ANTIGENIC RELATIONSHIP BEWEEN THE HANSEN BACILLUS AND OTHER MYCOBACTERIA

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Modern agar-diffusion techniques and immunoelectrophoresis are being used widely in the study of the mycobacteria. Some authors have used them to study the antigenic relationships between the different pathogenic and nonpathogenic mycobacteria ([1, 3, 7, 8]). Others have endeavored to devise sensitive serologic tests for diseases caused by such bacteria, since the old techniques do not have enough sensitivity to be of any practical value. In the reports published on these techniques applied to mycobacteria there has been very little concerned with the leprosy bacillus. This is easily understood, considering the lack of growth of the leprosy bacillus in artificial media, and its inability to infect laboratory animals, for which reasons any work in connection with it is extremely difficult.

The main purpose in introducing the gel-precipitation technique in our research was to obtain a serologic test for leprosy sufficiently sensitive to be applicable in clinical work. The difficulty of obtaining a pure antigen from leprosy material has, in the meantime, led us to test also antigens prepared from filtrates of cultures of various other mycobacteria, with which we were able to study certain aspects of the antigenetic relationships between *Mycobacterium leprae* and other pathogenic and nonpathogenic mycobacteria.

The present report deals only with the sera of rabbits immunized with the leprosy bacillus. Observations on human sera are in progress, and will be reported in the future.

MATERIALS

1. *Extract of the leprosy bacillus.*—A leproma was removed aseptically from an untreated lepromatous patient. After trituration by hand in a mortar, and in a Pfizer homogenizer at a low temperature, the suspension was centrifuged at 3,000 r.p.m. for 15 minutes in order to eliminate all the tissue debris. To the supernatant, which was very rich in bacilli, washed and sterilized sand was then added and the mixture was placed in a Mickle tissue disintegrator for 15 minutes ([6]). In this way the bacilli were almost entirely disintegrated. After centrifugation the clear extract, free from bacillary debris, was kept in a refrigerator.

2. *Antibacillus immune serum.*—The extract of the leprosy bacillus was mixed with Freund's adjuvant and inoculated into an adult rabbit in the following manner: subcutaneously 0.5 cc. in the right and left scapular regions, and 0.5 cc. in the right and left dorsal regions; intramuscularly, 0.5 cc. in the buttocks; and, finally, 0.3 cc. into the peritoneal cavity. After 4 weeks a booster effect was induced by injecting intradermally 0.3 cc. of the bacillus extract without the Freund adjuvant. After another week the rabbit was bled and the immune antiserum against the leprosy bacillus extract was separated.

3. *Mycobacterial antigens.*—Cultures were made in Youmans' liquid medium ([10]) of the following mycobacteria:

1. *M. tuberculosis bovis* (BCG)
2. *M. tuberculosis* H37Rv
3. *M. marianum*
4. *M. balnei*
5. *M. battey*
6. *M. butyricum*
7. *M. kansasii*
8. *M. marinum*
9. *M. platipoecillus*
10. *M. thamnopheos*

After 8 weeks the growths were abundant, and the cultures were filtered by the Seitz filter. The filtrates were dialyzed, concentrated by evaporation to one-tenth volume, and kept in a refrigerator at 4°C.

The antigens thus prepared were tested as to their content of proteins and carbohydrates. A total absence of proteins was ascertained by the biuret reagent and nitric acid. With reference to the carbohydrates, these were determined by the anthrone reagent ([5]). The amount of the carbohydrates present was not the same in all the antigens, this depending—at least partially—upon the rapidity of the growth of each particular culture in the medium used. The amounts encountered were compared with an equimolar mixture of galactose and mannose, and in Table 1 the results are expressed in mgm. per 100 cc. of antigen.

4. *Method of the agar double-diffusion test.*—For this test we used tubes of 7 mm. inside diameter, 12 cm. long. The inner wall of the tubes was previously coated with a fine film of agar by immersing them in melted agar, then draining and drying them. The agar used was from Difco, washed in distilled water and dissolved in the proportion of 1.5 per cent in physiologic serum containing 0.1 per cent of sodium azide.

Technique: (1) The melted agar was placed in a water bath at 48°C. The dilutions of antigens were then made with the proper agar in the proportion of 1:10, 1:20 and 1:40. One cc. of each antigen thus diluted was placed at the bottom of the tubes without touching the walls.

(2) When the bottom layer had hardened, another one was made by placing 0.4 cc. of pure agar over it. This is the reaction layer.

(3) When in turn this layer had hardened, 1 cc. of the immune rabbit serum (anti-leprosy bacillus extract), diluted 1:2, was added. The serum also contains 0.1 per cent of sodium azide, to avoid contamination.

(4) The tubes were placed in an incubator at 37°C.

RESULTS

(1) After 48 hours of incubation, bands of precipitation will have appeared as the result of the reaction between the leprosy-bacillus extract and the immune antiserum against that same extract.

