COMPLEMENT-FIXING ANTIBODIES IN THE SERA OF 534 LEPROMATOUS LEPERS UNDER TREATMENT WITH SULFONES

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Complement-fixing antibodies have been reported in leprous sera tests using antigens prepared from acid-fast bacteria (2-5, 7-9). It was found that the distribution of these antibodies is related to the activity of the infection, the highest incidence of strong, positive reactions occurring among lepromatous lepers (3-5, 7-8).

Regressive tuberculoid forms, from cases of lepromatous leprosy, present a moderate degree of reactivity in complement fixation tests with tubercle bacilli extracts (11, 13).

Since sulfone treatment resulted in a marked improvement in most lepromatous patients, it was highly desirable to investigate the level of antibodies in their sera. A more precise technic for antibody titration was employed and the results obtained in 534 lepers, in different stages of treatment, are presented.

TECHNIC

The methods employed in the titrations of antigen and serum are essentially those developed by WADSWORTH at al(16) in their studies on tuberculous sera. All reactions were evaluated in terms of the amount of complement required for 50-per-cent hemolysis under the conditions of the test; the preliminary incubation for fixation is 90 minutes in a 37°C water-bath and 15 minutes is allowed for hemolysis. Reagents are prepared as follows:

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ANTIGEN*

The aqueous extract of tubercle bacilli, prepared essentially by previously described methods (15), is precipitated at pH 4.0 in the cold. The precipitate collected by centrifugation is dissolved in water, keeping the volume within 10 to 15 fold concentration of crude extract; pH is adjusted to 8.0. The opalescent solution is shaken for 10 minutes with 2/3 its volume of a 5:1 mixture of chloroform and n-butanol. The treated solution is centrifuged at 5000 r. p. m. for 40 minutes and the supernate dialysed against distilled water; mertiolate (1:30000) is added and the dyalysate, lyophilized. The dry antigen is then extracted with alcohol-ether (1:1) mixture, at room temperature, dried again and dissolved in distilled water. Solution is not complete and insoluble material is removed by centrifugation. The clear, yellow solution is the antigen. It is stored in the cold in small capped flasks and heated at 56°C for 30 minutes before using.

SERUM

Blood was collected and centrifuged after clotting at room temperature; the clear serum is transfered to a tube, capped, and frozen at-30°C. On the day the test is to be performed, the serum is thawed at room temperature and inactivated in a water-bath at 37°C for one half hour.

COMPLEMENT

Sera from 30 male guinea-pigs were pretested individually to exclude those showing fixation with tubercle bacilli extract, in absence of immune serum. The approved sera were then pooled and stored in sealed tubes at -30°C. Complement was titrated according to the quantitative technic described elsewhere (1,15). Dilutions of complement was prepared to contain 6 units in 0.1 ml. After five or 10 minutes dilution is prepared to contain one unit, by adding to one part of the dilution containing 6 units five parts of borate-saline. Dilutions were checked, as indicated (15).

Complement dilutions are kept cold.

SHEEP CELLS

Sheep blood is collected and preserved in an equal volume of modified Alsever's solution (6). Blood cells are washed with saline;

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* The preparation of the antigen used in this work was done by one of us (C. P.) under the direction of Dr. Pangborn([12]) at N. Y. State Dep. Health, Albany, N. Y.

three washings are usually sufficient. After the final washing a 5% suspension is prepared by adding salt solution to the packed cells. The accuracy of the suspension is checked by spectrophotometric analysis of an aliquot of the lysed cells. In a Coleman Junior spectrophotometer, the 5% suspension diluted 1 :15 in distilled water has an optical density about 0.6 at wave-length 545 mu in 12 mm X 75 mm tubes.

ANTISHEEP AMBOCEPTOR

Rabbits are immunized by the technic of Ulrich & McArthur (14) with some minor modification (1). After animals are bled out, the blood is left overnight at 3° — 6°C ; serum is removed, centrifuged and preserved with an equal volume of CP glycerol.

DILUENT

A borate-buffered sodium chloride solution (15), pH 7.6-7.8, was used for dilutions of all reagents.

