenic principle
Active against various mice strains—no H-2 restriction
Not bound by anti-H-2 columns
Sensitive to chymotrypsin and papain
Resistant to DFP
About 15,000 to 20,000 daltons
Secretion
Low basal secretion in culture
Secretion increased by phagocytosis
Secretion increased by addition of activated T cells
Activated macrophages secrete less than stimulated macrophages

DNA synthesis (although this effect is clearly evident). Thymidine at high concentrations (10^{-2} to 10^{-4} M) is known to inhibit the biosynthesis of deoxycytidilic acid, thus restricting DNA synthesis. The EL-4 leukemia cells are, in fact, highly sensitive to thymidine, being inhibited by the amounts secreted by the macrophages, about 4×10^{-6} M thymidine, in a process reversed by 2-deoxycytidine. The source of the nucleotide needs studying. Opitz *et al.* gave evidence supporting a source from nuclear material phagocytosed by the macrophages. Although this is an obvious explanation, the possibility of endogenous synthesis and release has not been ruled out. Thymidine is not the only low-molecular-weight material released by macrophages. Another material, of about 600 daltons, has been found to inhibit cell growth.[54] We have found this material in small amounts affecting growth of EL-4 leukemia.

Conclusion

The monocyte macrophage system of cells comprises a highly mobile line capable of homing to places of nonimmunologic inflammation as well as forming important functional units with the lymphocytes. In this latter situation, the phagocytes appear to play a fundamentally basic role both in induction as well as in the effector side of the immune response. Phagocytes are highly capable of uptake and degradation of a number of unwanted materials; indeed, phagocytosis, the property by which these cells were identified, constitutes their fundamental *raison d'etre*. But, besides intervening in phagocytosis, the phagocytes make and secrete a large number of products, many of which have the potential to change their surrounding environment. It is likely that secretion by macrophages of a number of enzymes capable of modifying extracellular proteins must play a role in the reorganization of connective tissue during inflammation. At the same time, these cells have the potential for causing disease if, for example, a phlogogenic stimulus persists, leading to a continuous and repeated influx of macrophages into tissues with the persistent secretion of enzymes. Speculation along these lines has been made by Davies *et al.*,[11] based on the example of persistent granulomatous inflammation produced by poorly digestible streptococcal cell-wall proteins.

Of great interest is the secretion of mediators of the immunologic process. It is thought that two interrelated functions of phagocytes in immunologic process are to concentrate and remove antigen and to present some molecules for recognition of T and B lymphocytes. Previous studies have analyzed this question at depth. We now find that phagocytes can also secrete molecules which have the capacity to change the response of the lymphocyte. The secretion of lymphostimulatory molecules is under

regulation in the sense that it is controlled by important extracellular factors—a particle that is phagocytosed or an activated T cell. Furthermore, the extracellular factors only appear to operate on certain maturational steps of the phagocyte. The overall evidence suggests that the secretion of lymphostimulatory molecules, and of other molecules in general, is under a rather sophisticated control which could explain the various, diverse roles of phagocytes. Our data is consistent with the hypothesis that uptake of finite amounts of antigen by phagocytes of primary and secondary lymphoid organs leads to a burst of secretion of the lymphostimulatory molecules. In the thymus the reaction could play a critical homeostatic role in regulation of T-cell maturation in accordance with the activity of the phagocytes. In lymph nodes and spleen the secretion of the lymphostimulatory molecules could play a cooperative role during induction. We also speculate that a probable mechanism of action of immunologic adjuvants is the macrophage through the release of the lymphostimulatory factor mediated as described herein.

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IMMUNOLOGY
by The Williams & Wilkins Co.

Vol. 119, No. 5, November 1977
Printed in U.S.A.

■HESIS AND RELEASE OF THYMIDINE BY MACROPHAGES[1]

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ır of DNA synthesis and cell proliferation
·ophage supernatants was subjected to further
following conclusions were drawn: a. Bio-
ıe inhibitor appeared to be thymidine, judging
ration in three different thin-layer systems
iltration. b. The inhibitor was shown to be
novo by macrophage monolayers pulsed with
–l precursor of thymidine such as ^{14}C-formate.
ıt of inhibitor present in tissue culture fluids
sufficient to block the growth of the EL-4
line in a manner that could be prevented with
of 2'-deoxycytidine. The EL-4 line was consid-
ensitive to thymidine blockade than a number
ells tested.

