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ESTUDIOS SOBRE LA ESTRUCTURA ANTIGENICA DE M. tuberculosis: VARIACION EN LA EXPRESION ANTIGENICA, IDENTIFICACION DE ANTIGENOS INMUNODOMINANTES Y DEMOSTRACION DE ANTIGENOS GLICOPROTEICOS

Tesis que para obtener el grado de
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I. INTRODUCCION

La infección por M. tuberculosis afecta a 30 millones de personas en el mundo, con una mortalidad anual de 3 millones de casos y una expectativa de 10 millones de casos nuevos cada año (1,2). Como en otras enfermedades infecciosas, se ha considerado que la estrategia con mejores posibilidades de éxito en la erradicación de la tuberculosis es la prevención mediante la vacunación.

La vacunación con BCG ha dado resultados disímbolos. En algunos países, la protección inducida ha sido alta lo cual contrasta con lo observado en otras regiones. Un ejemplo notable fué en el sur de la India, donde la vacunación no disminuyó la frecuencia de la enfermedad; estos resultados han arrojado dudas sobre la esperanza inicial de que la vacunación con BCG podría ser la solución final al problema de la tuberculosis (3).

Otra estrategia para el control de la enfermedad es la circunscripción de la misma a través de las identificación y tratamiento de individuos infectados. Sin embargo, los métodos clásicos de diagnóstico, como la baciloscopía del esputo o de otras secreciones y el cultivo del bacilo, dejan sin identificar un número importante de enfermos (4).

Así mismo, las pruebas de inmunodiagnóstico como las intradermorreacciones y las serológicas están seriamente limitadas. Las intradermorreacciones son de gran utilidad en países con poca población tuberculosa pero dan un número inadmisiblemente alto de resultados falsos positivos y falsos

negativos en regiones con alta frecuencia de tuberculosis, lo cual es también cierto para las pruebas serológicas (5,6).

Las razones para lo anterior incluyen entre otras, la existencia de reactividad cruzada entre el bacilo de la tuberculosis con otras micobacterias y con microorganismos distantes filogenéticamente (7,8,9). También se presentan resultados falsos positivos atribuibles a la existencia de una memoria inmunológica producto de la exposición previa al bacilo de Koch y por vacunación con BCG; igualmente se dá una fracción de individuos infectados que no desarrollan reacciones de inmunidad celular o humoral detectables.

Ahora bien, las limitaciones de la vacunación con BCG y del inmunodiagnóstico podrían ser superadas con el uso de antigenos individuales. En efecto, dada las crecientes publicaciones al respecto, en la actualidad existe un gran interés por conocer correctamente la composición antigénica del bacilo de Koch. Esta línea de investigación podría además conducir al entendimiento de la patogenia de la tuberculosis, en particular de los mecanismos que ocasionan el daño celular y tisular. Sin embargo, a pesar de los esfuerzos realizados y de la introducción de nuevos métodos como son las tecnologías de hibridoma y DNA recombinante, aún no se cuenta con moléculas apropiadas para los propósitos anteriores.

1. INMUNOLOGIA DE LA TUBERCULOSIS.

La respuesta inmune a M.tuberculosis esta mediada por linfocitos T (10,11), la observación más clara es el desarrollo de hipersensibilidad celular en la mayoría de los individuos que entran en contacto con el bacilo; lo cual se ha demostrado por intradermorreacciones con PPD y por estudios in vitro de linfocitos sensibilizados (12,13). Que la respuesta confiere protección, lo sugiere el hecho de que los enfermos con respuesta de hipersensibilidad retardada disminuida o ausente presentan enfermedad progresiva, mientras que en los enfermos que presentan una respuesta enérgica, la enfermedad tiende a la resolución (14). La importancia de la respuesta celular está ampliamente documentada en modelos experimentales (15,16).

Durante la infección tuberculosa se producen anticuerpos en contra de la micobacteria y sus componentes (17,18), desconociéndose el papel que juegan estas moléculas en la infección. En experimentos de transferencia pasiva se ha observado que los anticuerpos no protegen (19). Además existe información que sugiere que los anticuerpos tienen un efecto negativo; Lenzini y col. observaron que el título de anticuerpos fué inversamente proporcional a la respuesta inmune celular es decir que en presencia de una gran cantidad de anticuerpos la respuesta celular fué menor (18).

Por otra parte, uno de los fenómenos más complejos de la infección por M.tuberculosis es la presencia de inmunosupresión. En etapas avanzadas, existe un predominio de linfocitos T

supresores (11,20). Se ha observado que los linfocitos de pacientes con tuberculosis meningea son incapaces de producir una linfocina despues de ser activados con fitohemaglutinina; este efecto se anula eliminando la población de linfocitos gamma radiosensibles (21). Además de los linfocitos se han encontrado otras células con actividad supresora en ratones inyectados con BCG y otras micobacterias (14,22,23). Se desconocen los factores involucrados en la inducción de los fenómenos anteriores; sin embargo, es posible que sean originadas por productos del bacilo cuya identificación sería de gran importancia (24).

Aunado a lo anterior, los componentes micobacterianos producen otros efectos sobre el sistema inmune. Estos incluyen: formación de granulomas (25), activación policlonal de linfocitos B, activación de macrófagos, fenómenos de autoinmunidad y el efecto adyuvante sobre la respuesta inmune (26,27). Esta gama de efectos producida por el bacilo de Koch pone de manifiesto la enorme complejidad de la relación huésped-parásito en la tuberculosis.

2. PURIFICACION Y CARACTERIZACION DE ANTIGENOS INDIVIDUALES

M. tuberculosis está constituido por una variedad de antígenos de diferente composición bioquímica. Algunos de ellos poseen epítopes especie-específicos, otros poseen epítopes de reactividad cruzada los que comparten con diferentes micobacterias y con microorganismos distantes filogenéticamente (7,8,9).

Una de las pioneras en el aislamiento y caracterización de antígenos micobacterianos fué Florence Seibert, quien sometió el filtrado del medio de cultivo de M. tuberculosis a precipitaciones ácidas y alcohólicas obteniendo tres proteínas que designó A,B,C y dos polisacáridos que denominó I y II (28). Desde entonces, se han utilizado para el fraccionamiento de antígenos micobacterianos numerosos métodos, que incluyen: cromatografía de intercambio iónico, cromatografía de exclusión molecular, gradientes de densidad por ultracentrifugación y punto isoeléctrico. La solubilización con sales y solventes se ha usado a menudo en combinación con los métodos anteriores (29).

Uno de los primeros trabajos que resultó en la obtención de un antígeno puro fué realizado por Kuwabara, quien cristalizó un componente del bacilo tuberculoso con actividad de tuberculina in vivo (30); este efecto estaba restringido a un hexapéptido tríptico; sin embargo, sus observaciones no pudieron ser reproducidas por otros investigadores (31,32,33). La utilización de cromatografía de afinidad con anticuerpos policlonales permitió a Daniel y col. aislar una proteína de 28 a 35 kDa de

filtrados de M. tuberculosis a la que denominaron antígeno 5 (34). Este antígeno despertó gran interés, pues parecía estar restringido a M. tuberculosis y M. bovis (35). Sin embargo, estudios posteriores demostraron reactividad cruzada amplia con otras micobacterias (36). Otros estudios importantes en la purificación y caracterización de antígenos fueron realizados por Nagai y col. quienes por cromatografía de intercambio iónico obtuvieron de filtrados de M. bovis una proteína de 18 kDa denominada MPB70, (37,38). Este antígeno representó el 10 % del total de las proteínas y fué especifico de BCG en pruebas de hipersensibilidad retardada. Posteriormente, estos investigadores aislaron de la misma bacteria una proteína de 23 kDA (MPB64) en la cual se demostró escasa reactividad cruzada con otras micobacterias e intensa con Nocardia asteroides (39). De importancia fueron los estudios de DeBruyn y col. quienes purificaron un antígeno proteico de 64 kDa de filtrados de cultivo deficientes en zinc de M. bovis BCG, utilizando cromatografía hidrofóbica, cromatografía de intercambio iónico con DEAE y cromatografía de exclusión molecular. Al ser probado en animales sensibilizados, este antígeno indujó eficientemente una reacción de hipersensibilidad retardada, así como un nivel alto de anticuerpos en animales inmunizados con la proteína purificada (40).

3. ANTICUERPOS MONOCLONALES EN LA PURIFICACION Y CARACTERIZACION DE ANTIGENOS MICOBACTERIANOS.

Los anticuerpos monoclonales han sido una herramienta útil en el estudio de la estructura antigenica de las micobacterias. En los últimos años se ha obtenido un considerable número de anticuerpos monoclonales que se han utilizado tanto en la purificación de antígenos, como en la definición de la especificidad de los mismos, (41,42). Además han servido como sondas para identificar clonas productoras de antígeno en bibliotecas genómicas de expresión de DNA de micobacterias (43,44).

Dentro de los antígenos aislados por cromatografía de afinidad con anticuerpos monoclonales, se encuentra una proteína de 38 kDa purificada a partir de extractos de M. tuberculosis por tres diferentes laboratorios (45,46,47).

Este antígeno despertó gran interés, ya que en base a estudios serológicos con animales inmunizados en contra de una variedad de micobacterias no tuberculosas, demostró ser específico a M. tuberculosis y M.bovis (45,46,48). A diferencia de lo observado con la respuesta humoral al antígeno de 38 kDa que permite distinguir individuos afectados de sanos (49), la respuesta T proliferativa al antígeno está igualmente presente en enfermos tuberculosos y en controles sanos vacunados con BCG, (50). Asi mismo, esta proteína induce reacciones de hipersensibilidad retardada en cobayos sensibilizados con M. tuberculosis, M. intracellulare y M. kansassi. Los resultados

sugieren que el antígeno contiene epítopes específicos detectados serológicamente y epítopes de reactividad cruzada para inmunidad celular (46,47,49).

Otro antígeno micobacteriano, una proteína de 10 kDa, fué aislada por Minden y col. utilizando anticuerpos monoclonales dirigidos contra epítopes comunes de M. bovis y M. tuberculosis. En el mismo estudio, y basados en la secuencia de aminoácidos del antígeno aislado, se preparó un péptido sintético capaz de inducir la síntesis de anticuerpos en conejos y reacciones de hipersensibilidad retardada en cobayos inmunizados con sonicados de H37 Rv y BCG, pero no en animales no inmunizados o inmunizados con micobacterias no tuberculosas. Lo sustancial de este trabajo radica en la posibilidad de usar péptidos sintéticos en lugar de moléculas nativas para estudiar la respuesta inmune a antígenos micobacterianos (51). Britton y col. aislaron un antígeno de 70 kDa de M. bovis BCG utilizando el monoclonal L7, esta proteína fué inmunoprecipitada con sueros de enfermos con lepra lepromatosa y fué capaz de inducir proliferación celular y secreción de interferón en células mononucleares de sangre periférica de individuos vacunados con BCG. La inyección intradérmica del antígeno purificado en los mismos sujetos produjó reacción de hipersensibilidad retardada (52).

4. DNA RECOMBINANTE EN EL ESTUDIO DE LA ESTRUCTURA ANTIGENICA DE MICOBACTERIAS.

El abordaje más reciente y que más expectativas ha despertado para la obtención y caracterización molecular de antígenos micobacterianos, es sin duda la expresión de DNA recombinante de micobacterias en un vector apropiado. Young y col. clonaron y expresaron el DNA de M.tuberculosis en E. Coli, usando como vector el fago Lambda AGt11. Para la identificación de las clonas recombinantes productoras de antígeno usaron los monoclonales TB78, TB71, TB68 y TB23 dirigidos contra proteínas de 33,41,16 y 18 kDa respectivamente, exceptuando TB71, los demás identificaron las clonas productoras (43). Usando la estrategia anterior Young y col. construyeron una biblioteca genómica en M. Leprae e identificaron clonas con anticuerpos monoclonales dirigidos contra proteínas de 65, 36,28 18 y 12 kDa, (44). En otro estudio, Thole y col. construyeron un banco de genes de M. Bovis BCG en E.Coli y usaron como vector de expresión del DNA al fago lambda EMBL3. Los antígenos expresados se identificaron con suero policlonal de conejo anti-BCG, observándose un alto porcentaje de clonas productoras de un antígeno de 64 kDa (53).

Unas cuantas proteínas recombinantes obtenidas mediante las técnicas de biología molecular han sido analizadas en lo concerniente a su actividad inmunológica, siendo alentador que algunas se comportan de manera similar a los antígenos nativos. Por ejemplo, Thole y col. utilizaron la proteína recombinante de 64 kDa de M. bovis BCG para determinar la presencia de anticuerpos en el suero humano. Se observó: que 80% de los sueros de

pacientes tuberculosos y 60% de los individuos vacunados con BCG tenían anticuerpos contra ella, al igual que el 30 % de los sueros de niños sanos (54).

Otro grupo de investigadores demostró que la proteína de 65 kDa fue capaz de estimular in vitro la proliferación de clonas de linfocitos T provenientes de individuos leprosos y de individuos con reacciones positivas a PPD (55). Así mismo se observó que las proteínas recombinantes de 65 y 14 kDa, indujeron la proliferación de clonas de linfocitos T obtenidas de individuos tuberculosos (56). Además de éstos, otros genes cuya expresión en E. coli ha sido posible, son los que codifican para antígenos de 38, 44, 17-19 kDa de M. tuberculosis (57).

Por otra parte, las técnicas de biología molecular han permitido realizar estudios orientados a definir relaciones entre antígenos similares de diferentes micobacterias. De esta manera, se ha demostrado la existencia de una homología entre los genes de M.tuberculosis, M. bovis y M. leprae que codifican para los antígenos de 65 y 70 kDa (9,58).

Hasta la fecha se han obtenido y caracterizado por medio de las diferentes metodologías varios antígenos; uno de los más importantes es sin duda, la proteína de 65 kDa. Los resultados de los diferentes trabajos realizados sobre la proteína indican que es altamente inmunogénica (8,54,55,56,59)). Además se ha observado que este antígeno junto con el de 70 kDa poseen epítopes especie-específicos y epítopes de reactividad cruzada con las demás micobacterias y otras bacterias no relacionadas y se ha

demostrado que estos antígenos son proteínas de "choque térmico" siendo una de las características más sobresalientes su alto grado de conservación (8,9,59,60,61,62,63,64,65).

Las implicaciones que se derivan de estas observaciones son importantes en lo que concierne a la respuesta inmune al bacilo de la tubeculosis, pues un contacto previo con otros microorganismos que posean estos antígenos podría influir sobre la respuesta inmune a la tuberculosis y en la vacunación con BCG. Además, es posible que un individuo sensibilizado responda en forma exagerada a los determinantes de reactividad cruzada presentes en bacterias no patógenas, lo cual podría dar origen a fenoménos de autoinmunidad. La relación tuberculosis-autoinmunidad está sustentada en muchos trabajos (64). Otro antígeno de interés ha sido la proteína de 38 kDa, que a diferencia de los anterior se encuentra únicamente en M. tuberculosis y M. bovis. Los trabajos realizados por nosotros y otros investigadores demuestran que esta proteína está asociada especificamente con la enfermedad (49,67,68).

En vista de lo considerado en esta revisión se decidió orientar esta tesis, primero al desarrollo de la metodología apropiada para el estudio de los antígenos micobacterianos (trabajo 1) y posteriormente, utilizar la capacidad adquirida para tratar de identificar antígenos que pudieran ser biológicamente relevantes (trabajos 2 y 3).

II. BIBLIOGRAFIA

1. Bloom, B.R., and Kodal, T. 1983. Selective primary health care: estrategies for control of disease in the developing word. V. Leprosy. Rev. Infect. Dis. 5:765- 780.

2. IUAT/WHO Study group. 1982. Tuberculosis control. Tubercle. 53:157-169.

3. Tuberculosis prevention trial, Madras. 1979. Trial of BCG vaccines in South India for tuberculosis prevention. Indian J. Med. Res. 70:349-363.

4. Daniel, T.M., and Debanne, S.M. 1987. The serodiagnosis of tuberculosis and the other mycobacterial diseases by enzyme-linked immunosorbent assay. Am. Rev. Resp. Dis. 135:1137-1151.

5. Bardana Jr. Ej., McClatchy, J.K., Farr, R.S.,and Minden, P. 1973. Universal ocurrence of antibodies to tubercule bacilli in sera from non-tuberculous and tuberculous individuals. Clin. Exp. Immunol. 13:65-77.

6. Nassau, E., Parson, E.R., and Johnson, G.D.1976. The detection of antibodies to M. tuberculosis by enzyme-linked immunosorbent assay (ELISA). Tubercule. 57:67-70.

7. Stanford, J.L 1983. Immunologically important constituients of mycobacteria: antigens P 85-127. In Ratledge and Stanford (ed). the biology of the mycobacteria. Vol Academic press Inc. (London) Ltd London.

8. Young, D.B., Ivanyi, J., Cox, J.H., and Lamb, J.R. 1987. The 65 kDa antigen of mycobacteria-a common bacterial protein?. Immunol. Today 8:215-219.

9. Garsia, R.J., Hellqvist, L., Booth, R.J., Radford, A.J., Britton, W.J., Astbury, I, Trent, R.J., and Basten, A. 1989. Homology of the 70-kilodalton antigen from Mycobacterium leprae and Mycobacterium bovis with the Mycobacterium tuberculosis 71-kilodalton antigen and with the conserved heat schock protein 70 of encaryotes. Infect. and Immun. 57: 204-212.

10. Hahn, H., and Kaufmann, S.H.E. 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. 3: 1221-1250.

11. Collins, F.M. 1982. The immunology of tuberculosis. Am. Rev. Resp. Dis. 125 (suppl). 3:42-49.

12. Lester, C.F., and Atwell R.J. 1958. The tuberculin skin reactive in active pulmonary tuberculosis. Am. Rev. Tuberc. 78:399-402.

13. Trnka, L., and Skvor, J. 1979. Direct migration inhibition test with leukocytes as indicator of mycobacterial antigens in the human body. Tubercle. 60:49-53.

14. Brown, C.A., Brown, I.N., and Sljivic, V.S. 1980 Active supression mask an underlying enhancement of antibody production in vitro by spleen cells from BCG infected mice. Immunology 40: 303-309.

15. Collins , F.M., Congdon, C.C., and Morrison, N.E. 1975. Growth of Mycobacterium bovis (BCG) in T t-lymphocyte depleted mice. Infect. Immun. 11:57-64.

16. Collins, F.M. 1979. Cellular antimicrobial immunity. CRC Crit. Rev. Microbiol. 7:27-91.

17. Minden, P., and Farr, R.S. 1969. Binding between components of the tubercule bacillus and humoral antibodies. J. Exp. Med. 130:931-954.