(2) The same anti-leprosy-bacillus-extract rabbit serum also reacted in varying degrees with all the antigens prepared from the filtrates of the ten mycobacteria listed above (see Table 1).

(3) The number of precipitation bands and their intensity was not the same in all cases.

Having ascertained the nonprotein nature of the antigens, a test was made with the same antigens after they had been autoclaved for 20 minutes at 1 atmosphere. The results were identical with the previous ones, both in intensity and in the number of bands. The correspondence between the carbohydrate level, determined by anthrone, and the inten-

TABLE 1.—*Comparative table showing the intensity of reaction in the agar-diffusion test and the level of polysaccharides of the antigens.*

Antigen	Agar diffusion in 48 hours[a]	Polysaccharides (mgm./%)
M. thamnopheos	+1	8.50
M. battey	+1	12.50
M. tuberculosis bovis (BCG)	+3	19.25
M. kansasii	+2	22.25
M. balnei	+3 +3	27.00
M. butyricum	+3 +3	32.50
M. marinum	+3 +3	33.00
M. tuberculosis H37Rv	+3 +3	62.00
M. marianum	+3 +3	84.00
M. platipoecillus	+3 +3	200.00
M. leprae	+3 +3	([b])

[a] Each plus mark (+) means a band of precipitation, and the number means the intensity of the band: 1-weak, 2-medium, 3-strong.

[b] Reference antigen, for which the polysaccharide determination was not made because of the tissue element, which problably contained such substance.

sity of the reaction between antigen and antibody in the agar-diffusion tubes, was confirmed. This fact proves the polysaccharide nature of the antigen used.

DISCUSSION

Without any doubt, there exists an antigenic relationship among the different mycobacteria. This fact has been amply demonstrated by various authors who tested antigens extracted from the mycobacteria themselves, or the filtrate of cultures in liquid media, against antisera obtained by inoculation of tuberculosis bacillus in rabbits. This present work does no more than confirm earlier reports, with the difference that we used antibodies obtained from an animal immunized by inoculation of an extract of the leprosy bacillus.

A difficulty arises, however, in the interpretation of the results from the quantitative point of view. In this connection we have to consider: (*a*) the intensity of the reaction, as indicated by the thickness of the precipitation band, and (*b*) the number of bands appearing and their significance.

With respect to the thickness of the precipitation band, this naturally depends upon the quantity of antigens and antibodies which enter into the reaction. However, we know that it is necessary that the quantities of both the antigen and antibodies be proportional, in order that the reaction may be visible in the form of a band of precipitation. A greater concentration of the antigen in relation to the antibody, or vice versa, causes the precipitate to redissolve and makes the reaction invisible. In our experiments we found that the rabbit antiserum produced by inoculation of the leprosy bacillus extract required dilution of the anti-

gens used in the proportions of 1:10, and 1:40. But, although the antigens were all prepared after the same length of time of incubation of the various bacilli on their media, i.e., after 8 weeks, the quantities of antigenic substances produced by the metabolic exchange between the germs and the media were naturally not identical for all of the mycobacteria cultured. This is why the precipitation reactions with the dilutions mentioned were not of the same intensity and were not the same in all of the antigen-antibody systems employed. It was even necessary, in some cases, to use more dilute antigens, as it also was necessary in other cases to use the antigens in greater concentration, to make the bands of precipitation of sufficient intensity for interpretation.

With respect to the number of precipitation bands our experience has shown the following facts: (*a*) in certain antigen-antibody systems there appear, from the first moment of visibility of the reaction, more than one band of precipitation whereas in others only one appears; (*b*) after a period of time, leaving the tubes at room temperature, in all of the systems there successively appear other bands of precipitation, always below those that appeared in the beginning.

These observations are in accord with the classical reports and theoretic explanations ([2, 4]) which are not discussed in this study, although we are gathering particular technical details for application in other investigations in progress.

The period of 48 hours for incubation of the reactions was considered optimal for this experiment, in view of observations made while using dilutions of the antigens and reading after varying periods of time.

According to our interpretation, only the bands which appear simultaneously at the beginning can, with certainty, be considered as representing the different antigenic fractions contained in the same antigen. The bands appearing later are, in our opinion, the results of the oversupply of antibodies which, diffusing beyond the precipitate, reacts with the antigen in the lower layers. We found that, in cases in which the initial precipitate is very dense because of the strong concentration of the antigen, these lower bands are not formed, undoubtedly because of the difficulty of diffusion through the precipitate; as they also are not formed when the antigen, because very dilute, is exhausted at the first contact with the antibody. There exists, however, a concentration which is ideal for successive formation of bands, which may be as many as 7, perfectly individualized, reaching even the lowest level of the agar in which antigen was diluted.