study from our laboratory showed that culture
ell cultures rich in macrophages contained an
NA synthesis (1). This inhibitor of DNA synthe-
d in tissue culture on the thymic leukemia cell
e EL-4 cells were inhibited in their growth and
porate radioactive thymidine into their DNA.
ı of growth was readily reversible after removal
ır, characterized as a small, dialyzable molecule,
eatment with phosphodiesterase and capable of
the tumor cells. Other investigators had, like-
l on low m.w. inhibitors of DNA synthesis from
ultures by using as test systems a variety of
oplastic cells (2, 3). Subsequent to these reports,
ıppeared with biochemical evidence pointing to
the possible inhibitor of DNA synthesis in mac-
re fluids (4, 5). These papers indicated that the
-DNA synthesis was not a true inhibition but a
ır the uptake of the radioactive nucleoside. Fur-
vas suggested that the thymidine may have been
the catabolism of cells ingested by the macro-

This report presents our biochemical analysis of the inhibitory material in macrophage cultures assayed as described by Calderon *et al.* (1), on EL-4 cells. The fact that the multiplication of the EL-4 cells was truly stopped made us question whether the presence of thymidine was the whole explanation for the inhibition. We reasoned that the concentrations of thymidine needed to produce true inhibition (or blockade) of DNA synthesis were too high to be derived from any cell source. Furthermore, in our cultures, there was no apparent phagocytosis of nuclear material by the macrophages. The concentration of thymidine capable of exerting blockade varies from cell to cell within the range of 10^{-2} to 10^{-5} M (6–9). (Thymidine blockage is readily reversed after the removal of the nucleoside, after which cells reassume proliferation in a synchronized fashion [10, 11]. A phosphorylated derivative of this nucleoside appears to prevent the conversion of cytidilate to deoxycytidilate [catalyzed by the appropriate ribonucleotide reductase] [6]. The lack of deoxycytidilate accordingly restricts the ability of the cells to synthesize DNA. As expected, the exogenous addition of deoxycytidine [CdR] allows for normal reassumption of cellular proliferation.)

We now report three matters: 1) the inhibitory molecule is indeed thymidine; 2) some of the thymidine, if not all, is actually synthesized and released by the macrophages; and 3) EL-4 cells are specially susceptible cells to thymidine blockade.

MATERIALS AND METHODS

Mice. Experiments were done with A/St or CD-1 mice of either sex, 8 to 10 weeks old at the initiation of the experiments (obtained from West Seneca Laboratories, Buffalo, N. Y., and Charles River Company, Wilmington, Mass., respectively). Male C57BL/6 mice of the same age (from Jackson Laboratories, Bar Harbor, Maine) were used to passage EL-4 leukemia cells.

Macrophage supernatants. Peritoneal exudate cells were harvested from mice injected 3 days previously with 1.5 ml of proteose peptone solution (Difco Laboratories, Detroit, Mich.) i.p. The mice were sacrificed and the cells collected after injecting i.p. 3 ml of minimal essential medium (Microbiological Associates, Bethesda, Md.) containing 10 units of heparin/ml, 1% *N*-2 hydroxyethylpiperazone-*N*-2-ethane sulfonic acid (HEPES,[5] Microbiological Associates), and 5% heat-inactivated fetal calf serum (FCS, Associated Biomedics, Inc., Buffalo, N. Y.). The cells were washed with the same medium (without heparin) and adjusted to concentrations usually of 5 to 10 × 10^6 cells/ml of medium. The latter was supplemented with 0.5 mM Na pyruvate, 2 mM L-glutamine, 0.75% Na bicarbonate, 50 units penicillin, 50 μg of streptomycin, 1% nones-

publication July 15, 1977.
publication August 10, 1977.
publication of this article were defrayed in part by
f page charges. This article must therefore be hereby
isement in accordance with 18 U.S.C. Section 1734
ıte this fact.
was supported by National Institutes of Health
91, AI 03260, and NCI 14723 and a grant from the
bacco Research, Inc., New York.
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[5] Abbreviations used in this paper: HEPES, N-2 hydroxyethylpiperazone-N-2 ethane sulfonic acid; TLC 1, methanol: concentrated hydrocholoric acid: water at 70: 20: 10 v/v; TLC 2, n-butanol: methanol: water: amonia; TMP, thymidine monophosphate.

) acids, and 10% FCS. The cells were cultured in
—s of 35 mm diameter. Cultures were placed in a
)ator at 37°C for 2 hr, washed vigorously at least
.o remove nonadherent cells: and further incu-
) 48 hr. At this time, culture fluids were removed,
—filtered through an 0.45-μm Millex disposable
illipore Corporation, Bedford, Mass.), and stored
ısed.