18. Lenzini, L., Rottoli, P., and Rottoli, L. 1977. The spectrum of human tuberculosis. Clin. Exp. Immunol. 27: 230-237.

19. Reggiardo, Z., and Milddlebrook, G. 1974. Failure of paddive serum transfer of immunity against aerogenic tuberculosis in rabits. Proc. Soc. Exp. Biol. Med. 145:173-175.

20. Chaparas, S.D. 1982. Immunity in tuberculosis. Bull. WHO. 60: 447-462.

21. Jaffe M.I., and Rabson, A.R. 1981. Supression of L.I.F. production but not blastogenesis in patients with tuberculous meningitis. Clin. Exp. Immunopathol. 18:245-253.

22. Wadee, A. H., Sher, G.L., and Rabson , A.R. 1980. Production of supressor factor by human adherent cells treated with mycobacteria. J. Immunol. 125:1380-1386,1980.

23. Kimplel, G.R., and Henney, C.S. 1978. BCG induced supressor cells. J. Immunol. 120:563-569.

24. Mehra, V., Brennan, P.J., Rada, E., Convit, J., and Bloom, B.R. 1984. Lymphocyte suppresion in leprosy induced by unique M. leprae glycolipid. Nature, 308:194-196.

25. Papadimitriu, J.M., and Spector, W.G. 1972. The ultraestructure of high-and low-turnover inflamatory granulomata. J. Path. 106:37-43.

26. Chaparas, S.D. 1982. The immunology of mycobacterial infections. CRC Crit. Rev. Microbiol. 9: 139-197.

27. Goren, M. B. Immunoreactive substances of Mycobacteria. 1982. Am. Rev. Resp. Dis. 125:50-69.

28. Seibert F.B. 1949. The isolation of three different proteins and two polysaccharides from tuberculin by alcohol fractions Their chemical and biological propiertes. Am. Rev. tuberc 59:86-101.

29. Daniel, T.M., and Bernard Janicki. 1978. Mycobacterial antigens: a review of their isolation, chemestry. and immunological properties. Microbiol. Rev. 42:84-113.

30. Kuwabara, S. 1975. Aminoacid sequence of tuberculin-active protein from Mycobacterium tuberculosis. J. biol. Chem. 250:2563-2568.

31. Savrda, J. 1980. Synthesis and biological assays of a peptide from a tuberculin-active protein. Infect. Immun. 30:686-693.

32. Sarvda, J. 1983. Synthesis and biological assays of peptides from tuberculin active protein. 40:1163-1169.

33. Toida, I., Yamamoto, S., Takuma, S., Suzuki, S., and Hirato, M. 1985. Lack of tuberculin activity odf synthetic peptide. Infect. Immun. 50:614-619.

34. Daniel, T.M., and Andersen, P.A. 1978. The isolation by immunoabsorbent affinity chromatography and physicochemical characterization of Mycobacterium tuberculosis antigen 5, Am. Rev. Res. Dis. 117:533-539.

35. Daniel, T.M., Ellner, J.J., Todd, L.S., McCoy, D.W., Payne, V.D.H., Andersen, P.A., and Bhe, F.T. 1979. Immunobiology and species distribution of Mycobacterium tuberculosis antigen 5, Infect. Immun. 24:77-82.

36. Daniel, T.M., Balestrino, E.A., Balestrino, O.C., Davidson, P.T., Debanne, S.M., Kataria, S., Kataria, Y.P., and Scocoza, J.B. 1982. The tuberculin specificity in humans of Mycobacterium tuberculosis antigen 5. Am. Rev. Res. Dis. 126: 600-606.

37. Nagai, S., Matsumoto, M., and Nagusuga, T. 1981. Specific skin reactive protein from culture filtrates of Mycobacterium bovis BCG. Infect. Immun. 31:1152-1160.

38. Harboe, M., and Nagai, S. 1984. MPB70, a unique antigen of Mycobacterium bovis BCG. Am. Rev. Resp. Dis. 129:444-452.

39. Harboe, M., Nagai, S., Patarroyo, M.E., Torres, M.L., Ramirez, C., Cruz, Nancy. 1986. Propiertes of proteins MPB-64, MPB70, and MPB80 of Mycobacterium bovis BCG. Infect. Immun. 52:293-302.

40. DeBruyn, J., Bosmans, R., Turneer, M., Maurice, W., Nyabenda, J., Van Vooren, J.P., Falmagne, P., Wiker, H.G., and Harbor, M. 1987. Purification, partial characterization, and identification of skin-reactive protein antigen of Mycobacterium bovis BCG. Infect. Immun. 55:245-252.

41. Engers, H.D., Abe, M., Bloom, B.R., Britton, W., Buchanan, T.M., Khanolkar, S.K., Closs, O., Gillis, T., Harboe, M., Ivanyi, J., Kolk, A.H.J., and Shepard, C.C. 1985. Results of the World Health Organization-Sponsored workshop onn monoclonal antibodies to Mycobacterium leprae. Infect. Immun. 48:603-605.

42. Engers, H.D., Houba, V., Bennedsen., Buchanan, T., Chaparas, S.D., Kadival, G., Closs O., David, J.R., Van EMbden, J.D.A., Godal, T., Mustafa, S.A., Ivanyi, J., Young D.B., Kaufmann, S.H.E., Khomenko, A.G., Kolk, A.H.J., Kubin, M., Louis, J.A., Minden, P., Shinnick, T.M., Trnka, L., and Young, R.A. 1986. Results of the World Health Organization-Sponsored workshop to characterization antigens recognized by Mycobacterium-specific monoclonal antibodies. Infect. Immun. 51:718-720.

43. Young, R.A., Bloom, B.R., Grosskinsky, C.M., ivanyi, J., Thomas, D., and Davis, R.W. 1985. Dissection of Mycobacterium tuberculosis antigens using recombinant DNA. Proc. Natl. Acad. Sci. USA. 82:2583-2587.

44. Young, R.A., Mehra, V., Sweetser, D., Buchanan, T., Clark-Curtiss, J., Davis, R.W., and Bloom, B. 1985. Genes for the major protein antigens of the leprosy parasite Mycobacterium leprae. Nature. 316:450-452.

45. Kadival, G.V., and Chaparas, S. 1987. Production, characterization, and specie specifity of five monoclonal antibodies to Mycobacterium tuberculosis. J. Clin. Microbiol. 25:76-80.

46. Young, D., Kent, L. Rees, A., Lamb, J., and Ivanyi, J. 1986. Immunological activity of a 38-Kilodalton protein purified from Mycobacterium tuberculosis. Infect. Immun. 54:177-183.

47. Worsaae, A., Ljungqvist, L., Haslov, K., Heron, I. and Bennedsen, J. 1987. Allergenic and blastogenic reativity of three antigens from Mycobacterium tuberculosis in sensitized guinea pigs. Infect. Immun. 55:2922-2927.

48. Andersen, A.B., Yuang, Z., Haslov, K., Vergmann,B., and Bennedsen J. 1986. Interspecies reactivity of five monoclonal antibodies to Mycobacterium tuberculosis as examined by immunoblotting and Enzyme-linked immunosorbent assay. J. Clin. Microbiol. 23:446-451.

49. Ivanyi, J., Krambovitis, E., and Keen, M. 1983. Evaluation of a monoclonal antibody (TB72) based serological test for tuberculosis. Clin. Exp. Immunnol. 54:337-345.

50. Kadival , G.V., Chaparas, S.D., and Hussong, D. 1987. Characterization of serological and cell mediated reactivity of a 38 kDa antigen isolated from Mycobacterium tuberculosis. J. Immunol. 139:2447-2451.

51. Minden, P., Houghten, R.A., Spear, J.R., and Shinnick, T.M. 1986. A chemically synthesized peptide which elicits humoral and cellular immune response to mycobacterial antigens. In - fect. Immun. 53:560-564.

52. Britton, W.J., Hellqvist, L., Basten, A., and Ingkis, A.S. 1986. Immunoreactivity of a 70 kD protein purified from Mycobacterium bovis bacillus Calmette-Guérin by monoclonal antibody affinity chomatography. J. Exp. Med. 164:695-708.

53. Thole, J.E.R., Dauwerse, G., Das, P.K., Groothuis,D.G., Schouls, L.M., and Van Embden, J.D.A.1985. Cloning of Mycobacterium bovis BCG DNA and expresion of antigens in Escherichia coli. Infect. Immun. 50:800-806.

54. Thole, J.E.R., Keulen, W.J., Kolk, A.H.J., Groothuis, D.G., berwald, L.G., Tiesjema, R. H., Van embden, J.D.A. 1987. Characterization, sequence determination and immunogenicity of a 64-kilodalton protein of Mycobacterium bovis BCG expressed in Eschericha coli K-12. Infect. Immmu. 55:1466-1475.

55. Emmrich, F., Thole, J., Van Embden, J., and Kaufmann, S.H.E. 1986. A recombinant 64 kilodalton protein of Mycobacterium bovis bacillus Calmette-Guerin specifically stimulates human T4 clones reactive to mycobacterial antigens. J. Exp. Med. 163: 1024-1029.

56. Oftung, F., Mustafa, A, S., Husson, R., Young, R.A., and Godal, T. 1987. Human T cell clones recognize two abundant Mycobacterium tuberculosis protein antigens expressed in Escherichia coli. J. Immmunol. 138:927-931.

57. Andersen, A.B., Worsaae, A., and Chaparas, 1988. S.D. Isolation and characterization of recombinant lambda gt11 bacteriophages expressing eight different mycobacterial antigens of potencial immunological relevance. Infect. Immun. 56: 1344-1351.

58. Shinnick, T.M., Sweetser, D., Thole,J., Van Embden, J. and Young, R.A. 1987 The etiologic agent of leprosy and tuberculosis share and immunoreactive protein antigen with the vaccine strain Mycobacterium bovis BCG. Infect. Immun. 55: 1932-1935.

59. Thole, J.R., Van Schooten, W.C.A., Keulen, W.J., Hermans, P.W.M., Janson, A. A.M., DeVires R.R.P., Kolk, A.H.J., and Van Embden. 1988. Use of recombinant antigens expressed in Echerichia coli K-12 to map B-cell an T-cell epitopes on the immunodominant 65-kilodalton protein of Mycobacterium bovis BCG. J. Infect. Immun. 56:1633-1640.

60. Buchanan, T., Momaguchi,H., Anderson, D.C., Young, R.A., Gillis, T.P., Britton, W. J., Ivanyi, J., Kolk, A.H.J., Closs, O., Blomm, B.R., and Mehra, V. 1987. Characterization of antibody-reactive epitopes on the 65-kilodalton protein of Mycobacterium leprae. Infect. Immun. 55:1000-1003.

61. Oftung, F., Mustafa, A.S., Shinnick, T.M., Houghten, A.R., Kvalheim, G., Degre, M., Lundin, K.E.A., and Godal, T. 1988. Epitopes of Mycobacterium tuberculosi 65-kilodalton protein antigen as recognized by human T cells. J. Immunol. 141:-2749-2754.

62. Anderson, D.C., Barry, M.E., and Buchanan, T. 1988. Exact definition of species-specific and cross-reactivite epitopes of the 65-kilodalton protein of Mycobacterium leprae using synthetic peptides. J. Immunol. 414:607-613.

63. Shinnick T.M. 1987. The 65-kilodalton antigen of Mycobacterium tuberculosis. J. Bacteriol. 169:1080-1088.

64. Young. D., Lathigra, R., Hendrix, R., Sweetser, D., Young, R. 1988. Stress proteins are inmune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. 85:4267-4270.

65. Mehlert, A., and Young,D.B. 1989. Biochemical and antigenic characterization of the Mycobacterium tuberculosis 71 Kd antigen a member of the 70 Kd heat-shock protein family. Mol. Microb. 3:125-130.

66. Shoenfeld, Y. and Isenberg, D.A. 1988. Mycobacteria and autoimmunity. Immunol. Today 9:178-182.

67. Espitia, C., Cervera, I., Gonzáles, R. and Mancilla, R. 1989. A 38 Kilodalton M. tuberculosis antigen associated with infection, its isolation and serologic evaluation. (En prensa).

68. Ranadive, S.N., Bhattacharya, S., Kale, M.K. and Bhattacharya, A. 1986. Humoral immune response in tuberculosis: initial characterization by immunoprecipitation of Iodine labelled antigens and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Clin. exp. Immunol. 64:277-284.

69. Hunter, S.W., Gaylord, H., and Brennan, P.J. 1986. Structure and antigenicity of the phosphorylates lipopolysaccharide antigens from the leprosy and tubercle bacilli. J. Biol. Chem. 261:12345-12351.

70. Mescher, M.F. & Strominger, J.L. 1976. Purification and characterization of a prokaryotic glycoprotein from the cell envelope of Halobacterium salinarium. J. Biol. Chem. 251:2005-2014.

71. Maeba, P.Y. 1986. Isolation of surface glycoprotein from Myxococcus xanthus. J. Bacteriol. 166:644-650.

72. Morris, E.J., Ganeshkumar, N., Song, M. & McBride, B.C. 1987. Identification and preliminary characterization of a Streptococcus sanguis fibrillary glycoprotein. J. Bacteriol. 169:164-171.

73. Pfannensteil, M.A., Muthukumar, G., Couche, G.A. & Nickerson, K.W. 1987. Amino sugars in the glycoprotein from Bacillus thuringiensis subsp. israelensis. J. Bacteriol. 169:796-801.

74. Kornfeld, K. and Kornfeld, S. 1976. Comparative aspects of glycoprotein structure. Ann. Rev. Biochem. 45:217-237.

75. Hamburger, J., Lustignam, S., Arap Siongok, T.K., Ouma, J.H. & Mahmound, A.A.F. 1982 Characterization of a purified glycoprotein from Schistosoma mansoni eggs: specificity stability and the involvement of carbohydrate and peptide moieties in its serologic activity. J. Immunol. 128, 1864-1869.

76. Sthal, P.D. Podman, J.S., Muller, M.J., and Schlesinger, P.H. 1988. Evidence for receptor-mediated binding of glycoproteins and glycoconjugates and lysosomal glycosidases by alveolar macrophages. Proc. Natl. Acad. Sci. USA. 75:1399-1403.

77. Warr, G.A. 1978. A. macrophage receptor for (mannose glucosamine). Glycoproteins of potencial importance in phagocitic activity. Biochem. Biophys. Res. 93:737-745.

78. Sung, S.J., Nelson, R.S. and Silverstein, C.S. 1983. Yest mannans inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. J. Cell. Biol. 93:160-166.

79. Willeman, T.E., Lemnarz, M.R. and Sthal, P.D. 1986. Identification of the macrophages mannose receptor as a 175 kda membrane protein. Proc. Natl. Acad. Sci. USA. 83:2501-2505.

80. Ofek, T., and Sharon, N. 1988. Lectinophagositosis: a molecular mechanisms of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. Infect. Immunol. 56:539-547.

III. RESULTADOS

THE ANTIGENIC STRUCTURE OF *Mycobacterium tuberculosis* EXAMINED BY IMMUNOBLOT AND ELISA. INFLUENCE OF THE AGE OF THE CULTURE AND OF THE OBTENTION METHOD ON THE COMPOSITION OF THE ANTIGENIC EXTRACTS.

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SUMMARY

In this study we have examined by immunoblot (IB) and enzyme-linked immunosorbent assay (ELISA) the humoral immune response in pulmonary tuberculosis. As a previous step, in an attempt to obtain the optimal antigen preparation for these studies, the influence of the culture age and of the obtention method on the composition of the extracts was analyzed. The highest number of antigenic bands was found in culture filtrates of 6 and 8 weeks; at these times two thick bands of 65 and 63 kilodaltons (kDa) were identified. These bands were absent from younger and older cultures. When analyzing the source of antigens, we found that culture filtrates contained more antigenic bands than sonic extracts. In view of these findings, cultures filtrates of 6 weeks of age were used as test antigens. With 19 tuberculous sera a total of 16 antigenic bands were observed by IB. The response was very heterogenous with respect to the intensity of the detected reactions and the number of reacting bands. The most frequently recognized bands were those of 31, 32, 38, 58 and 94 kDa. By ELISA with 49 tuberculous sera and with 48 control sera, a specificity of 0.98 and a sensitivity of 0.70 were obtained.

INTRODUCTION

Despite chemotherapy and BCG vaccination, tuberculosis is still an important health problem in Mexico and in other countries with similar social and economic structures (1,2). On the other hand, the emergence of conditions such as the acquired immunodefficiency syndrome which increase the risk of acquiring mycobacterial infections (3), has triggered the interest of better understanding this disease and its etiologic agent, the Koch bacillus.

At present, a goal in tuberculosis research is the dissection of the antigenic structure of the tubercle bacillus. We believe that this knowledge would increase understanding of the complex interactions between the bacillus and their hosts and to develop reliable methods of diagnosis and immunoprophylaxis. The latter seems to an important issue in view of the failure of BCG vaccination in countries with high rates of tuberculosis infection (4).

In this study we examined the antibody response to Mycobacterium tuberculosis protein antigens using IB and ELISA. As a previous step, searching for the optimal conditions for

these studies, the influence of the culture age and of the obtention method on the composition of test antigens was analyzed.

MATERIALS AND METHODS

BACTERIA AND ANTIGENS

Mycobacterium tuberculosis strain H37/Rv was obtained from the Departamento de Inmunología, Escuela de Ciencias Biológicas, Instituto Politecnico Nacional, Mexico City. Bacilli were grown in the synthetic medium of Proskawer-Beck-Youmans (5). At various times after culture, antigens were obtained by filtration of the culture medium, first through a Whatman # 3 filter and then through filters of 1.2, 0.45 and 0.22μ (Millipore Corp., Bedford, Mass), consecutively. Antigens were also obtained by physical disruption of whole bacteria. For this, the bacterial mass obtained by the above filtration procedure was washed with PBS containing Phenylmethyl-sulfonyl fluoride (0.006%), phydroxy-mercuribenzoate (0.04 %) and EDTA 2.5 mM and sonicated at 60 hertz for six 5 min periods in ice. This material was centrifuged at 20,000 rpm, and the supernatant containing the antigens released by the sonication procedure was passed through

a 0.22 μ filter. The sonic extract and culture filtrate proteins were precipitated with ammonium sulphate (0.5 g/ml) at 4 °C; this was followed by centrifugation at 10.000 rpm for 30 min and dialysis in PBS. The protein content was estimated with the Folin phenol reagent with bovine serum albumin (BSA) as a standard.

SERA.

Three 2 months old white New Zealand rabbits were injected, the first two weeks, with 6.5 mg of heat-killed H37/Rv bacilli and 3 mg of culture filtrate proteins suspended in 1 ml of Al(OH) at various subcutaneous sites of the neck and chest. Thereafter, weekly subcutaneous intramuscular injections of bacilli and proteins in PBS were given. The presence of serum antibodies was monitored by immunoelectrophoresis. When a maximal number of bands was observed, rabbits were exsanguinated and the serum was fractionated and stored at -70 °C until use. Sera from patients with pulmonary tuberculosis diagnosed by smear or culture were obtained from the Instituto Nacional de Enfermedades Respiratorias, Mexico City. Normal control sera were also obtained.