In our experience the most concentrated antigens were those obtained from the cultures of *M. tuberculosis* H37Rv, *M. balnei, M. marianum, M. platipoecillus, M. butyricum, M. marinum* and *M. tuberculosis bovis* (BCG). The filtrates of *M. battey, M. kansasii* and *M. tham-*

nopheos were poorer in antigens that reacted with leprosy bacillus anti-extract serum.

The antigens which gave two bands of precipitation simultaneously from the beginning were obtained from the following filtrates: *M. tuberculosis* H37Rv, *M. balnei, M. marianum* and *M. marinum.* The others—*M. battey, M. kansasii, M. butyricum, M. platipoecillus, M. thamnopheos* and *M. tuberculosis bovis* (BCG)—gave only one precipitation band.

SUMMARY

Circulating antibodies from rabbits inoculated with the leprosy bacillus were tested by agar double-diffusion technique against antigens of pathogenic and nonpathogenic mycobateria cultivated in synthetic Youmans' medium (*M. tuberculosis bovis* (BCG), *M. tuberculosis* H37Rv, M. *marianum, M. balnei, M. battey, M. butyricum, M. kanasii, M. marinum, M. platipoecillus, M. thamnopheos*).

Such antigens have been proved not to contain even negligible amounts of proteins as revealed by biuret and nitric acid tests. However, they proved to contain a great deal of polysaccharides as titrated by anthrone reagent, in comparison with an equimolar solution of galactose and mannose.

The amount of polysaccharides seems to be related with the growth of the mycobacteria under the conditions of the present work.

The agar double diffusion reaction has been positive with all of the mycobacteria-antigens used. There is closed interrelationship between the carbohydrate content of the antigens and the intensity of the antigen-antibody reaction. This seems to prove the polysaccharidic constitution of the antigens and the antigenic relationship between such mycobacteria and the leprosy bacillus.

The authors comment about the technique, the methods for evaluation results, and the possible utilization of such techniques for clinical purposes.

RESUMEN

Con la técnica de doble difusión de agar se comprobaron anticuerpos circulantes procedentes de conejos inoculados con el bacilo de Hansen contra antígenos de microbacterias patógenas y anapatógenas cultivadas en el medio sintético de Youmans (*M. tuberculosis bovis* (BCG), *M. tuberculosis H37Rv, M. marianum, M. balnei, M. battey, M. butyricum, M. kansasii, M. marinum, M. platipoecillus, M. thamnopheos*).

Quedó demonstrado que dichos antígenos no contienen ni siquiera cantidades menospreciables de proteínas según revlaron las reacciones del biuret y del ácido nítrico. Sin embargo, as demostró que contenían una gran porción de polisacáridos según valoró el reactive de antrona, comparado con una solución equimolar de galactosa manosa.

La cantidad de polisacáridos parece relacionarse con la proliferación de las micobacterias, en las condiciones del estudio actual.

La reacción de doble difusión de agar se mostró positiva con todos los antígenos micobacterianos usados. Existe una interrelación más íntima entre el contenido hidrocar-

bonado de los antígenos y la intensidad de la reacción de antígeno-anticuerpo. Esto parece demonstrar la composición polisacarídica de los antígenos y la relación antigénica entre dichas micobacterias y el bacilo de Hansen.

Los AA. comentan la técnica, los métodos usados para justipreciar los resultados y la posible utilización de esas técnicas para fines clínicos.

RESUMÉ

Les anticorps circulants de lapins inoculés avec le bacille de Hansen ont été testés, par la méthode de double diffusion sur agar, contre les antigênes de mycobactéries pathogénes et non pathogénes cultivées sur milieu synthétique de Youmans (*M. tuberculosis bovis* (BCG), *M. tuberculosis* H37Rv, *M.marianum, M. balnei, M. battey, M. butyricum, M. kansasii, M. marinum, M. platipoecillus, M. thamnopheos*).

Il été prové que de tels antigènes ne contiennent pas de protéines, même en quantités négligeables telles que peuvent les déceler les mêthodes au biuret et à l'acide ntirique. Par contre, ils se sont révélés contenir une fraction importante de polysaccharides, par rapport à une solution équimolaire de galactose et de mannose, lorsqu'on utilise l'anthrone comme réactif de titration.

La quantité de polysaccharides semble être en relation avec la croissance des mycobactéries telle qu'elle a été réalisée dans cette étude.

La réaction de double diffusion sur agar a été positive pour tous les antigènes mycobactériens utilisés. La relation ets plus étroite entre le contenu en hydrates de carbones des antigènes et l'intensité de la réaction antigénes-anticorps. Ceci semble démontrer la constitution polysaccharidique des antigénes et la relation antigênique de ces mycobactéries avec le bacille de Hansen.

Les auteurs discutent la technique et les méthodes d'appréciation des resultats, ainsi que l'emploi possible de telles techniques à des fins cliniques.

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