:o assays were used with EL-4 leukemia cells as

=1. This short-term assay measured the incorpora-
—ymidine by EL-4 cells in the presence of the
culture fluid, before and after fractionation. The
tured with the macrophage supernatants or frac-
in 0.5- or 1-ml volumes at a concentration of 5 ×
4 cells had been harvested from the peritoneal
=BL/6 mice inoculated 7 to 10 days previously with
cells. The cultures (in 12- x 75-mm plastic tubes
—tics, Oxnard, Calif.) received 0.5 to 1 μCi of ^{3}H-
—0 Ci/mM, New England Nuclear Company, Bos-
—and were incubated at 37°C in a CO_2 incubator for
)roacetic acid-insoluble material from cultured
ected on glass fiber filters in a sampling manifold.
' was measured in a liquid scintillation spectrom-
ults are means of triplicate cultures expressed as
—alue of 1 S.E.M.

2. This assay explored whether the macrophage
ȝ had actually reduced cell growth. 5 × 10^4 EL-4
ɜ incubated in different concentrations of macro-
—ıatants or its fractions in 1- or 0.5-ml volumes for
ells were then extensively washed, resuspended
in the presence of 1 μCi of ^{3}H-thymidine (2 Ci/
ed again for 4 hr at 37°C in a CO_2 incubator. The
' in trichloroacetic acid-precipitable material was
his assay was evaluated by adding high concen-
hymidine to cells and determining the number
—f washing required so that there would be no cold
—resent to cause a dilutional effect. We established
take of radioactive thymidine now reflected the
ells truly synthesizing DNA at that moment in
ıt which correlated well with the estimates of
umbers. On occasions, cell counts were performed
ıer hemocytometer and the viability of the cells
l by their ability to exclude trypan blue.

ling of the macrophage supernatant. In order to
ıether thymidine could be synthesized *de novo* by
s, cultures were pulsed with a radioactive precur-
dine, isotopically labeled formate (12). Therefore,
Na sodium formate (56 mCi/mM, New England
ıs cultured with 5 × 10^6 macrophages for various
me.

:al analysis of the inhibitor. Thin-layer chroma-
lacrophage supernatants or control media (both
:S) were concentrated 10 times by evaporation
ream of pure N_2. Two-microliter aliquots were
20- x 20-cm plastic sheets coated with 0.1 mm
N-300 (Macherey-Nagel Company, Duren, Ger-
cm from the edge, and dried under a stream of
he plates were placed in glass tanks and developed
g chromatography in the following mixture: meth-
trated hydrochloric acid:water at 70:20:10 v/v (TLC
is procedure usually took 2 hr. The plates were
with warm air, and the run was divided from the
ıe top in 16 equal areas (about 1 cm long). The
ım individual areas was scraped off with a spatula,
and solutes were eluted with medium. After centrifugation and filtering through a 0.45-μm Millex disposable filter unit, the biologic activity of each eluted fraction was assessed as per Assay 1. When the macrophage supernatants were radiolabeled, autoradiograms were first made of the plates by exposing them to photographic films (Kodak SB-5 x-ray film) for different lengths of time. To measure radioactive counts, the run was divided as indicated above and the scrapings placed into scintillation vials, suspended in Aquasol (New England Nuclear), and the radioactivity measured in a liquid spectrometer. In many experiments, certain areas from the chromatogram were removed, eluted with water, concentrated, and rechromatographed in a similar fashion, in a mixture of *n*-butanol, methanol, water, and ammonia, 60:20:20:1 v/v (TLC 2) (13), in a procedure that took 4 hr. The plates were dried and placed under vacuum for 24 to 48 hr. Autoradiograms were performed and both the assay for biologic activity and the assay for the measurement of radioactivity were conducted as above.

Sephadex G-25 column chromatography. One-hundred milliliters of macrophage supernatant were lyophilized, after which the active moiety was extracted with 100% ethanol. After evaporation of the ethanol under N_2, 4 ml of distilled water were added together with trace amounts of salts and radioactive markers. Two milliliters thereof were placed on a 90- x 1.5-cm glass column packed with G-25 fine Sephadex beads (Pharmacia, Uppsala, Sweden), and the column was developed with 0.015 M phosphate-buffered saline (PBS) with the aid of a peristaltic pump (set at 12 to 17 ml/hr) on an automatic fraction collector (LKB Ultrorac 7000, LKB Producter, Bromma, Sweden). Samples from these 2-ml fractions were used to establish positions of the inhibitory activity with both Assays 1 and 2. This was done after correction of the osmolarity by adding concentrated medium and FCS.

Macrophage extract preparation. To study the presence of inhibitor in the cell's interior, macrophages were removed with the aid of a rubber policeman, resuspended in a volume of fresh medium equal to that in which they were originally suspended, subjected to three cycles of freezing and thawing, spun down, and examined.