POLYACRYLAMIDE SODIUM DODECYL SULPHATE GEL ELECTROPHORESIS (PAGE-SDS) AND IMMUNOBLOT (IB).

Antigens were separated by PAGE-SDS under reduction with 2-mercaptoethanol following the method of Laemmli (6). Gel were stained with Coomasie blue. Some gels were subjected to densitometric analysis. For IB studies, gels were mounted in an electrontransference chamber (Idea Scientific, Corvallis, PA, USA) and transferred to nitrocellulose sheets (Bio-Rad, Richmond, CA) according to the method of Towbin et al (7). The efficiency of transfer of protein bands was monitored by staining a strip with Amido black. Thereafter, the nitrocellulose sheets with the transferred proteins were treated overnight with 3 % BSA in PBS-Tween 20, 0.3 %. After rinsing with PBS-Tween 20, strips were incubated with rabbit or human serum diluted 1/100 and 1/10 respectively. Incubation was carried out at room temperature for 3 h and after rinsing 5 times with PBS-Tween 20, the adsorbed antibodies were revealed by incubation with Protein A labelled with peroxidase (Sigma Chemical Co., St. Louis Mo,) diluted 1/1000 for 90 min. After rinsing, peroxidase was revealed with H_2O_2 and 4-chloro-1-naphtol for 15 min. Finally, strips were rinsed in tap water, dried and photographed.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Sera were processed in duplicate in a single session. Immulon II microtiter plates (Dynatech Industries, Inc., Mclean, VA,) were used. The conditions to better discriminate patients from controls were established by previous assays. Wells were sensitized with 0.5 mg of antigens in 100 µl of carbonate buffer, pH 9.6 at 37 °C for 5 h. This was followed by washings with PBS-Tween 20, 0.05 %. In order to block possible remaining active sites on the plastic, a 1 % BSA solution was applied to the wells for 30 min at 37 °C. Test sera diluted 1/1000 were applied to the wells for 1 h followed by rinsings and incubation with Protein A-peroxidase. After rinsing, peroxidase was revealed with orthophenylendiamine and 0.03% H_2O_2 in citrate 0.1M, pH 4.5 for 4 min at 37 °C. This reaction was stopped with 50 µl of sulfuric acid 1 N; optical density readings were made at 492 nm in a Behering automatic ELISA processor.

RESULTS

INFLUENCE OF THE AGE ON THE ELECTROPHORETIC PROTEIN PROFILE OF *M. tuberculosis* CULTURE FILTRATES (Fig. 1).

Culture filtrates of 2,4,6,8, and 10 weeks, all from the same original culture, were analyzed by PAGE-SDS under reducing conditions. At two weeks of culture, 18 Coomasie blue stained bands were observed with molecular weights varying from 100 to 29 kDa, (Lane A). The most abundant bands were those of 69,32 and 31 kDa. In 4- week-old cultures a similar protein profile was observed (Lane B). At 6 weeks of age, the number of protein bands was increased to 23 (Lane C). Among the new bands there were two thick ones of 65 and 63 kDa. An electrophoretic profile identical to that of 6 weeks was observed at 8 weeks of culture (Lane D). The protein profile of 10- week-old cultures was similar to that observed at 2 and 4 weeks with disappearance of the 65 and 63 kDa bands (Lane E).

COMPARATIVE ANALYSIS OF CULTURE FILTRATES AND SONIC EXTRACTS BY PAGE-SDS AND IB.

Culture filtrates and sonic extracts obtained both from the same 6 week old culture were analyzed. By PAGE-SDS it was

observed that both preparations shared the majority of protein band; however, in filtrates there were four Coomasie blue stained bands of high molecular weight (Fig. 2, B) which were absent from the sonic extracts (Fig. 2, A). The densitometric analysis of the gels helped to establish quantitative differences (Fig.2). The bands of 32 and 31 kDa were more abundant in the filtrates (asterisks) and the ones of 26 and 15 kDa in the sonic extracts (arrows). The most striking difference was in the 15 kDa band which represented 20 % of the densitograma area in the sonic extract and only 1.5 % in the filtrate. By IB using sera from rabbits hyperimmunized with M. tuberculosis, 18 antigenic bands were identified in sonic extracts and 25 in culture filtrates (Fig.3).

THE HUMORAL IMMUNE RESPONSE IN PULMONARY TUBERCULOSIS ANALYZED BY IB AND ELISA.

Since culture filtrates of 6 weeks of age contained the higher number of antigenic bands, they were used for these studies. With 19 tuberculous sera analyzed by IB, 16 antigenic bands were identified; the molecular weights of these bands ranged from 15 to 98 kDa (Fig.4). Table # I shows, by molecular

weight, the bands reactive with each serum with a semiquantitative estimation of the intensity of the reaction. The number of recognized bands varied from 3 to 15. There were 9 sera which reacted with ten or more bands The most frequently recognized bands were those of 31, 32, 38, 58, and 94 kDa which reacted with 15, 16, 13, 13, and 13 of the 19 sera examined, respectively.

ELISA studies were carried out with 49 sera of patients with pulmonary tuberculosis (Fig. 5). Twenty-five cases could be classified as active tuberculosis and 15 as inactive disease. Information concerning the extent of the disease, treatment and tuberculin reactivity were not obtained. Control sera were obtained from 48 healthy individuals. In patients, mean optical density was 0.640 and in controls 0.160. In order to estimate the sensitivity (fraction of patients above threshold) and the specificity (fraction of control sera below threshold), two standard deviations above the mean optical density of the control sera was chosen as threshold value. The sensitivity of the ELISA was 0.70 and the specificity 0.98. In patients with active tuberculosis, sensitivity was 0.70 and in inactive disease, 0.65.

DISCUSSION

The diagnosis of pulmonary tuberculosis is based on the demonstration of the tubercle bacillus on sputum smears or by culture. These methods fail in an estimated 25% of cases (8). In this group are children, who are usually poor sputum producers, and patients with minimal disease. These cases, plus the existence of tuberculosis outside the lungs, makes it necessary to have a reliable serodiagnostic test which would also be useful in carrying out epidemiologic studies. However, and despite the many efforts realized, there is not at present a widely accepted serologic test. Lack of reproducibility and inability to distinguish patients from healthy individuals seem to be the main limiting factors (9,10,11). There are, however, reports of tests with high specificity and sensitivity. These discrepancies could be related to the heterogeneity of the analyzed populations and to the methods and antigenic preparations used.

In this study we examined the humoral immune response in pulmonary tuberculosis. As a previous step we analyzed the influence of the age of the culture and of the obtention method on the antigen composition of the extracts. Both factors appear

to be important. A higher number of protein bands was observed in 6 and 8 week-old cultures. At these times two prominent antigens of 65 and 63 kDa were observed; these antigens were absent from younger and older cultures. These time-related differences in antigen expression have to be considered when immunologic studies are to be conducted. Moreover, one of the changing antigens, the 65 kDa band, has been shown to be a potent immunogen both in humans and in laboratory animals (15). That cultures of different ages vary in antigen composition has been noted before (16,17,18,19) but the antigens involved were not identified. The factors involved in the above phenomenon are poorly understood. It has been suggested that antigens released in early cultures are secretion antigens,while at later times cultures are enriched by degradation products (19). The possibility that the 65 and 63 kDa changing bands are degradation products is unlikely since they were not seen at 10 weeks when cell lysis would be expectedly higher than at 6 and 8 weeks. Our findings , as well as other observations (20), suggest the participation of factors which modulate the <u>in vitro</u> synthesis of antigens by <u>M. tuberculosis</u>.

At present, the usual sources of soluble mycobacterial antigens are culture filtrates and cell extracts obtained by physical disruption of whole bacteria by sonication or by the application of very high pressures (11). No significant differences have been noted between preparations obtained from either source using immunoelectrophoresis. However, by using the powerful methods of PAGE-SDS and immunoblotting, the number of antigenic bands was shown to be higher in culture filtrates than in sonic extracts. These methods also allowed the demonstration of significant quantitative differences in the expression of some antigens. Striking in this regard was a 15 kDa antigen, which represented by densitometry 20 % of the area in sonic extracts and only 1.5 % in culture filtrates. It is possible that the 15 kDa, which is released by the sonication procedure, is a component firmly anchored to the cell wall.

Our immunoblot studies aimed to analyze qualitatively the antimycobacterial antibody response in tuberculosis reveals a striking heterogeneity in band recognition from patient to patient. While there were sera reacting with a few bands, others reacted with a majority of blotted proteins. This analysis also

allowed the identification of immunodominant antigens which reacted with a majority of tested sera. Among these are the antigens of 32, 31, 38, 58 and 94 kDa. These antigens could be considered for isolation and evaluation as reagents for serodiagnosis and vaccination. The value of immunoblotting in the identification of immunodominant antigens in other pathogens is well documented (23,24).

It has been pointed out that the main limitation for the development of a reliable serodiagnosis test in tuberculosis is the existence of an antimycobacterial response in a significant fraction of noninfected individuals (9,10,11). This phenomenon has been adscribed to cross reactivity between M. tuberculosis and a variety of microorganisms (15,25). The very high sensitivity but relative low specificity of our ELISA disagree with the above view, suggesting rather that the main limiting factor is the absence of demonstrable circulating antibodies in a significant proportion of patients. Similar results have been reported en other works (27,27,28) including two performed in our country (29,30).

In summary, we have shown that the antigenic composition of

culture filtrates varies according to the age of the culture and that there are differences between culture filtrates and sonic extracts. These differences should be taken into account when the antigenic structure of M. tuberculosis is analyzed and serodiagnosis tests, as the ones reported in this study performed.

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REFERENCES

1.- Pacheco, C., Olvera, R. and Herrera, M., Sal. Pub. Mex., 23:25, 1980

2.- Bloom, B.R. and Kodal, T., Leprosy. Rev. Infect. Dis., 5:765, 1983

3.- Collins, F.M., Mycbacterium tuberculosis. Interactions with the immune system, edited by M. Bendinelli and M. Friedman p. 389, Plenum Press, New York, 1988

4.- Tuberculosis Prevention Trial: Trial of BCG vaccines in south India for tuberculosis prevention. Indian J. Med. Res., 70:349, 1979

5.- Youmans, G.P., J. Bact., 51:703, 1946

6.- Laemmli, U.K., Nature, 227:680, 1970

7.- Towbin, H., Staehelin, T. and Gordon, Proc. Natl. Acad. Sci. USA., 76:4350, 1979

8.- Kim, T., Blackman, R.S., Heatawole, K.M., and Rochester, D.F., Am. Rev. Resp. Dis., 129:264, 1984

9.- Parlett, R.C., Youmans, P., Rehr, C. and Lester, W., Am. Rev. Tuberc., 77: 462, 1958

10.- Nassau, E., Parsons, E.R. and Johnson, G.D., Tubercle, 57:67, 1976

11.- Affronti, L.F., Mycobacterium tuberculosis. Interactions with the immune system, edited by M. Bendinelli and H. Friedman p. 1, Plenum Press, New York, 1988

12.- Krambovitis, E., J. Med. Microbiol., 21:257, 1986

13.- Daniel, T.M. and Debanne, S.M., Amer. Rev. Respir. Dis., 135:1137, 1987

14.- Reggiardo, I., Vasquez, E. and Schnoper, L., J. Immunol. Methods., 34:55, 1980

15.- Young, D.B., Ivanyi, J., Cox, J.H. and Lamb, J.R., Immunol. Today, 8:215, 1987

16.- Castelnuovo, G.M., Duncan, M.E. and Bellezza, G., Tubercle, 45:246, 1964

17.- Turcotte, R. and Des Ormeaux, Y., Can. J. Microbiol., 18:637, 1972

18.- Wayne, L.G. and Srameck, H.A., Infect. Immun., 24:363, 1979

19.- Abou-Zeid, C., Smith, I., Grange, J.M., Ratliff, T.L., Seele, J. and Rook, G.A.W., J. Gen. Microbiol., 134:531, 1988

20.- De Bruyn, J., Bosmans, R., Turner, M., Weckx, M., Nyabenda, J., Van Vooren, J.P., Falmagne, P., Wiker, H.G. and Harboe, M., Infect. Immun., 55:245, 1987

21.- Daniel, T.M. and Janicki, B.W., Microbiol. Rev., 42:84, 1978

22.- Daniel, T.M., Good, R.C. and Janicki, B.W., Am. Rev. Respir. Dis., 112:639, 1978

23.- Erlich, H.A., Rodgers, G., Vaillancourt, P., Araujo, F.G. and Remington, J.S., Infect. Immun., 41:683, 1983

24.- Klatser, P.R., van Rens, M.M. and Eggelte, T.A., Clin. Exp. Immunol., 55:537, 1984

25.- Minden, P., McClatchy, J.K. and Farr, R.S., Infect. Immun., 6:574, 1972

26.- Benjamin, R.G., Debanne, S.M., Ma, Y. and Daniel, T.M., J. Med. Microbiol., 18: 309, 1984

27.- Daniel, T.M., Debanne, S.M. and van der Kuyp, F., Chest, 88:388, 1985

28.- Jagganath, C., Sengupta, D.N. and Bahadur, P., Tubercle, 64:201, 1983

29.- García-Ortigoza, E. and Gutierrez-Velázquez, A., Rev. Latinoam. Microbiol., 24:193, 1982

30.- Rojas-Espinoza, O., Quesada-Pascual, F., Anaya, N. and Estrada-Parra, S., Rev. Invest. Clin. (Mex), 30:121, 1978

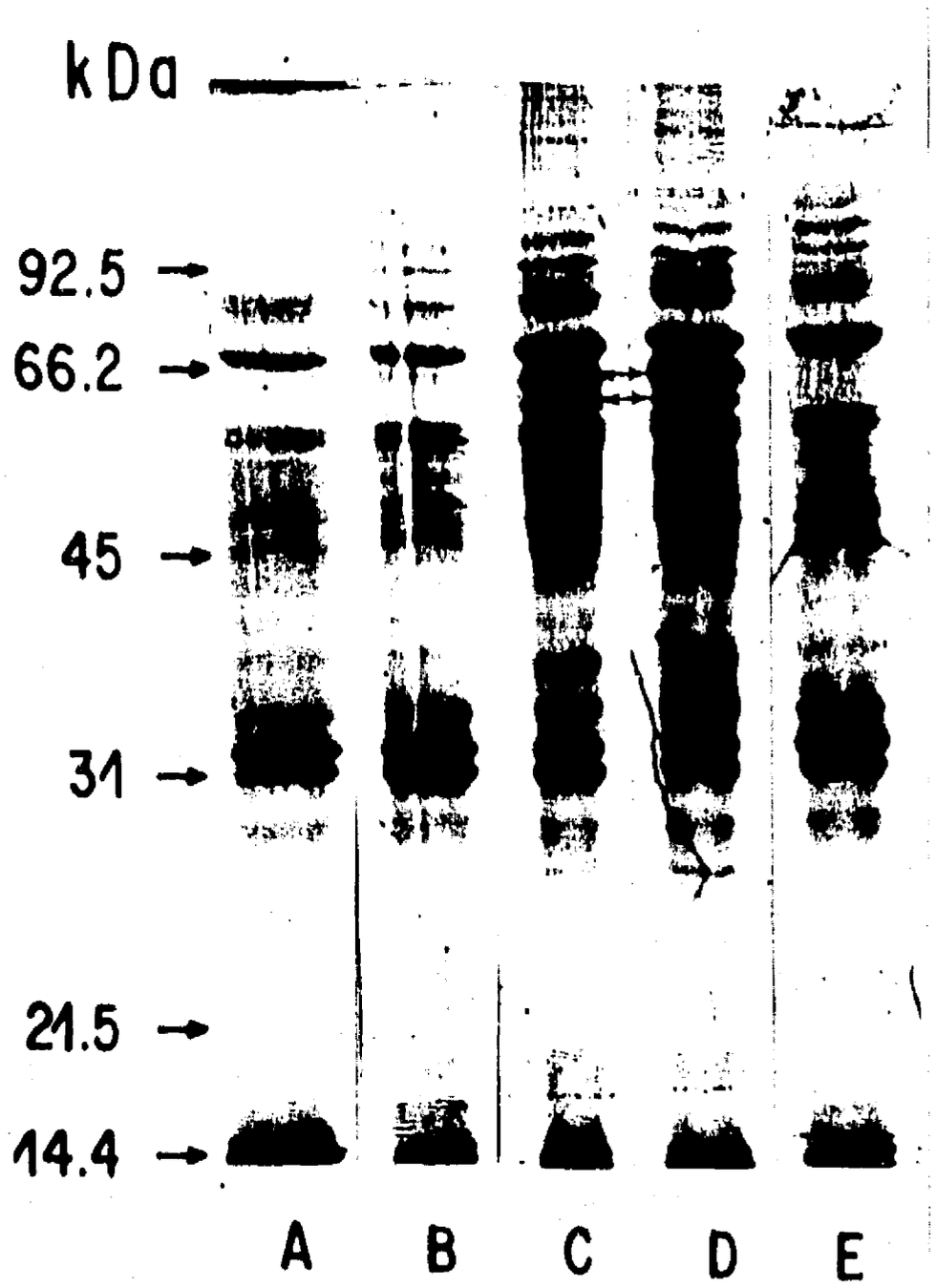

Figure 1

PAGE-SDS of culture filtrates of 2 (A), 4 (B), 6 (C), 8 (D) and 10 (E) weeks of age; gels were stained with Coomasie blue. Arrows indicate the 65 and 63 kDa bands expressed at 6 and 8 weeks. Molecular weights on kilodaltons (kDa) are indicated on the left.