PROCEDURE - ANTIGEN TITRATION

Varying dilutions of antigen were tested with different amounts of leprous serum in the presence of 6 units of complement. The reaction mixture, in a total volume of 0.3 ml, comprised, in addtion to the antigen dilution (0.1 ml), 0,05 ml of serum dilution and 6 units of complement (contained in 0.1 ml). The volume was completed by adding borate salt solution. After 90 minutes of incubation at 37°C, 0.2 ml of maximally sensitized sheep cells (15) were added to all tubes. Hemolysis was carried out at same temperature during 15 minutes. One ml of cold saline is added to each tube with partial hemolysis; the reaction tubes are centrifuged and the degree of hemolysis is determined in supernates by the aid of the spectrophotometer (1) .

Table I showns the results obtained in an antigen titration; there was no significant change in complement fixation when antigen concentration was increased from 1:100 to 1:28.5. More concentrated dilutions proved to be somewhat anticomplementary, in absence of serum. Dilutions of leprous serum were prepared to give reaction with six units of complement leaving some complement free to produce partial hemolysis. The amount of antigen used in this investigation was 0.1 ml of a 1:40 dilution tubercle bacilli extract previously made isotonic by adding the required amount of sodium chloride.

SERUM TITRATION

In the test proper, reagents were pipeted in the order indicated in table II. In each set of reactions, controls were included to evaluate the reactivity of antigen preparation with known reacting leprous serum and sera from normal patients. Complement, antigen, and serum controls were performed in the presence of six units of complement; before sensitized sheep cells were added, aliquots were taken and checked two contain one, two, and three units of complement. Evaluate the results by using table III. An example of titer determination is presented in table IV.

RESULTS

Quantitative complement fixation tests were performed in 534 cases of lepromatous leprosy, classified in three major groups.

Group I. Seventy-four cases of untreated leprosy, recently arrived at Sanatorio Padre Bento (Gopouva, São Paulo). All of them showed Hansen bacilli in cutaneous lesions and in the nasal mucous; 19 % presented titers less than 10;51% had titers between 10 and 100 and 30% ad titers higher than 100.

Group II. Two hundred and seventeen patients under sul-phone treatment for varying periods of time. Bacteriological smears from nasal mucosa failled to show acid fast bacilli ; however, they could be demonstrated in the cutaneous lesions of all lepers of this group. Complement fixation titers were distributed as follows : 33% less than 10; 52% between 10 and 100 and 15% higher than 100.

Group III. This group includes 243 lepers, treated by sulphone and has shown consistent negative smears from lesion and mucous. Clinical conditions were improved in such a way, that these patients were released from colony as "cured"; however they continue to receive treatment under the responsability of the Departamento de Profilaxia da Lepra. In this group titers less than 10 occurred in 70%; 27% had titers between 10 and 100 but only 3% had titers higher than 100. One patient with high titer was a relapsing case and was sent back to the colony.

The data suggest that activity of leprous infection in most of the studied cases was related to the presence of fixing antibodies in the blood. Individual titration in blood collected during the time the treatment is given can be valuable in determining the reduction of antibodies by effect of treatment. It will very desirable to have a serological method for leprosy, similar to the Wassermann test for syphilis.

In all patients studied in this survey, tuberculosis and kala-azar were ruled out, since these infections give intense reactions with

tubercle bacilli extract, in complement fixation tests (5,10). We could not exclude from these groups patients with Chagas' disease. Sera from Chagas' disease can present reaction with tubercle antigen. The interpretation of CF titers, obtained by using such an antigen with sera from countries where these diseases are prevalent, should be done with caution.

SUMMARY

Complement fixing antibodies were titrated in sera from 534 lepers; 74 were recent arrivals in the hospital; all of them had bacilli in lesions and mucous; 19% presented titers less than 10; 51% had titers between 10 and 100 and 30% had titers higher than 100. The second group included 217 patients under treatment; titers were distributed as follows; 33% less than 10;52% between 10 and 100 and 15% higher than 100. The third group was comprised of 243 *cured* cases; titers less than 10 occurred in 70%; 27% had titers between 10 and 100 and 3% was higher than 100.