RESULTS

Biochemical analysis of the inhibitor. All macrophage culture fluids inhibited ^{3}H-thymidine incorporation by and growth of EL-4 cells testing by either on Assay 1 or 2, i.e., short- or long-term cultures. The initial biochemical studies on macrophage supernatants indicated that the inhibitor behaved identically to thymidine. Both were readily adsorbed to charcoal from where they could be extracted with ethanol. Ion exchange chromatography revealed that the inhibitor was not negatively charged. The inhibitor migrated together with thymidine on paper chromatography and on Sephadex G-10 gel filtration eluted in the same fraction (these initial data will not be shown because the experiments were subsequently repeated in detailed form as reported below).

We describe now the biochemical analysis of supernatants from monolayers of peptone-induced peritoneal macrophages, aliquots of which were pulsed with radioactive formate. Our intention was to determine, by using the radioactive precursor molecule, whether the inhibitor molecule, now thought to be thymidine, was synthesized by the cells.

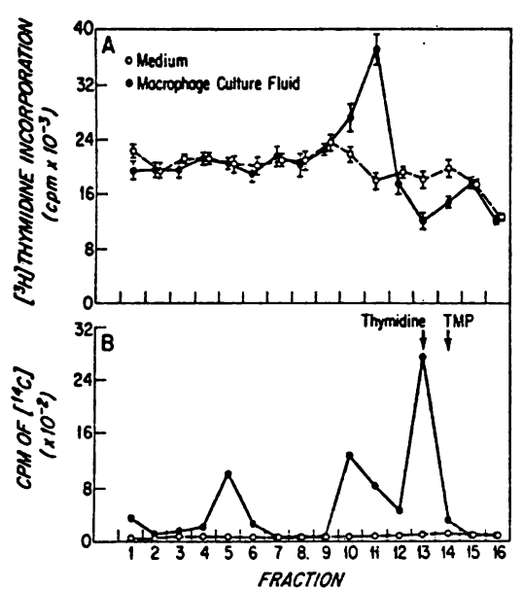
Culture fluids of macrophages exposed for 8 to 24 hr to ^{14}C-formate contained a number of radiolabeled products that could be separated and analyzed by various means. On thin-layer chromatography, by using as solvents TLC 1, three

—adioactive products could be detected. Their Rf values
espectively, 0.31, 0.63, and 0.81, as can be seen in Panel
gure 1. The third and largest fraction ("Peak 3") with
hest Rf value was in the same position as radioactive
—ine. Thymidine monophosphate (TMP) migrated some-
aster (Rf: 0.87). In this assay, the purine nucleosides
in the same position as the radioactive molecule(s) of
. Uridine and cytidine migrate with Rf's of 0.67 and
spectively (13).

nhibitor tested on biologic Assay 1 was detected in the
osition as thymidine and Peak 3 (Panel A). It was of
—t to find a simulator of ^{3}H-thymidine uptake as well, at
on close to the middle radioactive peak. This stimula-
tivity has not been consistently found, being absent
any of the macrophage culture fluids.

—etermine further the nature of the radioactive mole-
omigrating with the inhibitor, the active fraction was
concentrated, and run on a second thin-layer chroma-
ıy system, TLC 2. As can be seen in Figure 2, the
raction eluted from the TLC 1 now separates into four
ts, with Rf values, respectively, of 0.19, 0.50, 0.69, and
he inhibitory activity on Assay 1 as well as thymidine
onded with the fraction having an Rf of 0.69.

—eat of these experiments is shown in Figures 3 and 4.
re concerned whether other cell types also secreted the
or or contributed to its secretion and thus cultured
hages and lymphocytes separately or together in the
e of ^{14}C-formate. Figure 3 is the assay on thin-layer
—tography system of TLC 1 showing the three radioac-



re 1. Thin-layer chromatogram of a 10-fold concentrated atant of macrophages incubated for 18 hr with 10 μCi ^{14}C-e. Two microliters were spotted on the plate and run in the t mixture of methanol: HCl:H_2O (TLC 1). *A*, biologic activity cm fractions from either the macrophage supernatant (*closed* or control medium (*open circles*) eluted from the adsorbent ıedium and assayed on EL-4 cells in the presence of 0.5 μCi midine (assay 1). Each point represents the means of tripli-CA-precipitable counts incorporated by the cells with the ponding standard errors. The inhibitory activity is found in n 12 (peak 3). A stimulatory material is found in fraction 10. iolabeled products obtained from the same fractions measured quid scintillation spectrometer. *Closed circles*, supernatants ıacrophages incubated with ^{14}C-formate; *open circles*, medium ning ^{14}C-formate. Positions of ^{3}H-thymidine and ^{14}C-TMP are ed.

tive fractions. Figure 4 shows the separation of Fraction 3 containing the inhibitor in the system of TLC 2. Only macrophages secreted the radioactive molecule. Lymphocytes did not seem to contribute to this process. Again, the inhibitor was found only in the macrophage fluids in relationship to the fraction with Rf of 0.69 (Fig. 4).