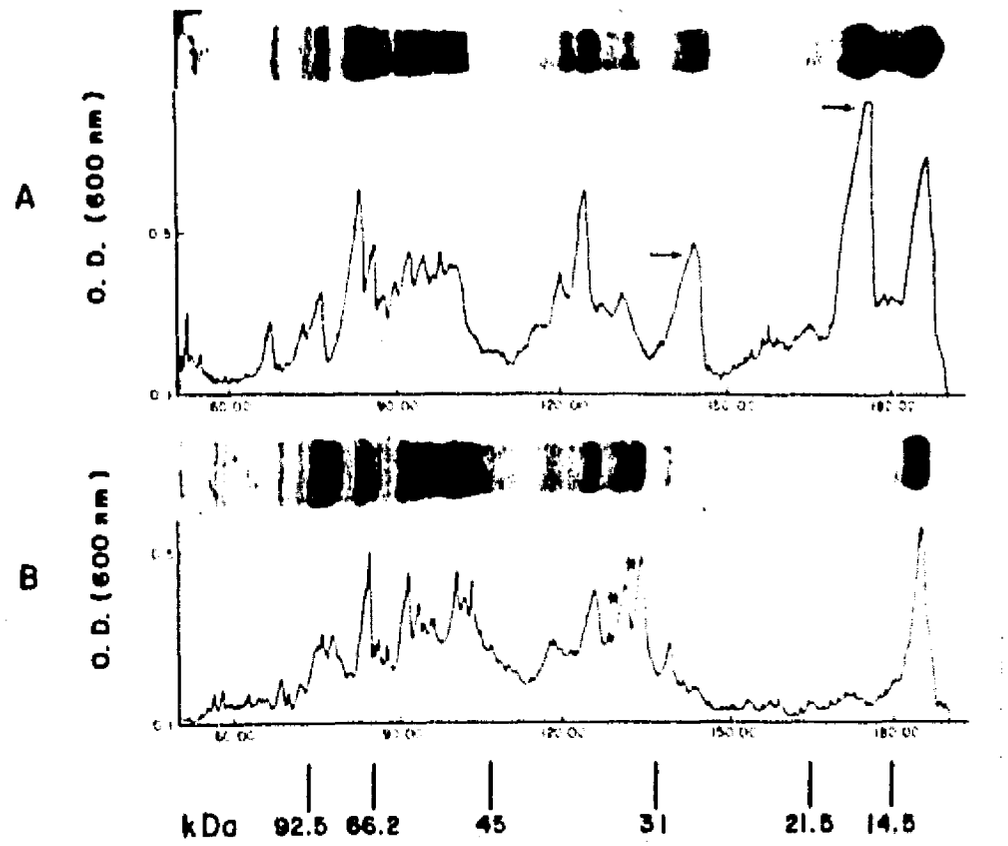

Figure 2

PAGE-SDS and corresponding densitograms to compare the protein profiles of sonic extracts (A) and culture filtrates (B). Arrows and asterisks indicate the main differences between both antigen preparations.

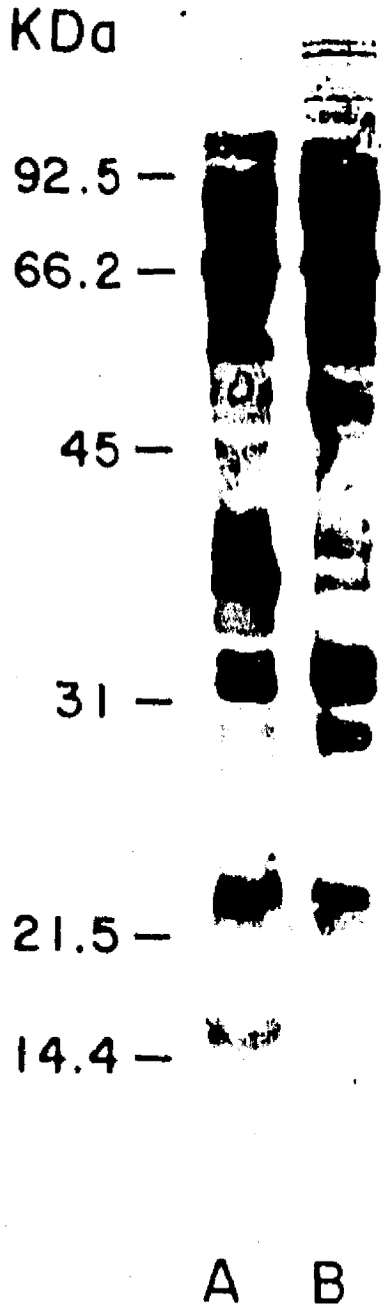

Figure 3

Immunoblot with serum of rabbits hyperimmunized wit M. tuberculosis using antigens obtained by sonication (A) or filtration (B).

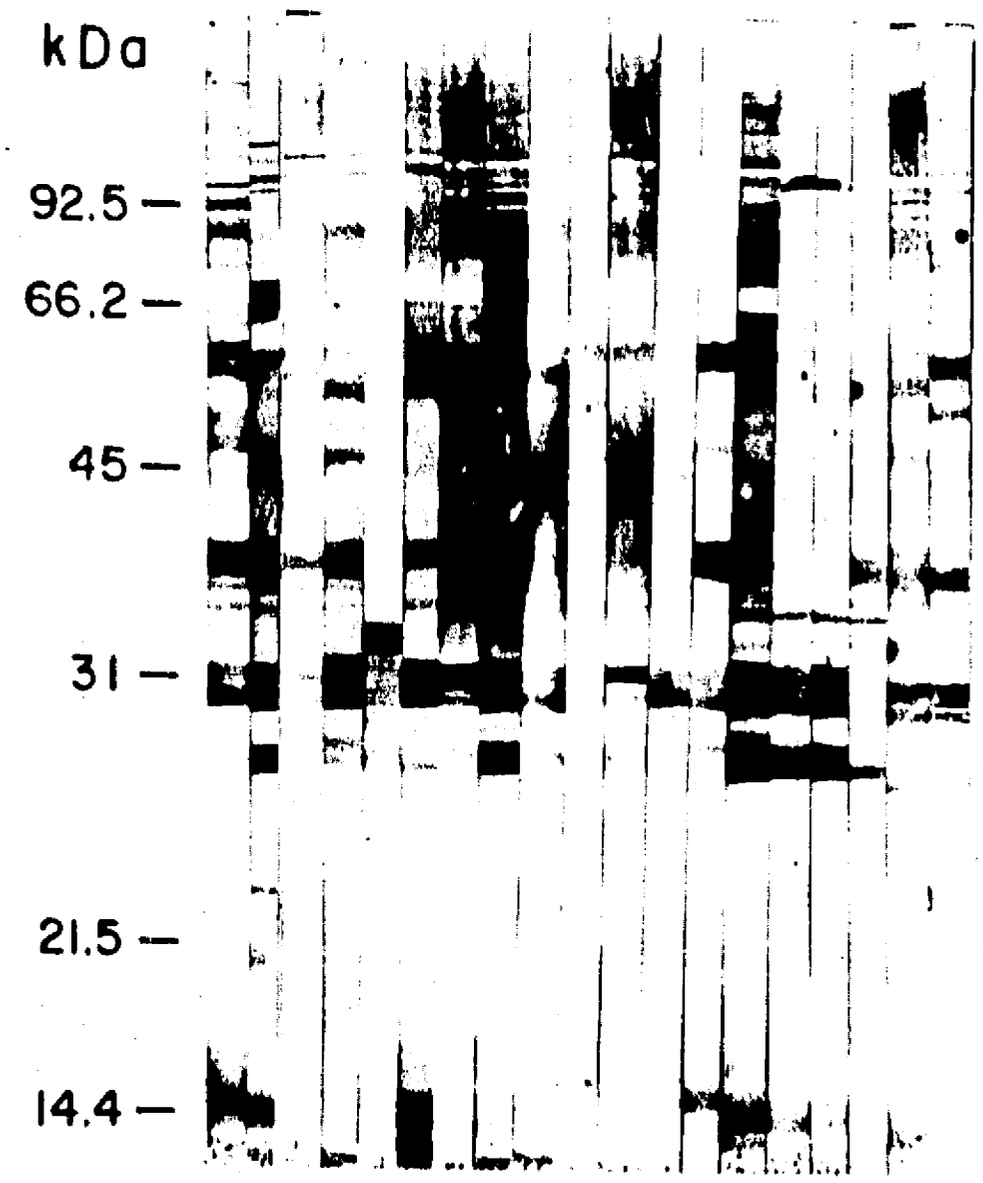

Figure 4

Immunoblot with 19 sera of patients with tuberculosis and 6-week-old culture filtrate antigens.

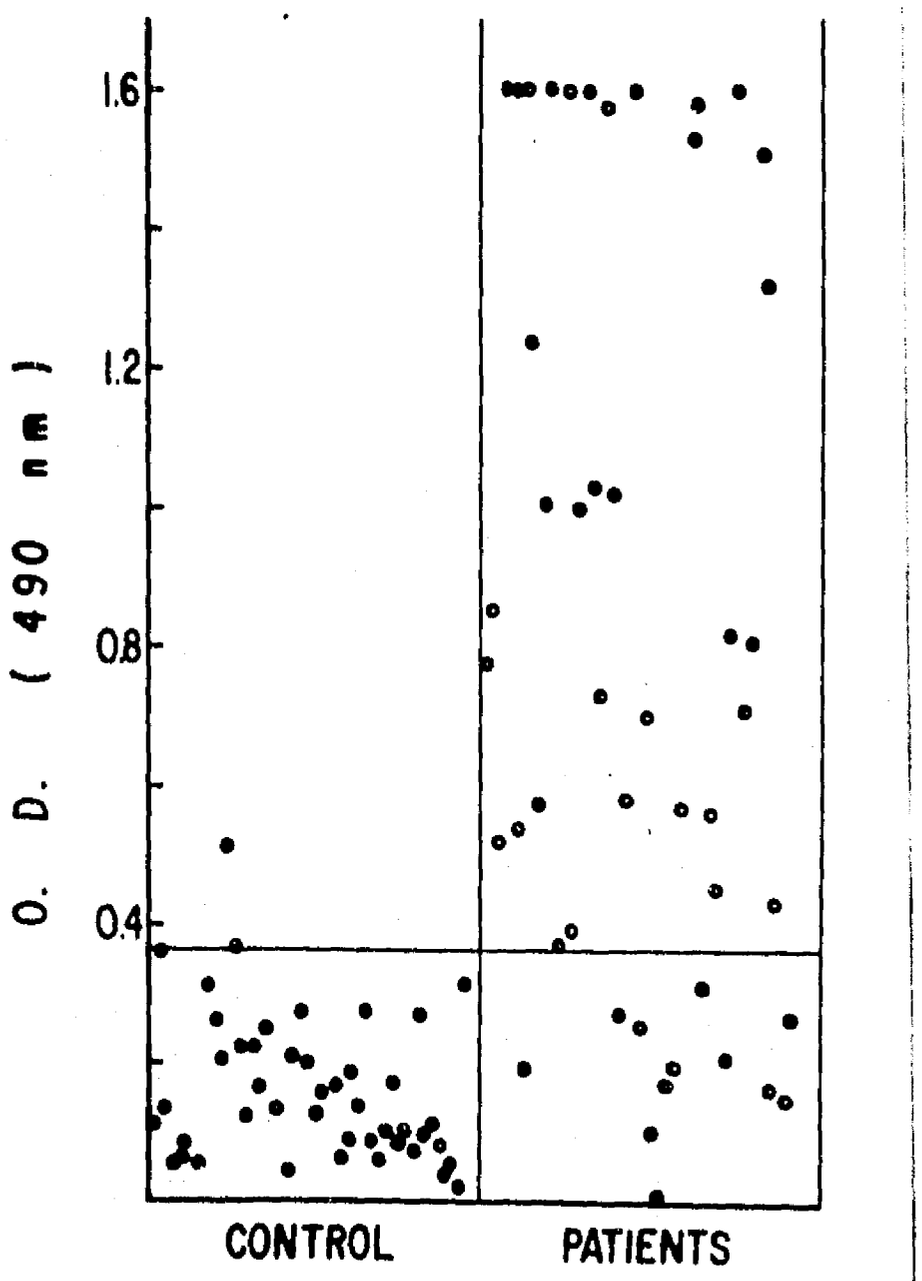

Figure 5

ELISA with 49 tuberculous sera and with 48 control sera.The threshold value (horizontal line) was set at 2 standard deviations above the mean optical density of the control group. Each circle represents a serum.

IMMUNOBLOT IN PULMONARY TUBERCULOSIS

ANTIGENICS BANDS (kDa)

Sera	98	94	92	77	69	65	60	58	53	38	35	32	31	27	21	15	TOTAL
1	-	++	+++	+	-	+	++	-	-	+++	+	++	++	-	-	++	10
2	+	+++	+	-	+++	++	++	++	+++	+++	+	+++	+++	++	+	+++	15
3	++	++	+	-	-	-	+	-	-	++	-	++	+	-	-	-	7
4	++	++	+	-	-	+	+	++	-	+++	+	+	+	++	-	-	11
5	-	++	-	-	-	-	-	+++	++	-	-	+++	+++	+	-	-	6
6	++	-	+++	-	-	++	+++	-	-	+++	+	+++	++	-	+	++	10
7	++	++	+++	++	-	++	+++	+++	-	+++	++	+++	+++	-	-	-	11
8	+++	++	+++	++	++	++	+++	+++	+++	+++	+++	+	+	++	-	-	14
9	-	++	-	++	-	-	++	+++	+++	-	-	-	-	-	-	-	5
10	-	-	-	++	-	-	-	+	+++	-	-	++	-	-	-	-	4
11	+++	-	++	-	-	-	-	+	+	++	-	-	-	-	+	-	6
12	-	++	-	-	-	-	+++	+	+	-	-	++	++	-	-	-	6
13	-	-	-	++	-	-	+++	-	-	+++	-	++	++	-	-	++	6
14	+++	-	+++	-	-	+++	+	-	++	+++	+	+++	+++	+++	+	+++	12
15	-	+++	+	-	-	-	-	+	-	-	+	+++	+++	+++	-	++	9
16	-	+++	-	+++	-	-	-	+	-	-	+	+++	+++	+++	-	+	8
17	-	-	-	-	-	-	-	-	-	++	+	-	-	++	-	-	3
18	+	++	+	++	-	+	-	++	++	++	-	+++	+++	-	-	++	11
19	+	++	+	++	-	+	++	++	-	+++	-	+++	+++	-	-	-	10
TOTAL	10	13	12	9	2	9	12	13	9	13	10	16	15	8	4	8	

Table 1

Analisis of the results of of the immunoblot studies of Figure 4. The molecular weight of reacting bands, in their recognition frequency, the sera tested the intensity of the reactions, and the bands recognized by each serum are indicated. -, no reaction; +, weak reaction; ++, moderate reaction; +++, intense reaction. kDa, kilodalton.

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A 38 KILODALTON *Mycobacterium tuberculosis* ANTIGEN ASSOCIATED WITH INFECTION. ITS ISOLATION AND SEROLOGIC EVALUATION.

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Key Words:.*Mycobacterium tuberculosis*, antigen purification

ELISA, immunoblot.

SUMMARY

To identify antigens that could be specifically associated with tuberculosis infection, the antibody response to Mycobacterium tuberculosis antigens of patients with pulmonary tuberculosis and of healthy individuals was compared by immunoblot. In healthy individuals, serum antibodies were found in the majority of cases. Bands of 60 and 32-31 kilodaltons (kDa) were the antigens more frequently recognized by antibodies of normal sera (55.8 and 64.7 %, respectively). In patients with pulmonary tuberculosis, the number and intensity of the developed antigen bands were much higher than in normal individuals. Antigens reacting preferentially with tuberculosis sera were also identified. Furthermore, a unique disease-associated protein antigen of 38 kDa was found to react with 57% of patients' sera but with none of the controls. This antigen was isolated by elution from nitrocellulose membranes and tested as an ELISA reagent in the serodiagnosis of pulmonary tuberculosis. A specificity of 0.96 and sensitivity of 0.68 were obtained.

INTRODUCTION

Isolated Mycobacterium tuberculosis constituents are required not only for immunodiagnosis and immunoprophylaxis but also for dissection of the complex interactions between the tubercule bacillus and its host. However, and despite the fact that purification and characterization of mycobacterial antigens has been a very active field of research for several decades (reviewed by Daniel & Janicki, 1978), criteria to select biologically significant antigens are lacking. Usually, the feasibility of isolation is the main consideration. Recent methodological advances could help to select potentially important antigens for isolation. Immunoblot has been shown to be a powerful tool for identification of antigens inducing B cell responses (Erlich et al, 1983; Klatser, van Rens & Eggelte, 1984), and recent adaptations of this method (Abou-Zeid et al., 1987; Lamb & Young, 1987) may help to identify, among the complex mosaic of mycobacterial antigens, those eliciting T cell responses.

The goal of the present study was to identify and then isolate potentially significant mycobacterial antigens. For this purpose, the reactivity patterns of mycobacterial antigens with sera of patients with pulmonary tuberculosis and of healthy controls were compared by immunoblot. A 38 kDa protein antigen reacting only

with sera of patients was identified, isolated, and tested as an ELISA reagent in the diagnosis of pulmonary tuberculosis.

MATERIALS AND METHODS

Antigens.

Mycobacterium tuberculosis strain H37/Rv was grown in the synthetic medium of Proskawer and Beck, modified by Youmans (1946). After 4-6 weeks of culture, the medium was filtered and precipitated with 90 or 45% $(NH_4)_2SO_4$. Phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) was added before dialysis in PBS, pH 7.4. Protein was estimated with the Folin phenol reagent.

Sera.

Serum was obtained from white New Zealand rabbits immunized with heat-killed H37/Rv bacilli mixed with culture filtrate proteins in incomplete Freund's adjuvant. Sera from pulmonary tuberculosis patients and from healthy individuals were obtained. Patients' sera were obtained from the Instituto Nacional de Enfermedades Respiratorias, Mexico City.

PAGE-SDS electrophoresis and immunoblot.

A discontinuous buffer system (Laemmli, 1970) was used with a 3% stacking gel and a 10% separating gel. Gels were run with and without reduction with 2-mercaptoethanol. For immunoblot, gels were mounted in a protein transfer apparatus (Idea Scientific Co., Corvallis, OR) and transferred to nitrocellulose paper

sheets (Bio-Rad Laboratories, Richmond, CA) by the method of Towbin, Staehlin & Gordon, (1979). The sheets were treated with 3% BSA in PBS-Tween 20 0.3%, overnight at 4°C. Strips were incubated for three or five h at room temperature with test sera diluted 1/10 in PBS-Tween 20. After washing, the strips were treated for 90 min with horseradish peroxidase-labelled Protein A (Sigma); H_2O_2 and 4-chloro-1-naphtol were used to reveal peroxidase. Immunoblot was also carried out with monoclonal antibodies TB71 and TB72 (Kindly donated by J. Ivanyi, Hammersmith Hospital, London, UK). Bound antibodies were revealed with a peroxidase-labelled goat anti-mouse IgG antiserum (Cappel Laboratories, Cochranville, PA).

Analysis of Immunoblots.

Immunoblots were analyzed by a graphic procedure that simplifies band identification (Larralde et al., unpublished observations). Briefly, a "master" immunoblot pattern was constructed which included all the different antigen bands (ordered by molecular weights) appearing at least once in the blots when reacted with all sera. With this pattern that included all antigenic bands for the whole population under study, the bands in the strips, each representing an individual serum, were scored. Then, the frequency with which a given band reacted with sera of individuals with pulmonary tuberculosis was plotted against the frequency of that same antigen band when reacted with control sera.

Purification of the 38 kDa antigen.