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RESUMO

Reações de fixação de complemento, com antígeno preparado de bacilos da tuberculose, foram praticadas em soros de 534 leprosos. A reatividade sérica foi medida em unidades de complemento necessárias para 50% de hemólise, quando 0,05 ml de sôro reagia com antígeno diluído para dar reações de máxima intensidade. O título do sôro foi determinado empregando seis unidades de complemento e partes alíquotas de sôro. Considerando haver proporção entre complemento necessário para 50% de hemólise e a quantidade de sôro presente na reação, o título foi determinado com sôro diluído, em presença de antígeno e seis unidades de complemento.

Os pacientes puderam ser classificados em três grupos distintos: o primeiro grupo compreendia 74 leprosos recentemente internados no Sanatório Padre Bento (Gopouva, São Paulo), com baciloscopia positiva nas lesões e no muco nasal; neste grupo sõmente 19% apresentaram títulos menores que 10, 51% dêles estavam entre 10 e 100, e 30% eram maiores de 100. O segundo grupo compunha se de 217 pacientes submetidos a tratamento por sulfonas, com baciloscopia positiva apenas nas lesões; neste grupo 33% dos títulos eram menores que 10;52% estavam entre 10 e 100 e 15% eram maiores que 100. O terceiro grupo era formado de doentes em alta, mas ainda sob tratamento, apresentando baciloscopia negativa nas lesões e no muco nasal. A maior percentagem de títulos menores que 10, totalizando 70% dos 243 doentes examinados, caracterizou êste grupo; títulos maiores que 100 em apenas *3%* e títulos entre 10 e 100 compreenderam 27% dos casos.

Nos grupos examinados, tuberculose e calazar foram excluídos clinicamente, pois soros de pacientes com essas infecções costumam reagir intensamente com antígeno tuberculoso, em testes de fixação do complemento. Soros chagásicos podem também reagir com antígeno preparado de bacilo da tuberculose e nesses grupos doença de Chagas não pôde ser excluída clinicamente. Em alguns casos, portanto, os títulos podem ser a soma da reação específica

(de lepra) com a reação cruzada (de doença de Chagas). Os dados apresentados sugerem uma relação entre atividade do processo leproso e o nível dos anticorpos fixadores de complemento e aconselham o levantamento de curvas sorológicas individuais como método de avaliação do tratamento sulfônico.

SUMMARIO IN INTERLINGUA

Anticorpores complemento-fixante esseva titrate in le seros de 534 leprosos. Septanta-quatro habeva recentemente arrivate al hospital, e omnes habeva bacillos de Hansen in cutanee lesiones e in muco nasal. In iste grupo, 19% habeva titros de minus que 10;61% habeva titros inter 10 e 100; e 30% habeva titros de plus que 100. Un secunde gruppo includeva 217 leprosos sub tractamento per sulfon. In iste gruppo, le titros esseva distribuite como seque: 33% - minus que 10;62%a - inter 10 e 100; e 15% - plus que 100. Un tertie gruppo comprendeva 243 patients sub tractamento e monstrante constantemente resultados negative in examines bacteriologic de lesiones cutanee e de muco nasal. Titros de minus que 10 esseva trovate in 70% de iste gruppo; 27% habeva titros de inter 10 e 100; e 3% habeva titros de plus que 100.

Le datas suggere un certe relation inter le activitate del infection leprose e le capacitate complemento-fixante del sero leprose con le antigen preparate con bacillo tuberculotic.

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TABLE I

REACTIONS OBTAINED WITH VARYING AMOUNTS OF TUBERCULE ANTIGEN AND LEPROUS SERUM IN TESTS WITH SIX UNITS OF COMPLEMENT

Serum dilutions	Tubercle antigen dilutions						
	1:28.6	1:33.3	1:40.0	1:50.0	1:66.7	1:100	1:200
	Per cent hemolysis						
1:4.0	5	10	15	15	15	25	35
1:5.0	35	40	40	45	45	55	75
1:6.67	60	65	65	65	70	85	95
1:8.0	80	85	90	90	95	100	100
Antigen controls	Per cent hemolysis						
1 U.	15	25	20	90	45	40	45
2 U	85	90	90	90	95	95	95
3 U	95	100	100	100	100	100	100