(In a third chromatography system involving tertiary pentyl alcohol:formic acid:water [60:40:1 v/v] (14), the active fraction eluted from the TLC 1 chromatography migrated without further fractionation together with the inhibitory activity and marker thymidine to an Rf value of 0.57 [data not shown].)

Macrophage supernatant, together with the ^{14}C active fraction from TLC 1 and trace amounts of ^{3}H-thymidine were fractionated on a G-25 Sephadex column. The elution profile of the inhibitor as well as the markers is shown on Figure 5. Most of the ^{14}C synthetic products eluted with the salt peak.

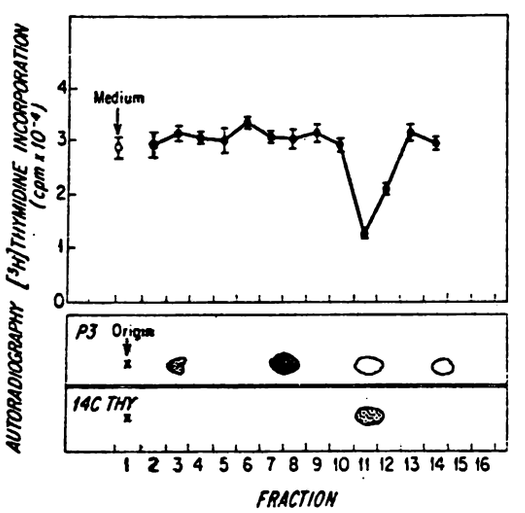


Figure 2. The fractions containing the inhibitory molecule from the chromatograms in Figure 1 were eluted with water, pooled, concentrated, and separated on a second thin-layer chromatography with a solvent mixture consisting of *n*-butanol:methanol:water:ammonia. The upper panel shows the position of the inhibitory activity assayed as in Figure 1. The lower panel shows a diagram of autoradiograms of the test plate and a control run with pure ^{14}C-thymidine.

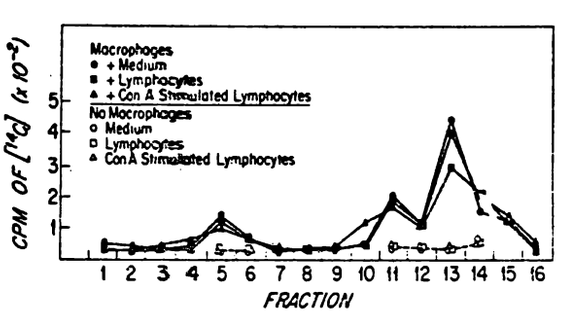
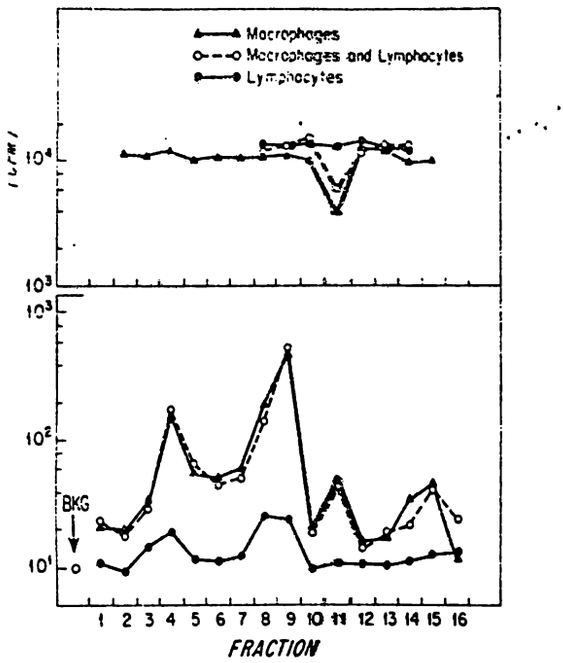
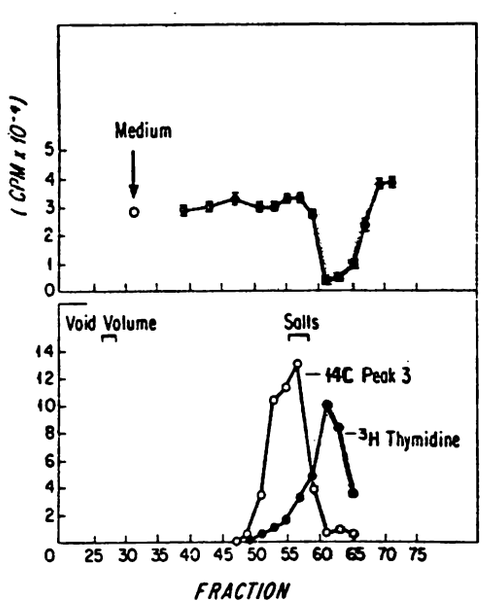