Isolation of the immunodominant 38 kDa antigen identified by immunoblot was carried out in two steps. Based on previous observation showing a thick 38 kDa band on supernatants of culture filtrates precipitated with 45 % $(NH_4)_2SO_4$, we attempted its isolation by alcohol fractionation (Seibert, 1946). Briefly, to the supernatant 0.5 g/ml $(NH_4)_2SO_4$ was added. The precipitate was dyalized and its pH adjusted to 4.0. The supernatant of this precipitation was adjusted to pH 7.0 and sufficient alcohol added to give a 30 % alcohol concentration. The supernatant was brought to pH 4.0. Finally, to the supernatant obtained, alcohol was added to give a 70 % concentration. The various precipitates and the last supernatant were analyzed by PAGE-SDS and a preparative gel of the fraction containing the 38 kDa antigen was transferred to nitrocellulose. The desired band was located by staining strips excised from the sides of the sheet with Amido black. Thereafter, a horizontal strip containing the antigen band was excised and treated with 40% acetonitrile at 37°C for 3 h (Parekh et al., 1985).

Enzyme-linked immunosorbent assay (ELISA).

Sera were processed in one session with the same batches of ELISA reagents and included two replicates per each serum. Immulon II microtiter plates (Dynatech Industries, Inc., McLean, VA) were used. The conditions to better discriminate patients from controls were established by preliminary assays. Wells were sensitized with 0.5 µg of antigen in 100 µl of carbonates

buffer, pH 9.6, at 37°C for 5 h. This was followed by washing with PBS-Tween 20, 0.05%. Blocking of possible remaining active sites on the plastic was done with 1% BSA in PBS for 30 min 37°C.Test sera diluted 1/1000 were applied to the wells for one hour at 37°C. Protein A labelled with horseradish peroxidase (Sigma) was added to reveal bound antibodies. Peroxidase was developed by adding 100 µl of 1 mg/ml orthophenylendiamine and 0.03% H_2O_2 in citrate buffer 0.1 M, pH 4.5, for 4 min at 37°C. The reaction was stopped with 50 µl of 1 N sulphuric acid. Optical density readings at 492 nm were done in a Behering automatic ELISA processor.

RESULTS

Immunoblot analysis of the antibody response in patients with pulmonary tuberculosis and in healthy individuals.

By PAGE-SDS, the antigen preparation used for these studies contained 35 Coomasie blue stained bands ranging from 122 to 14 kDa (not shown). Only two of the 49 tuberculosis sera tested gave negative results. The remaining 47 sera recognized a total of 25 bands (Fig. 1). Positive reactions were detected with 30 of the 33 control sera tested, with a total of 14 reacting bands seen; these reactions were of considerably lesser intensity than those observed with tuberculosis sera. By using a graphic procedure to analyze immunoblots, a meaning to reacting bands could be adscribed according to their positions in the plot (Fig. 2). There were 4 bands located more or less equidistant to both

axes which were antigens reacting similarly with both control and pathologic sera. Of these, a 32-31 kDa doublet band was found to react with 90% of tuberculosis sera and with 55.8% of normal sera. Another antigen band with similar reactivity was a 60 kDa antigen which was recognized by 82% of pathologic sera and by 64.7% of normal sera. There were bands favored by pathologic sera which were located close to the axes of the tuberculosis sera. Most of these were low frequency bands; however, there were two high frequency bands of 29 and 38 kDa. The 38 kDa band was unique in that it reacted with antibodies of 53% of sera of patients with tuberculosis but with none of healthy control sera. Immunoblot performed with a fraction highly enriched in the 38 kDa antigen obtained by alcohol fractionation (vide infra) allowed a better discrimination between control and tuberculosis sera (not shown). The "background" response on control sera was abolished, and reactivity of tuberculosis sera with the 38 kDa antigen was higher (57%). Some impurities were observed.

Purification of the 38 kDa antigen (Fig. 3).

The PAGE-SDS profile of the various fractions obtained by alcohol fractionation are shown in Fig. 3. A single 38 kDa band was observed on Coomasie blue stained gels of the supernatant obtained after precipitation with 70% alcohol at pH 4 (Lane F). Since immunoblot with tuberculosis sera and with anti-M. tuberculosis rabbit antisera disclosed some impurities (not shown), further purification steps were carried out. A preparative 10% PAGE-SDS gel loaded with 1.5 mg of the fraction

enriched in the 38 kDa antigen was tranferred to a nitrocellulose sheet and eluted with acetonitrile. At the end of the procedure, from 200 to 250 µg of the starting material were recovered. On Coomasie blue stained gels of the eluted material, a single 38 kDa band was observed (Lane G). The purity of the isolated antigen was confirmed by immunoblot with a polyvalent antiserum obtained from rabbits hyperimmunized with M. tuberculosis which revealed a single 38 kDa band free of other antigenic contaminants (Lane H).

Demonstration of epitopes recognized by monoclonal antibodies TB71 and TB72 on the 38 kDa antigen.

Recently, monoclonal antibodies which react with a 38 kDa protein antigen of M. tuberculosis have been obtained (Young et al., 1986). On the antigen preparation obtained by 90% $(NH_4)_2SO_4$ precipitation and on the enriched fraction obtained by alcohol fractionation, monoclonal antibodies TB71 and TB72 reacted with a single 38 kDa band on blots (not shown). In contrast, the antigen isolated by elution from nitrocellulose sheets reacted with TB71 but it did not bind the TB72 antibody (not shown).

The antibody response to the isolated 38 kDa antigen in pulmonary tuberculosis analyzed by ELISA (Fig. 4).

These studies were performed with 49 tuberculosis sera. It was possible to classify 25 cases as active tuberculosis and 15 as inactive. Information about BCG vaccination tuberculin reactivity, extent of disease, and length of treatment was not obtained. The control group consisted of 48 healthy individuals.

In patients the mean optical density was 0.620 and in healthy individuals 0.152. In order to estimate the sensitivity (fraction of tuberculosis sera above threshold) and specificity (fraction of control sera below threshold) of the ELISA, a threshold value of two standard deviations above the mean optical density of the control group was chosen. A specificity of 0.96 and sensitivity of 0.68 were obtained. In the group of patients with active tuberculosis, sensitivity was 0.68 and in inactive disease, 0.60.

DISCUSSION

The development of a reliable serodiagnosis test for tuberculosis has been made difficult by cross reactions between the tubercle bacillus and other microorganisms (Minden et al., 1972; Kadival, Chaparas & Hussong, 1987; Young et al., 1987). Also contributing to this failure is the fact that a fraction of the general population can be sensitized by previous resolved exposures to M. tuberculosis or by BCG vaccination. Therefore, identification and isolation of species and disease-specific antigens seems to be a prerequisite to develop an accurate immunodiagnostic test.

In the past the antibody response to mycobacterial antigens has been analyzed by measuring such a response with a variety of tests (Toussaint et al., 1969; Reggiardo et al., 1981; Winter & Cox, 1981; Daniel & Debanne, 1987). With the introduction of immunoblot (Towbin et al., 1979), a novel approach has been made possible. By this method, patterns of reactivity based on the

number and the electrophoretic mobility of the recognized antigens and on the intensity of the antigen-antibody reactions on the paper, can be assesed. Despite this great potential, immunoblot has seldom been used to systematically analyze the response to mycobacterial antigens in patients with tuberculosis (Coates et al., 1986).

In this study we examined by immunoblot the antibody response to M. tuberculosis antigens in sera of individuals with pulmonary tuberculosis and in healthy individuals. An anti- mycobacterial antibody response was documented in a majority of healthy individuals. This "background" response was preferentially directed against antigen bands of 60 and a 32-31 kDa. These antigens were also recognized by most tuberculosis sera, suggesting that they are either widely distributed cross reacting antigens or antigens involved in sensitization due to previous encounters with the tubercle bacillus or by BCG vaccination. The practical implication derived from these observations is that they rule out these mycobacterial antigens as candidates for isolation and testing as serodiagnosis reagents. Otherwise, our immunoblot studies revealed striking differences between normal and pathologic sera. With tuberculosis sera, the intensity and multiplicity of the reacting bands were much higher. Furthermore, immunodominant disease-associated antigens were identified. Among these, a 38 kDa antigen was unique in that it reacted with 57 % sera of patients with pulmonary tuberculosis but in no instance with control sera.

The purification of M. tuberculosis antigens has been very frustating when using classical physical-chemical methods (reviewed by Daniel & Janicki, 1978). However, the introduction of hybridoma and DNA recombinant technologies has opened a new perspective in this field of research. By monoclonal antibody affinity chromatography, several mycobacterial antigens have been purified (Minden et al., 1984; Kadival et al., 1987; Young et al., 1986). Recombinant DNA expressed in bacteria now makes it possible to obtain antigens in sufficient amount to evaluate their biological potential (Young, et al., 1985). In this work we have shown that individual mycobacterial antigens can be successfully isolated from complex antigen mixtures transferred onto nitrocellulose membranes by elution with acetonitrile. The purification procedure was successful in that it gave an homogenous and undegraded product with retained antigenicity. Indeed, a 38 kDa band free of other antigenic contaminants was seen by immunoblot with a polyvalent rabbit antiserum against M. tuberculosis. However, the epitope recognized by monoclonal antibody TB72 was lost during isolation, showing that elution with acetonitrile is not an entirely inocuous procedure.

The purified 38 kDa antigen was evaluated as an ELISA reagent in the diagnosis of pulmonary tuberculosis. Specificity was very high (0.96) but sensitivity was only 0.68. These results reinforce the immunoblot data showing that an antibody response

to this antigen is unlikely in non infected individuals and that a substantial number of patients makes no antibodies against the 38 kDa antigen. These results also appear to indicate that a reliable serodiagnostic test in tuberculosis will be difficult to achieve even with the use of disease-related antigens such as the 38 kDa protein. Rather than false positive results, the main limiting factor seems to be a poor or absent antibody response in a fraction of infected individuals, thus resulting in low sensitivity indexes. Similar results have been obtained in other studies using isolated antigens (Reggiardo & Vazquez, 1981; Balestrino et al., 1984).

The disease-associated antigen identified in this work seems to be a glycoprotein (Espitia & Mancilla, 1989) which carries the TB72 and TB71 epitopes of a 38 kDa protein antigen isolated from M. tuberculosis extracts by Young et al.(1986). The role of these epitopes has been investigated by others in a competitive radioimmunoassay (Ivanyi, Krambovitis & Keen, 1983). It was found that monoclonal antibody TB72, but not TB71, gave positive values in 74% of patients with active tuberculosis. These findings have been taken as indicating immunodominance of the TB72 epitope in the antibody response to the 38 kDa antigen. However, our ELISA data, showing positive values in 68% of patients despite the absence of the TB72 epitope on the purified antigen used, suggest the participation of additional epitopes in the antibody response to the 38 kDa antigen in patients with tuberculosis.

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REFERENCES

Abou-Zeid, C., Filley, E., Steele, J. & Rook, G.A.W. (1987) A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes *in vitro* after converting bands cut from Western blots into antigen-bearing particles. J. Immun. Meth. 98, 5.

Balestrino, E.A., Daniel, T.M., de Latini, M.D.S., Latini, O.A., Ma, Y. & Scocozza, J.B. (1984) Serodiagnosis of pulmonary tuberculosis in Argentina by enzyme-linked immunosorbent assay (ELISA) of IgG antibody to *Mycobacterium tuberculosis* antigen 5 and tuberculin purified protein derivative. Bull. WHO 62, 755.

Coates, S.R., Hansen, D., Schechter, G., Slutkin, G., Hopewell, P., Affronti, L. & Echenberg, D.F. (1986). Identification of *Mycobacterium tuberculosis* antigens in Seibert fractions by immunoblotting. J. Clin. Microbiol. 24, 126.

Daniel, T.M. & Debanne, S.M. (1987) The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. Am. Rev. Respir. Dis. 135, 1137.

Daniel, T.M. & Janicki, B.W. (1978) Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol. Rev. 42, 84.

Erlich, H.A., Rodgers G., Vaillancourt, P., Araujo, F.G. & Remington J.S. (1983) Identification of an antigen-specific

immunoglobulin M antibody associated with acute Toxoplasma infection. Infect. Immun. 41, 683.

Espitia, C. & Mancilla, R. (1989). Identification, isolation and partial characterization of M. tuberculosis glycoprotein antigens. Clin. Exp. Immunol.

Ivanyi, J., Krambovitis, E. & Keen, M. (1983) Evaluation of a monoclonal antibody (TB72)-based serological test for tuberculosis. Clin. Exp. Immunol. 54, 337.

Kadival, G.V., Chaparas, S.D. & Hussong, D. (1987) Characterization of serologic and cell-mediated reactivity of a 38 kDa antigen isolated from Mycobacterium tuberculosis. J. Immunol. 139, 2447.

Klatser, P.R., van Rens, M.M. & Eggelte, T.A. (1984) Immunochemical characterization of Mycobacterium leprae antigens by the SDS-PAGE Immunoperoxidase technique (SGIP) using patients' sera. Clin. Exp. Immunol. 55, 537.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680.

Lamb, J.R. & Young, D.B. (1987) A novel approach to the identification of T-cell epitopes in Mycobacterium tuberculosis using human T-lymphocyte clones.Immunology 60,1.

Minden, P., Keller, P.J., Freed, J.H., Nielsen, L.D., Brennan, P.J., McPheron, L. & McClatchy, J.K. (1984) Immunological

evaluation of a component isolated from Mycobacterium bovis BCG with a monoclonal antibody to M. bovis BCG. Infect. Immun. 46, 519.

Minden, P., McClatchy, J.K., Cooper, R., Bardana, Jr., E.J. & Farr, R.S. (1972) Shared antigens between Mycobacterium bovis (BCG) and other bacterial species. Science 176, 57.

Parekh, BS., Metha, H.B., West, M.D. & Montecarlo, R.C. (1985) Preparative elution of proteins from nitrocellulose membranes after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal. Biochem. 148, 87.

Reggiardo, Z., Aber, V.R., Mitchison, D.A. & Devi, S. (1981) Hemagglutination test for tuberculosis with mycobacterial glycolipid antigens. Am. Rev. Respir. Dis. 124, 21.

Reggiardo, Z. & Vazquez, E. (1981) Comparison of enzyme-linked immunosorbent assay and hemagglutination test using mycobacterial glycolipids. J. Clin. Microbiol. 13, 1007.

Seibert, F.B. (1949) Isolation of three different proteins and two polysaccharides from tuberculin by alcohol fractionation: their chemical and biological properties. Am. Rev. Tuberc. 59, 86.

Toussaint, A.J., Fife, E.H., Parlett, R.C., Affronti, L.F., Wright, G.L., Reich, M. & Morse, W.C. (1969) A soluble antigen fluorescent antibody test for serodiagnosis of tuberculosis infection. Am. J. Clin. Pathol. 52, 708.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76, 4350.

Winter, W.D. & Cox, R.A. (1981) Serodiagnosis of tuberculosis by radioimmunoassay. Am. Rev. Respir. Dis. 125, 582.

Youmans, G.P. (1946) A method for determination of the culture cycle and the growth rate of virulent human type tubercle bacilli. J. Bact. 51, 703.

Young, D.B., Ivanyi, J., Cox J.H. & Lamb, J.R. (1987) The 65 kDa antigen of mycobacteria-a common bacterial protein? Immunol. Today 8, 215.

Young, R.A., Bloom, B.R., Grosskinsky, C.M., Ivanyi, J., Thomas, D. & Davis, R.W. (1985) Disecction of Mycobacterium tuberculosis antigens using recombinand DNA. Proc. Natl. Acad. Sci. USA 82, 2583.

Young, D., Kent, L., Rees, A., Lamb, J. & Ivanyi, J. (1986) Immunological activity of a 38-Kilodalton protein purified from Mycobacterium tuberculosis. Infect. Immun. 54, 177.

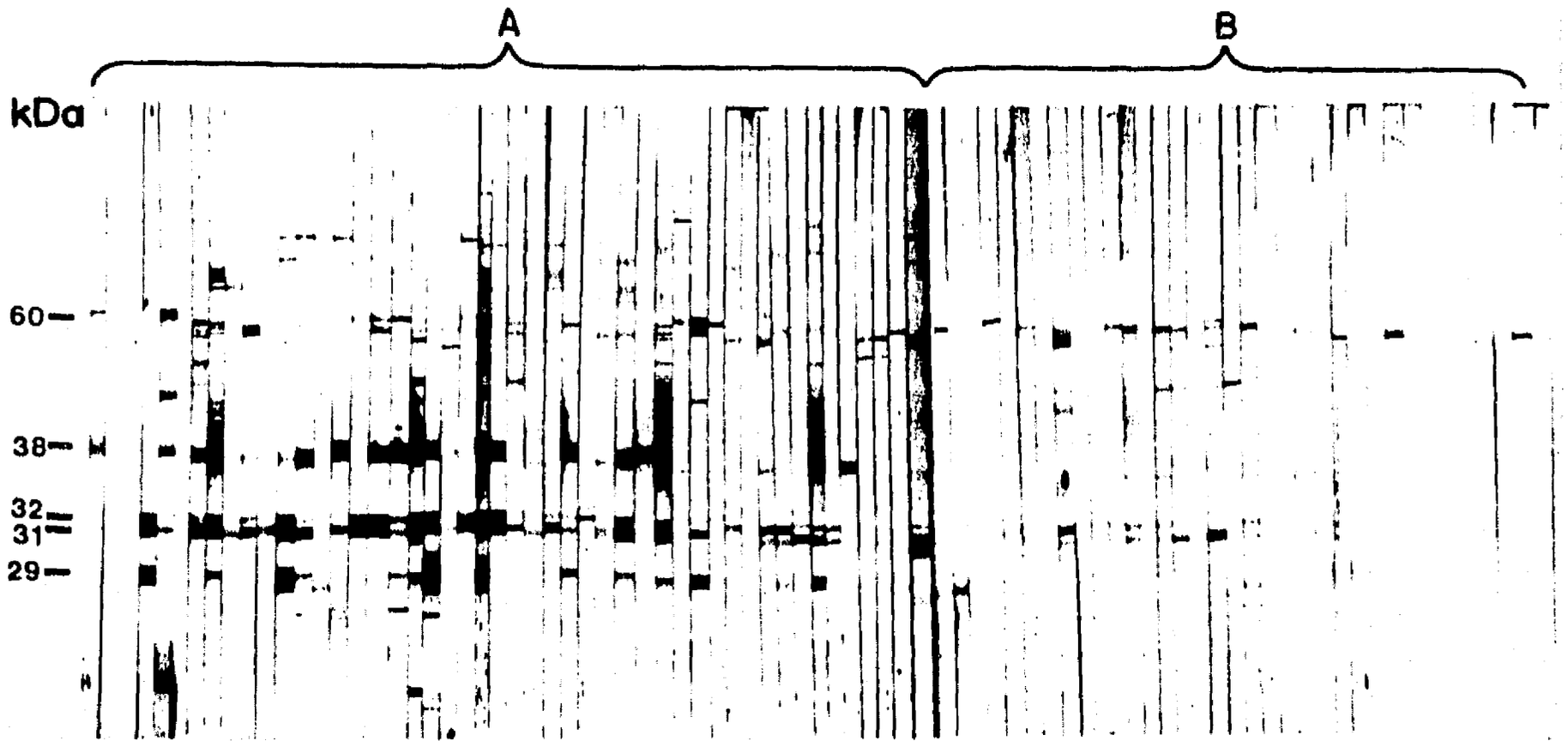

Figure 1

Immunoblot analysis of the antibody response to mycobacterial antigens on 49 sera of patients with pulmonary tuberculosis (A) and on 33 sera of healthy individuals (B). On the left, the molecular weight of the bands more frequently recognized are indicated on kilodaltons (kDa).