Serum control
1 U 45-50
2 U 100-100

Cells control: 0

Complement control
1 U 35-45
2 U 100-100

TABLE II

TECHNIC OF TEST

Tube nº	Leprous serum		Borate saline ml	Equivalent dilutions 1:	Mixture of antigen, serum and complement*	Fixation	Sensitized cells	Period for hemolysis
	dil. 1:	amount ml						
1	1.0	0.05		1.0	0.25	90 minutes	0.2	15 minutes
2		0.03	0.02	1.67	0.25	in a	0.2	in a
3	2.0	0.05		2.0	0.25	water-bath	0.2	water-bath
4		0.04	0.01	2.5	0.25	at 37°C	0.2	at 37°C
5		0.03	0.02	3.33	0.25		0.2	
6	4.0	0.05		4.0	0.25		0.2	
7		0.04	0.01	5.0	0.25		0.2	
8		0.03	0.02	6.67	0.25		0.2	
9	8.0	0.05		8.0	0.25		0.2	
10		0.04	0.01	10.0	0.25		0.2	
11		0.02	0.03	16.7	0.25		0.2	

Add 1.0 ml of cold salt solution to tubes showing partialhemolysis, centrifuge and read optical density of supernates, at wave length 545 mu, in a Coleman Junior photo-colorimeter. Convert optical density to hemolysis per-cent by the aid of a factor or by using a calibrated galvanometer scale panel.

* Mix one volume of antigen (diluted according to the dose previously determined), one volume of complement dilution (containing 6 units in 0.1 ml) and a half volume of borate-saline. Keep in cold, before using.

TABLE III

VALUES FOR TITERS IN TESTS WITH TUBERCLE ANTIGEN, SIX UNITS OF COMPLEMENT, AND LEPROUS SERUM DILUTED TO 1 IN:

% hemolysis	1.0	1.67	2.0	2.5	3.33	4.0	5.0	6.67	8.0
10	7.9	13.19	15.80	19.75	26.31	31.60	39.50	52.69	63.20
15	6.9	11.52	13.80	17.25	22.98	27.60	34.50	46.02	55.20
20	6.4	10.69	12.80	16.00	21.31	25.60	32.00	42.69	51.20
25	6.0	10.02	12.00	15.00	19.98	24.00	30.00	40.02	48.00
30	5.7	9.52	11.40	14.25	18.98	22.80	28.50	38.02	45.60
35	5.5	9.19	11.00	13.75	18.32	22.00	27.50	36.69	44.00
40	5.3	8.85	10.60	13.25	17.65	21.20	26.50	35.35	42.40
45	5.2	8.68	10.40	13.00	17.32	20.80	26.00	34.68	41.60
50	5.0	8.35	10.00	12.50	16.65	20.00	25.00	33.35	40.00
55	4.8	8.02	9.60	12.00	15.98	19.20	24.00	32.02	38.40
60	4.7	7.85	9.40	11.75	15.65	18.80	23.50	31.35	37.60
65	4.5	7.52	9.00	11.25	14.99	18.00	22.50	30.02	36.00
70	4.4	7.35	8.80	11.00	14.65	17.60	22.00	29.35	35.20
75	4.2	7.01	8.40	10.50	13.99	16.80	21.00	28.01	33.60
80	4.1	6.85	8.20	10.25	13.65	16.40	20.50	27.35	32.80
85	3.9	6.51	7.80	9.75	12.99	15.60	19.50	26.01	31.20
90	3.7	6.18	7.40	9.25	12.32	14.80	18.50	24.70	29.60

Note. When the dilution is a 10 fold value of one given in the table, multiply the corresponding value in the table by 10; e. g. for 35% hemolysis and serum diluted to 1:25, the titer will be 135.5.

TABLE IV

Calculation of titer

Tube nº	Serum dilution	% hemolysis	Titer of serum as read from table III
6	4.0	15	27.60
7	5.0	40	26.50
8	6.67	65	30.02
9	8.0	90	29.60

The titers should present agreement within 25% between the maximum value obtaneid (taken as titer of the serum) and that just preceding or just following the maximum.