Figure 3. Thin-layer chromatography profile of supernatants from dishes either planted with 5 × 10^6 macrophages/ml or empty, to which were added medium or syngeneic lymphocytes. Lymphocytes were obtained from the spleens of A/St mice. After removing cells adherent to plastic, the lymphocytes were incubated at 5 × 10^6/ml in the presence or absence of 1 μg/ml concanavalin A (Con A) (Sigma, St. Louis, Mo.). After 72 hr of incubation, the cells were washed, counted, and the viability assessed by their ability to exclude trypan blue. Aliquots containing 4 × 10^5 viable cells were added to the dishes, which were pulsed with 10 μCi ^{14}C-formate for 6 hr. Cellfree supernatants were obtained, concentrated 10 times, and 2-μl aliquots were run in a mixture of methanol:HCl:water. Each point represents the amount of radioactivity present in fractions 1 through 16, measured in a liquid scintillation spectrometer.



. This is a similar analysis to that depicted in Figure 2. phages (5×10^6) were incubated with 100% viable lympho- hr in the presence of ^{14}C-formate. The culture fluid was ed 10-fold and run in the thin-layer chromatography . 1 of methanol:HCl:H_2O (of Figs. 1 and 3). The active ere eluted and run in the chromatography system of thanol:water:ammonia (TLC 2). *Upper panel*, inhibitory the various fractions in assay 1; *lower panel*, distribution activity. Fraction 11, representing about 5% of the total ty, contained the inhibitor.



Elution pattern of a 25-fold concentrated macrophage t chromatographed on Sephadex G-25. The supernatant ed as indicated in the *Materials and Methods*. *Upper* gic activity of each of the fractions tested on assay 1; , position of several markers, which include the radioac- n 3 from the chromatogram represented in Figure 1 and ne.

tory activity (the upper panel) and marker thymi- ll as a small but a significant amount of ^{14}C counts ed somewhat thereafter, probably reflecting some interaction between the thymidine molecules and dex beads.

Figure 6 is a repeat of the above experiment but this time assaying for inhibition of EL-4 growth (assay No. 2). There was growth inhibition of the cells by molecules that eluted in the same fraction as the thymidine marker. Also shown are the effects of 2'-deoxycytidine when added to the EL-4 cells in the presence of the inhibitor. The inhibitory effect was reversed by 2'-deoxycytidine, clearly indicating that the blocking agent is thymidine, affecting DNA synthesis by way of thymidine blockade.

Kinetics and rate of secretion of the inhibitor. The rate of synthesis of the inhibitor by macrophages and secretion into the supernatant over a period of 20 hr is shown in Figure 7, where it is compared with the amount of inhibitor extractable from the same cells at equivalent points in time. There is a steady appearance of inhibitor in the supernatant, whereas no inhibitory activity could be recovered from the cell extracts. In other experiments, production of inhibitor was steady for at least 72 hr.

Figure 8 shows the thin-layer chromatography profile of cell

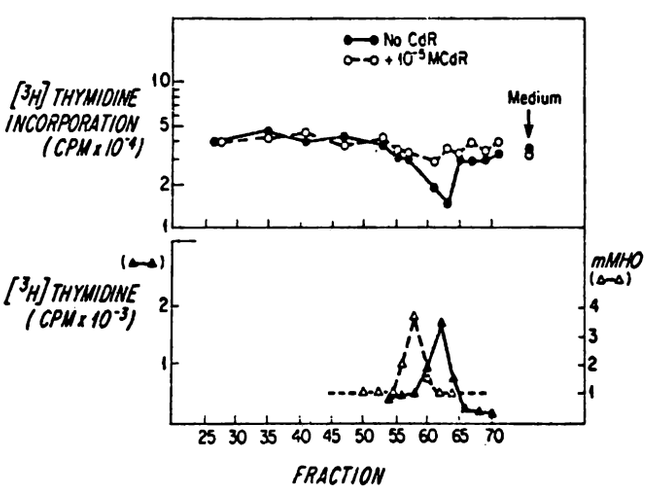


Figure 6. This experiment employed the same protocol as described for the experiment of Figure 5, but the biologic activity was assayed as per assay 2 in the presence or absence of 10^{-5} M 2'-deoxycytidine. The lower panel shows the position of the salts (measured by conductivity) and marker thymidine. CdR is cytidine deoxyriboside, and its position in the chromatogram is shown in the lower panel.