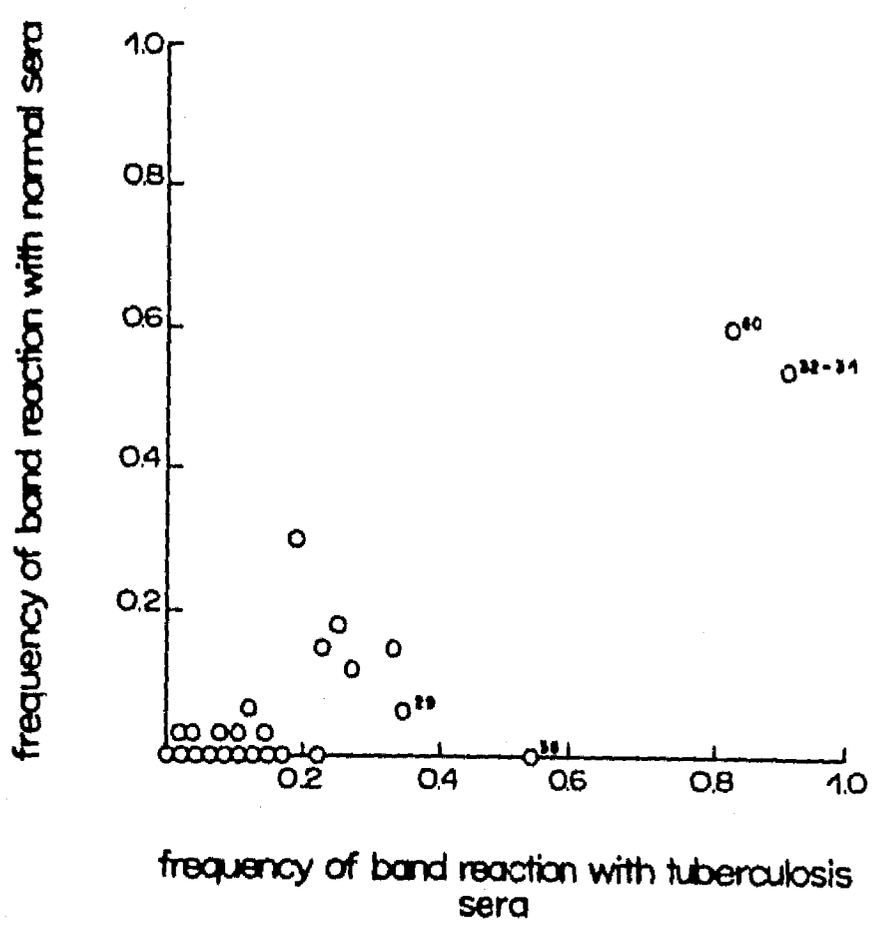

Figure 2

Graphic analysis of the immunoblot studies shown on Fig. 2. The frequency of reaction of tuberculosis sera with a given antigen was plotted against that of control sera. The molecular weight on kDa of the antigens more frequently recognized is indicated.

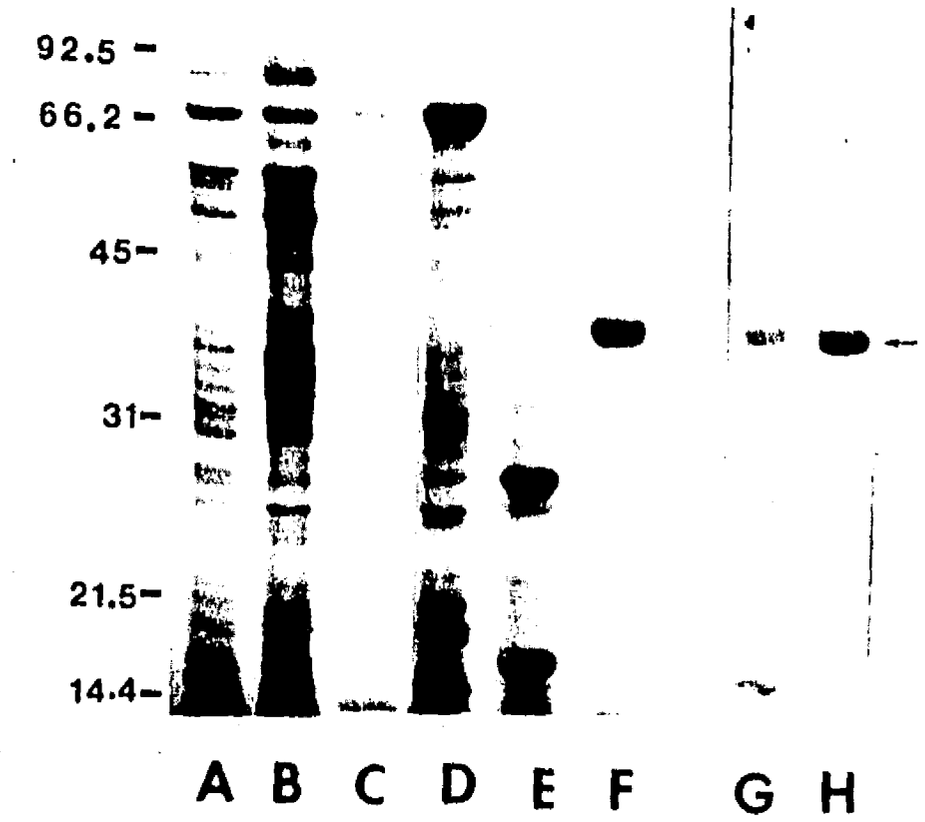

Figure 3

Isolation of the 38 kDa antigen from supernatants of culture filtrates precipitated with 45% $(NH_4)_2SO_4$. Lane A, starting material; Lane B, precipitate at pH 4; Lane C. precipitate at pH 7 and 30% alcohol; Lane D, precipitate at pH 4; Lane E, precipitate at pH 4 and 70% alcohol; Lane F, supernatant of the precipitate at pH 4 and 70% alcohol. Gels were stained with Coomasie blue. From fraction of Lane F the 38 kDa antigen was isolated by elution with acetonitrile as described in Materials and Methods. Lane G, SDS-PAGE of the isolated antigen stained with Coomasie blue. Lane H, isolated antigen tested by immunoblot with a polyvalent hyperimmune anti- M. tuberculosis antiserum raised in rabbits.

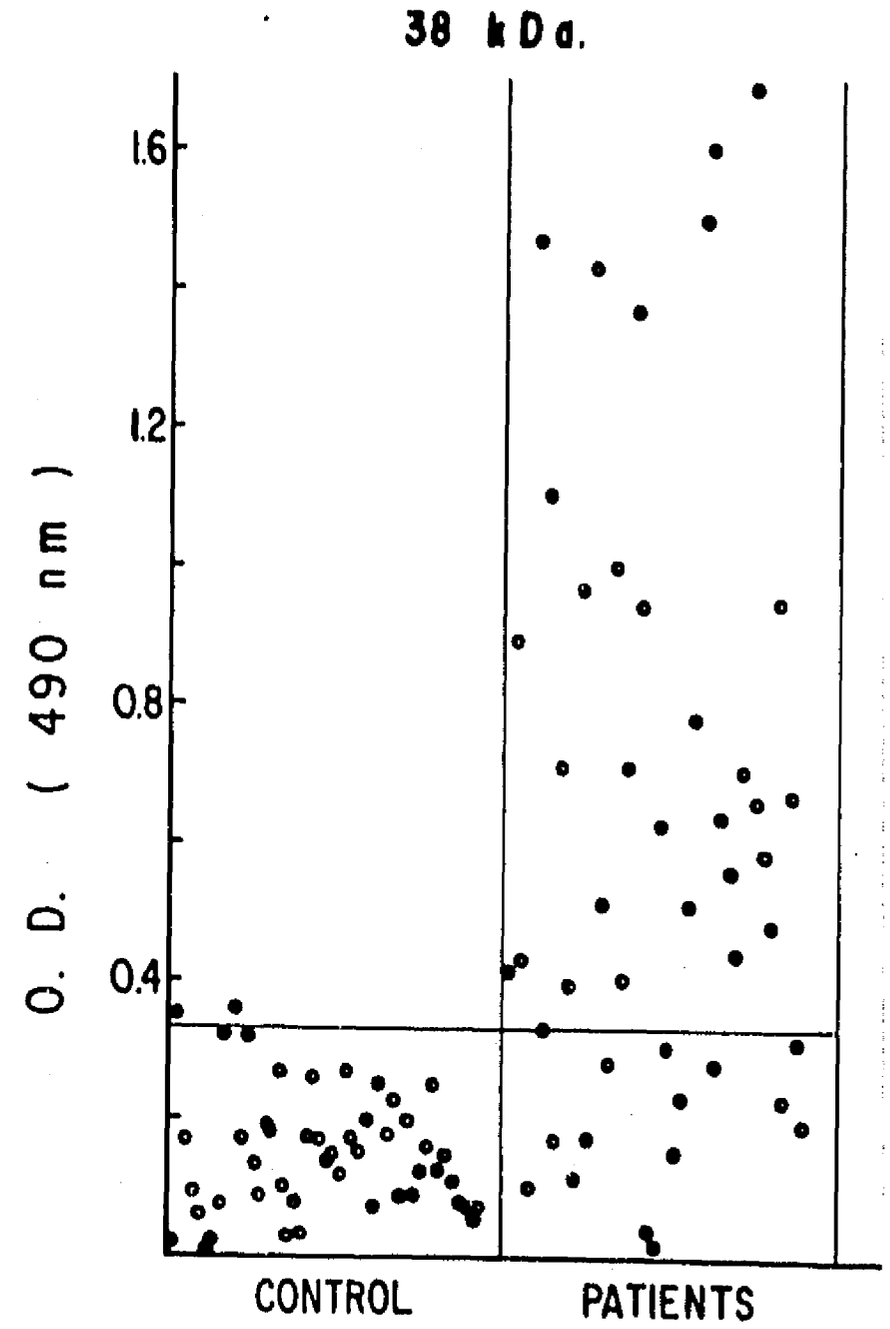

Figure 4

Serum antibody titers against the isolated 38 kDa antigen by ELISA in 48 healthy individuals (control) and in 49 individuals with pulmonary tuberculosis (patients). A threshold value of 2 standard deviations above the mean optical density of the control group was chosen (horizontal line). Each circle represents an individual serum.

IDENTIFICATION, ISOLATION AND PARTIAL CHARACTERIZATION OF *Mycobacterium tuberculosis* GLYCOPROTEIN ANTIGENS.

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SUMMARY

In *Mycobacterium tuberculosis* culture filtrates, three Concanavalin A (Con A)-binding bands of 55, 50 and 38 kilodaltons (kDa) were identified by labelling blotted proteins with a Con A-peroxidase conjugate. Binding was inhibited by the competitor sugar α-methyl D-mannoside and by reduction with sodium *m*-periodate. Bands of 55, 50 and 38 kDa stained with Coomasie blue were sensitive to digestion with proteases, thus indicating that they are proteins. Glycoproteins were isolated by lectin affinity chromatography or by elution from nitrocellulose membranes. On the isolated form, the 55-50 kDa doublet glycoprotein was 65.4% protein and 34.6% sugar. The purified 38 kDa molecule was 74.3 % protein and 25.7 % carbohydrate. By immunoblot, antibodies against mycobacterial glycoproteins were demonstrated in immunized rabbits and in patients with pulmonary tuberculosis, but not in healthy individuals. Treatment with sodium *m*-periodate abolished binding of rabbit antibodies to the 38 kDa glycoprotein. Reactivity of the 55-50 kDa doublet glycoprotein was not altered by reduction. By immunoblot with monoclonal antibodies TB71 and TB72, a carbohydrate-dependent and a carbohydrate-independent epitope could be identified on the 38 kDa glycoprotein.

INTRODUCTION

Glycoproteins represent a large group of conjugated proteins of wide distribution and considerable biological significance (reviewed by Marshall, 1972). They are often located in the extracellular milieu or expressed at the cell surface and carry out, in both cases, important roles in cell-cell interactions (Marshall, 1972). Examples of biologically important glycoproteins are the immunoglobulins, many different hormones, and surface receptors present in a variety of cells (Neufeld & Ashwell, 1980).

Data generated during the past several years show that glycoproteins are also important in infectious and parasitic processes. It has been shown that glycoproteins of some pathogens are immunodominant antigens (Nogueira, Unkeless & Cohn, 1982; Robey et al., 1986; Dalton & Strand, 1987), which makes these molecules potentially suitable for immunodiagnosis and vaccination. Of equal interest are the observations implicating glycoproteins as infectivity and virulence factors (Moskophidis & Müller, 1984; Alves et al., 1986; Robey et al., 1986).

The above data provoked our interest in finding out if any of the many Mycobacterium tuberculosis components were glycoproteins, and in investigating an eventual role for these mole-

cules in infection. By Concanavalin A (Con A)-based methods, we have identified and isolated three carbohydrate-associated proteins of 55, 50 and 38 kilodaltons (kDa) on unheated culture filtrates. Antibodies against these glycoproteins were demonstrated in serum of immunized rabbits and in humans with pulmonary tuberculosis.

MATERIALS AND METHODS

Bacteria and antigens.

Mycobacterium tuberculosis strainS H37/Rv and H37/Ra were grown and their culture filtrate protein antigens obtained as described elsewhere (Espitia et al., 1989). The protein content was estimated with the Folin reagent and total sugar by the phenol sulfuric acid method.

Sera.

Sera were obtained from white New Zealand rabbits immunized with M. tuberculosis in incomplete Freund's adjuvant. Sera from patients with confirmed pulmonary tuberculosis were obtained from the Instituto Nacional de Enfermedades Respiratorias, México, D.F. Control sera were obtained from healthy individuals.

Polyacrylamide sodium dodecyl sulfate gel electrophoresis (PAGE-SDS) and immunoblot.

The discontinuous buffer system described by Laemmli (1970)

was used with a 3% stacking gel and a 10% separating gel. After electrophoresis, gels were either stained with Coomasie brilliant blue or mounted in a protein transfer apparatus (Idea Scientific Co., Corvallis, OR), and the separated proteins were transferred to nitrocellulose paper sheets (Bio-Rad Laboratories, Richmond, CA) as described elsewhere (Towbin, Staehelin & Gordon, 1979). Immunoblot using human and rabbits' sera and monoclonal antibodies TB71 and TB72 (Kindly donated by J. Ivanyi, Hammersmith Hospital, London, UK) was performed as described (Espitia et al, 1989).

Schiff's stain and labelling of electrontransferred proteins with peroxidase-labelled Con A.

Schiff's stain of PAGE-SDS gels was performed according to a published method (Zacharius et al., 1969). Con A-peroxidase labelling of electrontransferred proteins was performed as described elsewhere (Glass II, Briggs & Hnilica, 1981) Briefly, after transfer of PAGE-SDS separated proteins, nitrocellulose sheets were treated with a 3% BSA solution for one h at 40°C. The BSA solution had been previously treated with sodium *m*-periodate 10 mM to reduce possible contaminating carbohydrate residues. Strips were immersed in the 3% BSA solution containing Con A (5 µg/ml), 1 mM Ca Cl and 1 mM Mg Cl. Control strips were reacted with the above Con A-peroxidase solution plus α-methyl *D*-mannoside 0.2 M.

Isolation of glycoproteins by Con A affinity chromatography and by elution from nitrocellulose sheets.

Sixty or seventy mg of protein antigens were mixed with 10 ml of Con A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and, after overnight incubation at 4°C or for one h at room temperature with shaking, unbound proteins were washed out in two steps. Sepharose was mounted on a column and rinsed with PBS until readings at 280 nm were less than 0.02, and then with NaCl 0.5 M until readings approached zero. Bound material was eluted with α-methyl D-mannoside 0.01 M. Purification of a glycoprotein identified in supernatants of culture filtrates precipitated with 45% $(NH_4)_2SO_4$ was done by alcohol fracctionation (Seibert, 1949) followed by elution from nitrocellulose sheets (Parekh et al., 1985; Espitia et al., 1989). Some protein samples were subjected to proteolytic digestion and periodate reduction. For digestion, 50 µg of proteases type IV (Sigma) in PBS (pH 7.4) were mixed with 30 µg of protein for one h at 37°C. Reduction was carried out with sodium *m*-periodate as described elsewhere (Rapp & Ross, 1986). At the end of the above procedures samples were transferred to the electrophoresis cocktail. Occassionally, digestion and reduction were done after transfer of proteins into nitrocellulose.

RESULTS

Demonstration of glycoproteins by Schiff's stain and by labelling with Con A (Figure 1.)

Similar observations were made with strains H37/Rv and H37/Ra. On PAGE-SDS reducing gels of proteins precipitated

from culture filtrates with 45% $(NH_4)_2SO_4$, about 25 well-defined bands varying from 90 to 14 kDa were observed (Lane A). Schiff's stain of these gels revealed an ill-defined zone of strong reactivity from about 40 to 60 kDa (Lane B). These findings prompted the use of a more sensitive method to identify glycoproteins. Culture filtrate proteins were electrophoresed, transferred to nitrocellulose paper, and reacted with Con A conjugated to peroxidase. A thick band of strong reactivity which corresponded to that revealed by the Schiff's reagent was observed (Lane C). Glycoproteins were also sought by Con A-peroxidase labelling in the supernatant of the 45% $(NH_4)_2SO_4$ precipitate after alcohol fractionation. In the supernatant obtained after precipitation with 70% alcohol at pH 4, a Coomasie blue stained band of 38 kDa (Lane D) was labelled by the Con A-peroxidase conjugate after blotting (Lane E). In the remaining fractions obtained by alcohol fractionation, Con A binding was not observed (not shown).

Isolation of glycoproteins by lectin affinity chromatography and by elution from nitrocellulose membranes.

From culture filtrate proteins precipitated with 45% $(NH_4)_2SO_4$ subjected to affinity chromatography with Con A-Sepharose 4B, two fractions were obtained (Fig. 2). The peak obtained by rinsing with PBS after incubation represented molecules lacking the correct sugar moieties to bind to Con A. The fraction eluted by a 0.01 M solution of α-

methyl D-mannoside represented specifically bound molecules. The eluted material represented approximately 1% of the total material applied to the column. The unfractionated and fractionated materials were analyzed by PAGE-SDS. The unfractionated material contained about 33 well-defined Coomasie blue stained bands (Fig. 3, lane A); a similar banding pattern was present on the unbound material (Fig. 3, lane B). On the material eluted from the Con A column, a major band of 55 kDa and a minor one of 50 kDa were detected on Coommasie blue stained gels (Fig. 3, lane C, arrows). To further test the affinity of these molecules for the lectin, bound and unbound fractions were electrophoresed, blotted onto nitrocellulose and treated with Con A-peroxidase. Reaction of the 55 and 50 kDa bands was only observed on the specifically eluted material (Fig. 3, lane D,); this reaction was blocked when α-methyl D-mannoside 0.2 M was added to the incubation medium (Fig.3, lane E) and by reduction with sodium m-periodate (Fig. 3, lane F). The protein and sugar contents of the isolated 55-50 kDa doublet glycoprotein were 65.4 and 34.6%, respectively.

Isolation of the 38 kDa Con A binding band was not possible by lectin affinity chromatography but, as previously described (Espitia et al. 1989), alcohol fractionation followed by elution with acetonitrile was successful. By PAGE-SDS, a single Coomasie blue stained band of 38 kDa was observed on the eluted material (Fig. 3, lane G, asterisk). The isolated band retained the ability to react specifically with Con A on blots (Fig. 3, lane H), which was blocked also

by α-methyl D-mannoside (Fig. 3, lane I) and by reduction (Fig. 3, lane J). The isolated 38 kDa antigen represented 16.6% of the starting material and was 74.3% protein and 25.7 % sugar. After digestion with proteases, all three glycoprotein bands were no longer detected on Coommasie blue stained gels or by blotting with Con A-peroxidase (not shown).

The antibody response to Con A binding glycoproteins (Figure 4).

Purified glycoproteins were tested by immunoblot with sera of rabbits hyperimmunized with M. tuberculosis. When unfractionated antigens were used, about 30 reacting bands were seen (Lane A). On the fraction eluted from the Con A column, rabbit antiserum recognized only the bands of 55 and 50 kDa (Lane B). Immunoblot analysis of the glycoprotein eluted with acetonitrile from nitrocellulose membranes revealed a single 38 kDa band reacting with rabbit antiserum (Lane D, arrow). Treatment with proteases eliminated antibody binding to all three glycoproteins (Lanes C and E). Periodate reduction did not significantly alter reactivity of the 55-50 kDa glycoprotein with anti-mycobacterial antiserum (not shown). In contrast, the ability of the 38 kDa molecule to bind rabbit antibodies was eliminated by periodate oxidation (Lane F). An antibody response against purified glycoproteins was investigated by immunoblot in sera of patients with pulmonary tuberculosis and in healthy individuals. Antibodies

to the 55 and 50 kDa glycoproteins were found in 7 of 18 tuberculosis sera tested (38.8%) but in none of 7 controls (not shown). The antibody response to the 38 kDa glycoprotein in tuberculosis is the subject of a separate paper (Espitia et al., 1989).

Demonstration of the TB71 and TB72 epitopes on the 38 kDa glycoprotein (Figure 5).

Monoclonal antibodies TB72 and TB71 reacted with the 38 kDa glycoprotein by immunoblot (Lanes A and D, arrow). Binding of TB72 was abolished by proteolytic digestion and by reduction of the blotted antigen with sodium *m*-periodate (Lanes B and C). Binding of TB71 was also abolished by proteolytic digestion (Lane E) but it was not affected by periodate (Lane F).

DISCUSSION

In the present study we demonstrated that M. tuberculosis possessed Con A binding glycoproteins. Indeed, a 38 kDa band and a doublet band of 55-50 kDa reacted specifically with the peroxidase-labelled lectin on blots. These glycoproteins were isolated and high total sugar content demonstrated on both of them. These molecules were shown to react with serum antibodies of hyperimmunized rabbits and of humans with pulmonary tuberculosis. Furthermore, in a separate paper, data suggesting that the 38 kDa glycoprotein is a disease-associated antigen are presented (Espitia et al., 1989).

Information on the chemical nature of Con A binding proteins was sought by digesting samples with proteases and by reduction with sodium *m*-periodate. Digestion resulted in extensive hydrolysis of all three putative glycoproteins, and therefore they were not detectable on Coomasie blue stained gels or on blots using anti-mycobacterial antibodies or Con A-peroxidase. Since periodate oxidation might cleave carbohydrate vicinal hydroxyl groups without altering the structure of polypeptide chain (Woodard, Young & Bloodgood, 1985), this method has been a useful tool in analyzing the contribution of carbohydrates to the structure and properties of glycoproteins (Hamburger et al., 1982; Woodard et al., 1985; Mitchell, Smith & Lehner, 1987). In this study, reduction neither altered the electrophoretic mobility nor the reactivity of glycoproteins with Coomasie blue on gels. Reduction, however, abolished binding to Con A on blots, thus indicating that this property depends upon the presence of sugar moieties.

Because it has been reported for glycoproteins of other pathogens that the carbohydrate chains may represent a major antigenic portion of the molecule (Hamburger et al., 1982; Alexander & Elder 1984; Ramasamy & Reese, 1985; Mitchell et al., 1987), we made a preliminary characterization of the contribution of sugars to the antigenicity of mycobacterial glycoproteins. This was done by examining their ability to react with antibodies after periodate reduction. Reaction of the 55-50 kDa doublet band with rabbit antimycobacterial

antibodies was unaffected, revealing that rabbit's response to this glycoprotein is mainly directed to polypeptide epitopes or to periodate resistant carbohydrate moieties (Woodard et al., 1985). In contrast, reduction of the 38 kDa glycoprotein resulted in a complete loss of binding, thus demonstrating an important contribution of sugar to the antigenicity of this protein. This contribution could be twofold; first, it is possible that carbohydrate themselves are antigenic or, secondly, that they contribute to stabilization of antigenic polypeptide conformations (Ramasamy & Reese, 1985; Alexander & Elder, 1984).

The presence of carbohydrate-dependent epitopes on the 38 kDa antigen was further substantiated by immunoblot using monoclonal antibodies TB71 and TB72. These antibodies, which recognize non-overlapping epitopes on a 38 kDa protein antigen isolated from M. tuberculosis (Young et al., 1986), reacted with the similar weighted glycoprotein herein described indicating they are the same molecule. It is of interest that after periodate reduction, the TB72 epitope was lost while that recognized by TB71 was unchanged. These observations, by demonstrating the presence of carbohydrate-dependent and carbohydrate-independent epitopes in the 38 kDa antigen, give further support to the view that it is a glycoprotein.

Although the above observations are strong indications that the Con A binding molecules identified in M. tuberculosis are

glycoproteins, some considerations are necessary. Glycoproteins in bacteria are rather rare. True glycoproteins, that is to say proteins with covalently attached carbohydrate moieties, have been well-characterized in Halobacterium salinarium (Mescher & Strominger, 1976), and in Bacillus thuringiensis (Pfannensteil, et al., 1987). Less well-characterized glycoproteins have been reported in Streptococcus sanguis (Morris et al., 1987), in Streptomyces albus (Larraga & Muñoz, 1975), in Mixococcus xanthus (Maeba, 1986), and in some bacteria of pathogenic interest such as Escherichia coli (Okuda & Weinbaum, 1968), Treponema pallidum (Moskophidis & Müller, 1984), and Streptococcus mutans (Mitchell et al., 1987). Regarding the presence of glycoproteins in mycobacteria, the information is scarce. A 50 kDa glycoprotein was isolated from Mycobacterium kansasii by lectin affinity chromatography (Ma & Daniel, 1983). In Mycobacterium leprae, antigens of 12 and 33 kDa were considered to be glycoproteins because they reacted with the Schiff's reagent and were sensitive to pepsin (Chakrabarty, Maire & Lambert, 1982).

The results obtained in several of the above-cited studies and in the present work show that, in prokaryotes, there are carbohydrate-protein complexes and that they are probably covalently linked. In the mycobacterial glycoproteins that we have identified, covalent attachment of sugars is strongly suggested because glycoproteins could still be detected by Con A binding after they had been isolated, boiled in sodium

dodecyl sulfate, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. However, further chemical characterization will be necessary in order to determine whether they are true glycoproteins as found in eukaryotic cells or heteropolysaccharide-protein complexes as described in some bacteria (Wu & Heat, 1973). The latter consideration is particularly critical since in the 40-60 kDa zone where the glycoproteins migrate, lipoarabinomannan has been demonstrated in extracts of M. tuberculosis and M. leprae (Gaylord et al., 1987).

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REFERENCES

Alexander, S. & Elder, J.H. (1984) Carbohydrate dramatically influences reactivity of antisera to viral glycoprotein antigen. Science 226, 1328.

Alves, M.J.M., Abuin, G., Kuwajima, V.Y. & Colli, W. (1986) Partial inhibition of trypomastigote entry into cultured mammalian cells by monoclonal antibodies against a surface glycoprotein of Trypanosoma cruzi. Mol. Biochem. Parasitol. 21, 75.

Chakrabarty, A.K., Maire, M.A. & Lambert, P.H. (1982) SDS-PAGE analysis of M. leprae protein antigens reacting with antibodies from sera from lepromatous patients and infected armadillos. Clin. Exp. Immunol. 49, 253.

Dalton, J.P. & Strand, M. (1987) Schistosoma mansoni polypeptides immunogenic in mice vaccinated with radiation attenuated cercariae. J. Immunol. 139, 2474.

Espitia, C., Cervera, I., Gonzales, R. & Mancilla, R. (1989) A 38 kDa Mycobacterium tuberculosis antigen associated with infection. Its isolation and serologic evaluation. Clin. Exp. Immunol.

Gaylord, H., Brennan, P.J., Young, D.E. & Buchanan, T.M. (1987) Most Mycobacterium leprae carbohydrate-reactive monoclonal antibodies are directed to lipoarabinomannan.

Infect. Immun. 55, 2860.

Glass II, W.F., Briggs, R.C. & Hnilica, L.S. (1981) Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose sheets. Anal. Biochem. 115, 219.

Hamburger,J., Lustigman, S., Arap Siongok, T.K., Ouma, J.H. & Mahmoud, A.A.F. (1982) Characterization of a purified glycoprotein from Schistosoma mansoni eggs: specificity, stability and the involvement of carbohydrate and peptide moieties in its serologic activity. J. Immunol. 128, 1864.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London). 227, 680.

Larraga, V. & Muñoz, E. (1975) Molecular organization in bacterial cell membranes. Specific labelling and topological distribution of glycoproteins and proteins in Streptomyces albus membranes. Eur. J. Biochem. 54, 207.

Ma, Y. & Daniel, T.M. (1983) Isolation, characterization and specificity of a glycoprotein antigen from Mycobacterium kansasii. Am. Rev. Respir. Dis. 128, 1059.

Maeba, P.Y. (1986) Isolation of surface glycoproteins from Myxococcus xanthus. J. Bacteriol. 166, 644.

Marshall, R.D. (1972) Glycoproteins. Amer. Rev. Biochem. 41, 673.

Mescher, M.F. & Strominger, J.L. (1976) Purification and characterization of a prokaryotic glycoprotein from the cell envelope of Halobacterium salinarium. J. Biol. Chem. 251, 2005.

Mitchell, C.G., Smith, R. & Lehner, T. (1987) Recognition of carbohydrate and protein epitopes by monoclonal antibodies to a cell wall antigen from Streptococcus mutans. Infect. Immun. 55, 810.

Morris, E.J., Ganeshkumar, N., Song, M. & McBride, B.C. (1987) Identification and preliminary characterization of a Streptococcus sanguis fibrillary glycoprotein. J. Bacteriol. 169, 164.

Moskophidis, M. & Müller, F. (1984) Molecular characterization of glycoprotein antigens on surface of Treponema pallidum: comparison with nonpathogenic Treponema phagedenis biotype Reiter. Infect. Immun. 46, 867.

Neufeld, E.F. & Ashwell, G. (1980) Carbohydrate recognition systems for receptor-mediated pinocytosis. In The Biochemistry of Glycoproteins and Proteoglycans (ed. by William J. Lennarz) p. 241. Plenum Press, New York and London.

Nogueira, N., Unkeless, J. & Cohn, Z. (1982) Specific glycoprotein antigens on the surface of insect and mammalian

stages of Trypanosoma cruzi. Proc. Natl. Acad. Sci. USA. 79, 1259.

Okuda, S. & Weinbaum, G. (1968) An envelope-specific glycoprotein from Escherichia coli B. Biochemistry 7: 2819.

Parekh, B.S., Metha, H.B., West, M.D. & Montecarlo, R.C. (1985) Preparative elution of proteins from nitrocellulose membranes after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal. Biochem. 148, 87.

Pfannensteil, M.A., Muthukumar, G., Couche, G.A. & Nickerson, K.W. (1987) Amino sugars in the glycoprotein from Bacillus thuringiensis subsp. israelensis. J. Bacteriol. 169, 796.

Ramasamy, R. & Reese, R.T. (1985) A role for carbohydrate moieties in the immune response to malaria. J. Immunol. 134, 1952.

Rapp, V.J. & Ross, R.F. (1986) Antibody response of swine to outer membrane components of Hemophilus pleuropneumoniae during infection. Infect. Immun. 54, 751.

Robey, W.G., Arthur, L.O., Matthews, T.J., Langlois, A., Copeland, T.D., Lerche, N.W., Oroszlan, S., Bolognesi, D.P., Gilden, R.V. & Fischinger, P.J. (1986) Prospect for prevention of human immunodefficiency virus infection: purified 120-kDa envelope glycoprotein induces neutralizing antibody. Proc. Natl. Acad. Sci. USA 83, 7023.

Seibert, F.B. (1949) Isolation of three different proteins

and two polysaccharydes from tuberculin by alcohol fractionation: their chemical and biological properties. Am. Rev. Tuberc. 59, 86.

Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350.

Woodard, M.P., Young Jr., W.W. & Bloodgood, R.A. (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Meth. 78, 143.

Wu, M. & Heat, E.C. (1973) Isolation and characterization of lypolysaccharydes protein from Escherichia coli. Proc. Natl. Acad. Sci. USA. 70, 2572.

Young, D., Kent, L., Rees, A., Lamb, J. & Ivanyi, J. (1986) Immunological activity of a 38-Kilodalton protein purified from Mycobacterium tuberculosis. Infect. Immun. 54, 177.

Zacharius, R.M., Zell, T.E., Morrison, J.H. & Woodlock, J.J. (1969) Glycoprotein staining following electrophoresis on acrylamide gels. Anal Biochem 30, 148.

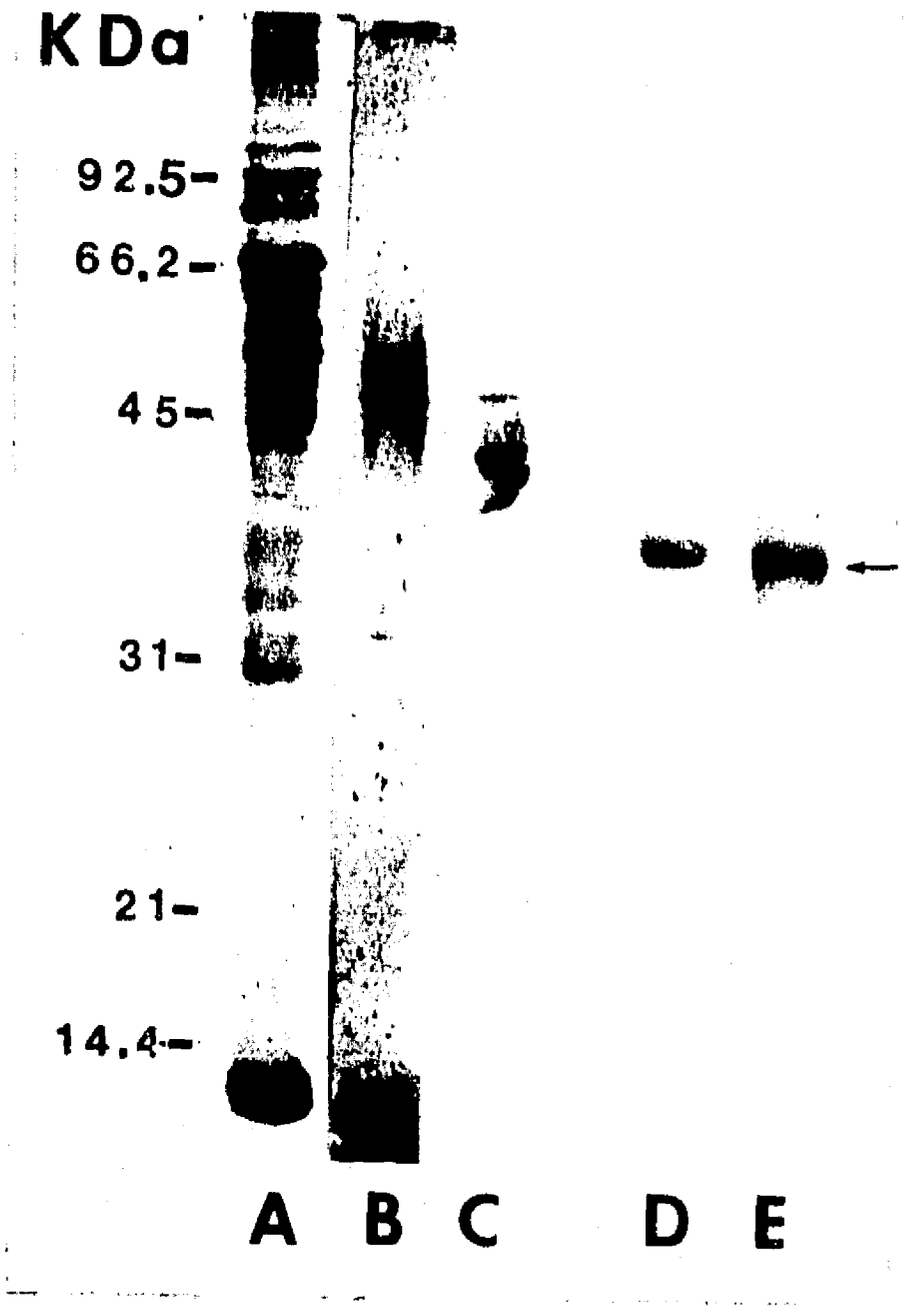

Figure 1.

Identification of glycoproteins among mycobacterial proteins precipitated with 45% $(NH_4)_2SO_4$. Lane A, PAGE-SDS of precipitated proteins; Lane B, Schiff's stain; Lane C, Con A-peroxidase labelling of blotted proteins. Identification of a 38 kDa glycoprotein in the supernatant of culture filtrates precipitated with 45% fractionated by alcohols (Seibert, 1949). In the supernatant obtained after 70 % alcohol precipitation, a 38 kDa band reactive with Coomasie blue (Lane D) and with Con A was observed (Lane E). Molecular weight standards (Bio-Rad) on kDa are indicated on the left.