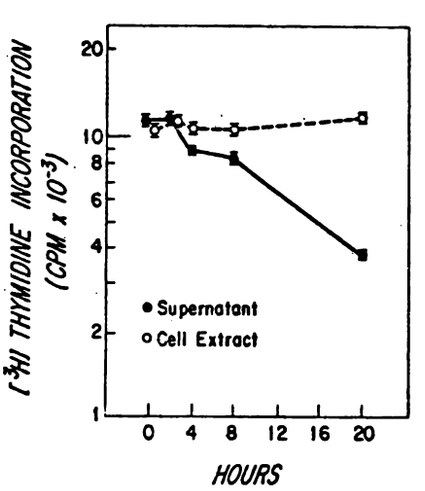
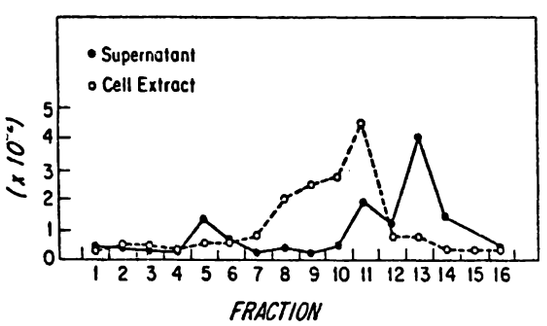


Figure 7. The graph shows the amounts of inhibitor in macrophage culture fluids and cell extracts with time of culture. Supernatants and cell extracts were tested at 50% v/v on EL-4 cells in the presence of ^{3}H-thymidine as per assay 1. Points represent the means of triplicate TCA-precipitable counts incorporated by the cells with the corresponding standard errors.

and culture fluids of macrophages after pulsing with ıate. The characteristic profile of the culture fluid can with the Peak 3 containing the active molecule. The ılular profile was distinctly different, showing no large of radioactivity corresponding to the active fraction.

ɟic activity of thymidine. The previous work clearly d that the inhibitory material was thymidine. The s of inhibitor secreted could be quantified by Assay 1, g as standards the inhibition produced by increasing s of thymidine. Although there is some variation from ıent to experiment, 10^7 macrophages released into 1 ml natant an amount equivalent to 0.4 to 1 μg of thymi- 24 hr. This certainly could explain the inhibition of ^{3}H- ne uptake by simple dilution of the isotope, but it d difficult to explain a drop in EL-4 cell proliferation by concentrations of thymidine as low as 1 μg/ml (4 ×

ıis reason, we examined the sensitivity of EL-4 cells to ıne blockade and compared these cells to normal and ımor cells. Table I summarizes experiments with Assay ıows the amount of thymidine necessary to inhibit the of different cell lines by 50% in a manner that could be ed by the addition of 10^{-5} M 2′-deoxycytidine. The EL-4 ere extremely sensitive to thymidine blockade and ɜ blocked by concentrations of 1×10^{-6} M, an amount ent to that being present in macrophage supernatants.



e 8. Comparison of radiolabeled products in the supernatant :act from 5×10^6 macrophages (in 1 ml), pulsed with 10 μCi ıate. Fluids were concentrated 10 times, and 2 μl were run -layer chromatography with methanol:HCl:H_2O as solvent. int represents the amount of ^{14}C counts present in fractions ɟh 16.

TABLE I

hibition of ^{3}H-thymidine incorporation by cold thymidine in different cell lines[a]

Cell	Strain	I 50
	C57BL	1×10^{-6} M
	SJL	2.5×10^{-4} M
	DBA/2	2.5×10^{-4} M
	DBA/2	2×10^{-4} M
stimulated n cells	A/St	3×10^{-3} M
imulated n cells	A/St	3×10^{-3} M

rious tumor cells or mitogen-activated spleen cells were cul- s in assay 2 with various amounts of thymidine. After 48 to 72 ılture, the cells were extensively washed and then incubated r with 1 μc of ^{3}H-thymidine. The values in the last column ɔ the concentration of thymidine that inhibited by 50% the of the cells. Except for the mitogen-stimulated spleen cells, ıwth of the inhibited cells was reversible by the addition of 2′- ytidine to the medium.

Other tumor cell lines and mitogen-stimulated normal lymphoid cells could be blocked only with amounts that exceeded that active for EL-4 by more than 100-fold.