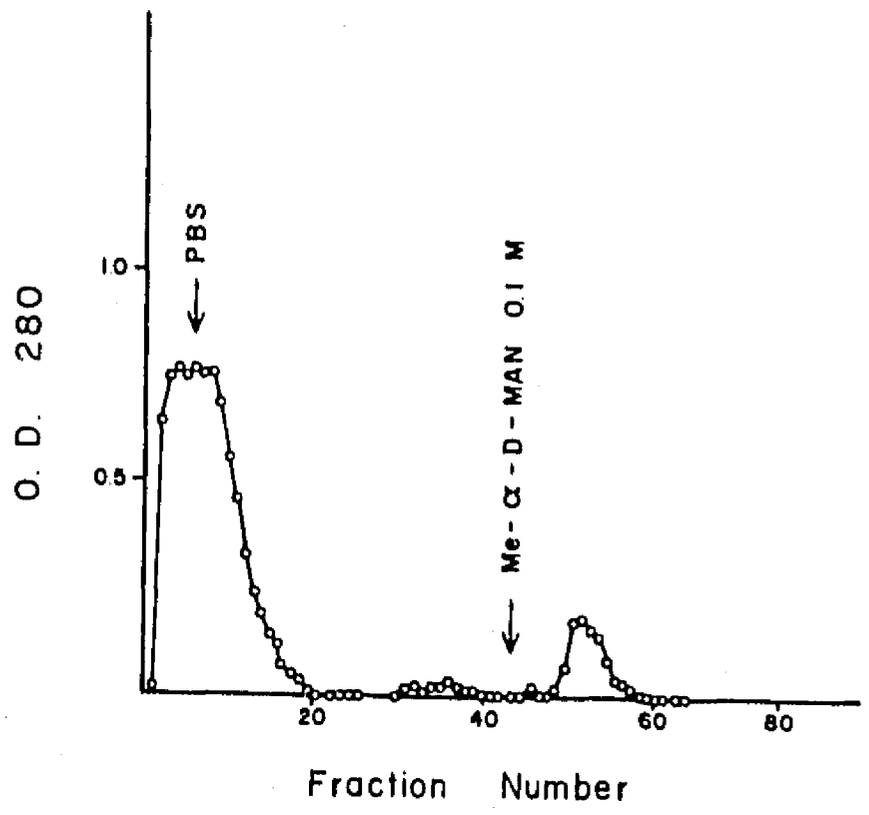

Figure 2

Con A affinity chromatography of 45% $(NH_4)_2SO_4$ precipitated proteins. The specifically bound material (second peak) was eluted with α-methyl D-mannoside 0.01 M (arrow). Unbound material (first peak) was washed out with PBS.

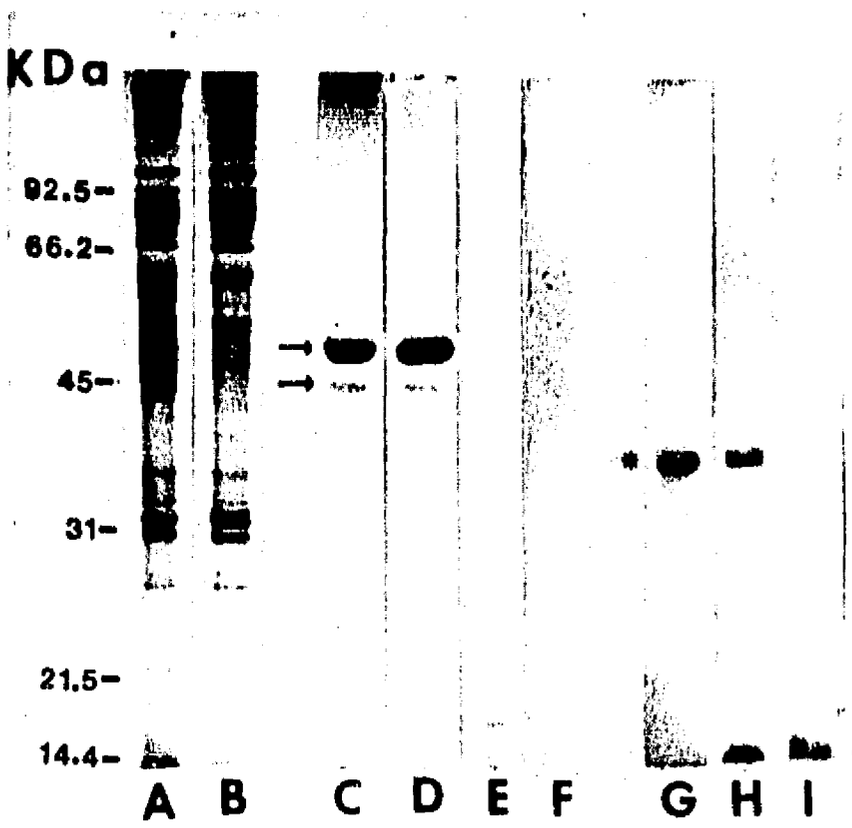

Figure 3.

PAGE-SDS analysis and Con A-peroxidase labelling of purified glycoproteins. Lane A, Coomasie blue staining of myco-bacterial proteins before lectin fractionation. Lane B, Coomasie blue staining of proteins which failed to bind to the Con A column. Lane C, Coomasie blue staining of the material eluted from the Con A column with α-methyl D-mannoside. Lane D, Con A-peroxidase labelling of glyco-proteins eluted from the Con A column; labelling was eliminated with α-metyl D-mannoside (Lane E) and by reduction with sodium m-periodate (Lane F). Arrows indicate the 55 and 50 kDa bands. Coomasie blue staining (Lane G, asterisk) and Con A-peroxidase labelling (Lane H) of the material eluted from nitrocellulose membranes with acetonitrile. Con A binding was abolished by α-methyl D-mannoside (Lane I) and by reduction (Lane J).

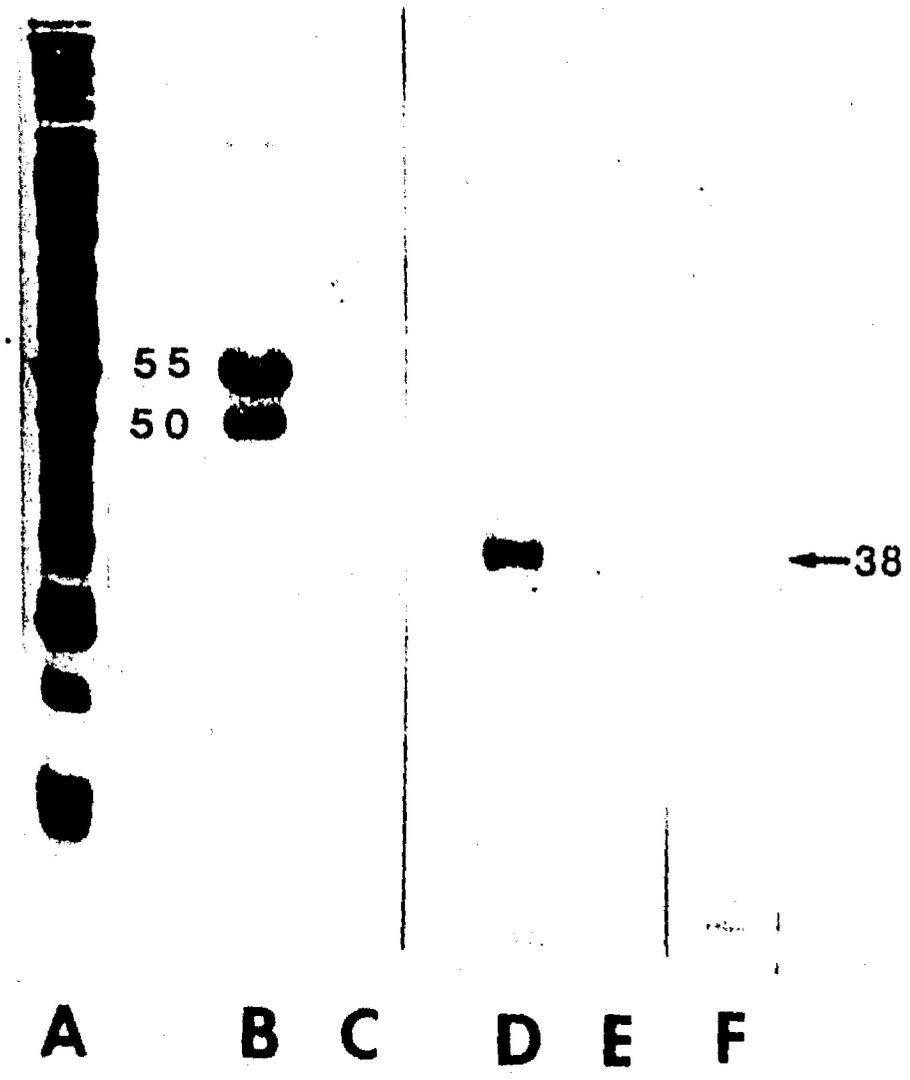

Figure 4.

Immunoblot of purified glycoproteins with anti-*M. tuberculosis* rabbit antiserum and the effects of proteolytic digestion and periodate reduction on antibody binding. Lane A, untreated whole mycobacterial antigens. Lane B, untreated glycoproteins purified with Con A. Lane C, glycoproteins of Lane B digested with proteases; the positions of the 55 and 50 kDa bands are indicated. Lane D, reaction of rabbit antibodies with the untreated 38 kDa glycoprotein (arrow) isolated by elution from nitrocellulose sheets. Lane E, immunoblot of the purified 38 kDa glycoprotein after digestion with proteases (Lane E) and after periodate reduction (Lane F).

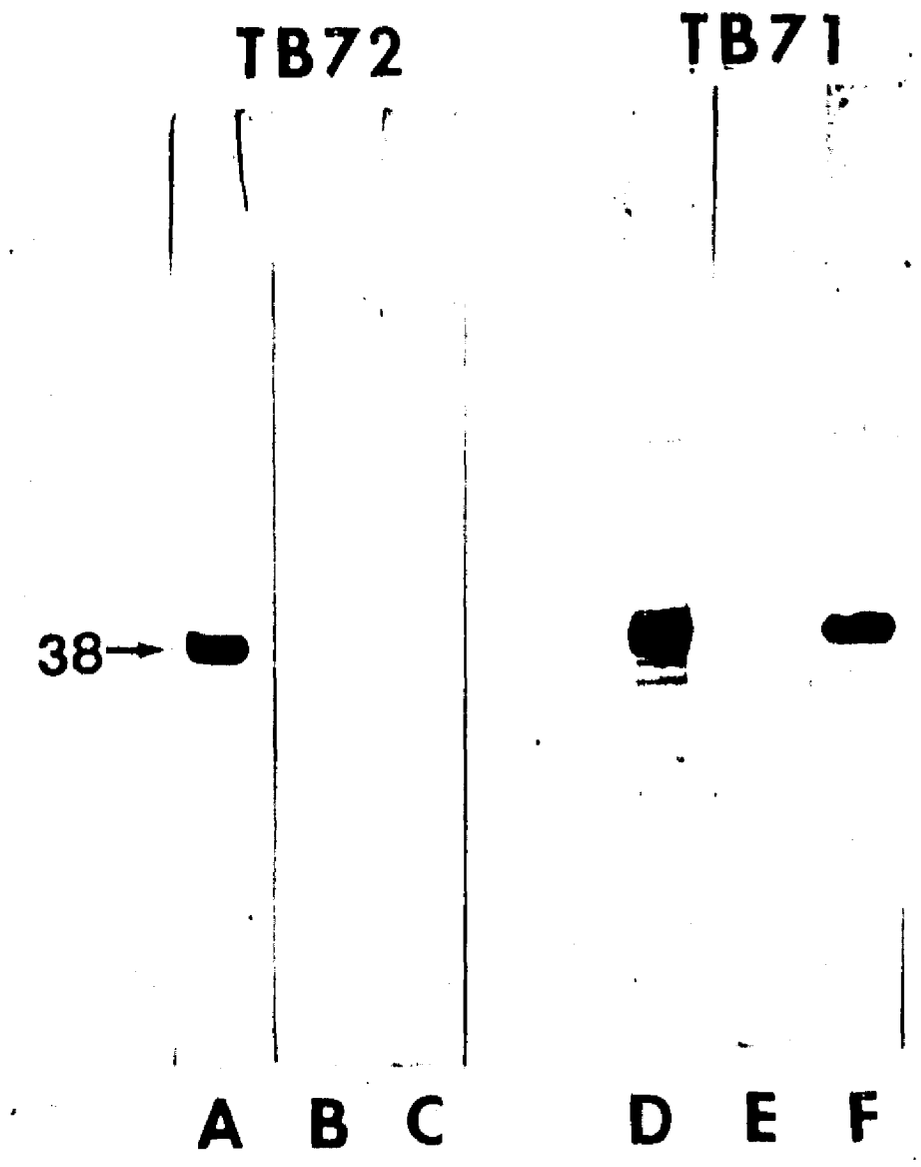

Figure 5.

Binding of monoclonal antibodies TB72 and TB71 to the 38 kDa glycoprotein and the effects of digestion with proteases and periodate reduction. Lanes A and D, untreated glycoprotein. Lanes B and E, after digestion. Lanes C and F, after reduction. Digestion and reduction were carried out after transfer of the 38 kDa glycoprotein onto nitrocellulose.

IV. DISCUSION GENERAL

La identificación, aislamiento y caracterización de antígenos de M. tuberculosis ha sido una de las áreas prioritarias de la investigación en tuberculosis. Los diferentes trabajos realizados en este campo han tenido como objetivo principal la obtención de antígenos, los cuales podrían ser de gran utilidad para el estudio de la compleja interacción huésped-bacteria y como reactivos de inmunodiagnóstico e inmunoprofilaxis.

Las estrategias para llevar a cabo estos objetivos pueden ubicarse en dos fases; en la primera donde se situan los trabajos de esta tesis, se intenta identificar y caracterizar componentes micobacterianos biológicamente relevantes mediante la utilización de la metodología apropiada para cada caso en particular. La siguiente fase consiste en desarrollar métodos que permitan obtener antígenos en cantidad suficiente para estudios más amplios. Esto último se puede lograr con la síntesis química de antígenos individuales seleccionados o de epítopes importantes, lo cual ofrece ventajas indudables sobre el cultivo masivo de bacterias. La aplicación de esta metodología permitiría además la síntesis de compuestos con epítopes provenientes de diferentes moléculas nativas, lo cual podría incrementar las posibilidades de obtener reactivos útiles.

Otra estrategia para obtener antígenos en cantidades importantes es la tecnología del DNA recombinante, la cual se introdujo al estudio de las micobacterias hace 5 años (43). A pesar

estos sistemas es mínimo comparado con la gran diversidad de antígenos presentes en las micobacterias. Además de esta, existen otras limitaciones importantes como son el hecho de que las proteínas expresadas pudieran no necesariamente ser los antígenos involucrados en protección o ser útiles para el inmunodiagnóstico. En las moléculas recombinantes podrian además estar ausentes porciones funcionales o ser imposible la expresión de constituyentes importantes no proteicos. Es necesario tener en mente las consideraciones anteriores con el fin de buscar estrategias alternativas.

Los trabajos 2 y 3 de esta tesis se centraron en la identificación y purificación de antígenos individuales ya que creemos que su uso podría se superior al de mezclas antigénicas complejas. Algunas de las ventajas son la siguientes: (a) El control de calidad de un antígeno individual es más simple, (b) se reduce la posiblidad de inducir respuestas inmunes no deseables o reacciones tóxicas (c) la evaluación de antígenos individuales es de más fácil interpretación, siendo posible identificar las causas de su éxito o fracaso.

Un primer paso en nuestra investigación, fué analizar la composición antigénica de los extractos de M. tuberculosis de acuerdo al tiempo de cultivo y método de obtención y posteriormente definir algunos antígenos potencialmente significativos, en base a sus características inmunológicas y a su composición bioquímica.

Los resultados de este análisis indicaron la necesidad de de homogenizar las fuentes de antígeno a utilizar. En este trabajo únicamente demostramos diferencias antigénicas cuantitativas debidas a dos variables, sin embargo, existen otros factores que podrían contribuir a la heterogeneidad de los extractos, como por ejemplo: el tamaño y viabilidad del inoculo inicial en la resiembra, el número de pases in vitro y la estabilidad de las preparaciones crudas.

Con el fin de definir antígenos potencialmente significativos, estudiamos la respuesta inmune humoral a proteínas micobacterianas por inmunoblot utilizando sueros de enfermos tuberculosos e individuos sanos. Esto nos llevo a la identificación de proteínas de diferentes pesos moleculares altamente inmunogénicas, destacando entre ellas una de 38 kDa cuyo reconocimiento parece marcar un estado de infección por M. tuberculosis. En vista de los resultados, se purificó este antígeno y se probó en un inmunoensayo. Los resultados utilizando extracto total y la proteína pura fueron similares en especificidad y sensibilidad, existiendo una buena correspondencia en los valores de densidad optica utilizando una u otra preparación antigénica.

El análisis de la proteina de 38 kDa incluyó solamente grupos polares de sujetos enfermos y sanos. Es necesario analizar la influencia de otros factores como son la extensión de la enfermedad, la presencia de hipersensibilidad retardada al PPD, vacunación con BCG, el tratamiento etc., antes de poder establecer el valor real de este antígeno como reactivo de serodiagnóstico.

Una característica interesante de esta proteína es la presencia de manosa en su composición, la cual fue detectada por su capacidad de unirse a Con A. La unión a la lectina, ocurrió únicamente cuando la molécula habia sido transferida a papel de nitrocelulosa siendo infructuosos todos los intentos para obtenerla por cromatografía de afinidad con Con A. Esta conducta paradójica podría deberse a que en el procedimiento de electroforesis y transferencia se exponen sitios reactivos los cuales se encuentran ocultos en la molécula en solución. Este mismo comportamiento hacia Con A ha sido descrito para lipoarabinomanan (69).

La identificación de glicoproteínas en este trabajo es una observación original, pues proteínas con estas características no se habian identificado antes en M. tuberculosis y en bacterias en general solo en unas cuantas especies (70,71,72,73). Por otra parte existen observaciones que indican que esta moléculas son muy importantes en fenómenos de comunicación intercelular y que pueden ser muy inmunogénicas (74,75).

La presencia de manosa en las glicoproteínas micobacterianas que hemos estudiado, hace que sea de interés estudiar la participación de estas moléculas en el reconocimiento e internalización de los bacilos por las células fagocíticas, ya que se han descrito receptores para dicho azúcar en macrófagos alveolares y se ha observado que estos son capaces de fagocitar partículas y paredes celulares de levaduras ricas en manosa (76,77,78,79,80).

Por último y como una perspectiva de trabajo a realizar, planeamos evaluar la participación de los antígenos de M. tuberculosis purificados en el desarrollo de inmunidad celular, lo que podría conducir a la identificación de epítopes T capaces de inducir una respuesta inmune protectora.