DISCUSSION

The most important feature of this paper is the demonstration that macrophages produce and secrete thymidine and most likely other nucleosides. Indeed, the inhibitor of ^{3}H-thymidine incorporation and cell proliferation present in macrophage supernatants described in our earlier work has clearly been found to be thymidine in agreement with the results of others. This conclusion was reached after biochemical characterization of the inhibitory molecule by thin-layer chromatography and gel filtration. Furthermore, the inhibition of cell proliferation could be reversed in the presence of 2′-deoxycytidine, a phenomenon that is known to occur in thymidine blockade. Finally, the molecule was synthesized *de novo* by cells pulsed with precursors of thymidine metabolism such as ^{14}C-formate.

Biochemical analysis on three thin-layer chromatography systems failed to separate the inhibitory activity from a radiolabeled product synthesized by the cells, both of which comigrated with the same Rf values as thymidine. The solvent systems employed rule out other naturally occurring nucleosides and bases (13), which migrate to Rf positions distinct from thymidine. Gel filtration on G-25 Sephadex also proved the identity between the inhibitor and the radiolabeled product. The fact that both eluted after the salt peak suggests some sort of nonspecific interaction with the gel and points to a property of thymidine that selects it among other small m.w. substances. Ion-exchange chromatography provided additional evidence that the molecule was not negatively charged, thereby ruling out the possibility of it being a nucleotide, even a cyclic one.

Besides thymidine, a large list of substances are known to inhibit the uptake and transport of nucleosides; and, with the possible exception of prostaglandins, none of them occur in mammalian species (for example, theophylline, papaverine (15), colchicine (16), artificial nucleoside analogues (17), etc.). Several of these molecules, including prostaglandins (15), are thought to exert their action through an elevation of intracellular levels of cyclic AMP (17), which, in itself, affects nucleoside transport. However, the inhibitor isolated in this study does not seem to be a prostaglandin, since these are negatively charged, synthesized from unsaturated (essential) fatty acids, and do not incorporate one-carbon metabolites into their molecule (18).

Our results indicate that macrophages secrete thymidine continuously as a part of an active synthetic process. Chan and co-workers have recently shown that cell lines, lacking pyrimidine salvage enzymes such as thymidine kinase, will excrete nucleosides synthesized *de novo* (19). Preliminary evidence in collaboration with Drs. R. Davidson and E. Kaufman shows that macrophages are deficient in thymidine kinase, which explains why these cells are releasing nucleosides. The lack of nucleoside kinase, leading to the inability to phosphorylate the corresponding substrate, may also deprive the cell of an important mechanism of feedback inhibition of nucleoside synthesis *de novo*. Our findings do not negate the conclusion of Opitz *et al.* (4), who suggested that the thymidine from macrophages may derive from enzymatically degraded nuclear material from phagocytized dead cells. In fact, we have also found that this *can* be a significant source of thymidine in experiments where macrophages were fed opsonized nucleated cells.

ient experiments. there is no evidence of phagocyto-
ear material. About one-fourth of the cells of the
ould have been phagocytized in order to release the
found in the medium. which is clearly not the case.
ess. we cannot rule out a contribution of thymidine
degraded nuclear material inasmuch as our data
stimated the total endogenous thymidine pool.
vivo significance of the synthesis and excretion by
ymidine into the outside milieu is not known and
be quite unique for macrophages. A possible bio-
might be in the cytostasis that macrophages might
rtain cells or bacteria in an environment of high cell
ion. An obvious implication of these studies con-
caution that should be used when interpreting data
ire systems in which macrophages or products
ibit cell proliferation and/or DNA synthesis. This is
mportant since some cells appear to be particularly
o thymidine, e.g., the EL-4 leukemia cell, whose
locked by amounts of thymidine that are in keeping
present in macrophage supernatants. With an I 50
r of 10^{-6} M, EL-4 proved to be more sensitive than
ines studied in this paper and by other investiga-
ugh doses in the range of 10^{-3} M to 10^{-4} M thymi-
been widely employed to block cells, 50% growth
doses down to 10^{-4} to 10^{-5} M have been found in
ne (6-8) and human (9) cell lines. The reason for
ensitivity to thymidine blockade by the EL-4 line is
but seems to be a property of this particular cell
n of thymocytes or of cells of the H-2^b haplotype
hown).
ard to the capacity of entire macrophage superna-
ffect the proliferation of cells, this seems to be
on two critical parameters: 1) a balance between
and stimulatory molecules; inhibitors include not
sides as shown herein but may also include prosta-
id cyclic nucleotides, as well as possible macromo-
terials; stimulatory materials include a mitogenic
) and possible low m.w. materials (Fig. 1); and 2)
of the target cells. Thus, EL-4 are particularly
hibition because of their sensitivity to thymidine;
thymocytes are not affected by the concentrations
ie and readily respond to the mitogenic protein,
i stimulation of DNA synthesis.

dgment. We thank Drs. Harold Amos, Richard
and Elliot Kaufman for their advice throughout
of this work.

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