THE BEHAVIOR OF THREE DIFFERENT KINDS OF ANTIBODIES IN TUBERCULOSIS: ANTIPROTEIN, ANTIPOLYSACCHARIDE, AND ANTIPHOSPHATIDE

II. Human Tuberculosis

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In the preceding study (3, 4) evidence was obtained that, of the three different kinds of antibodies in tuberculous serum, antiprotein, antipolysaccharide, and antiphosphatide, only the antiphosphatide as measured by the phosphatide hemagglutination technique indicates the activity of experimental tuberculosis infection. In the present study, in order to verify whether this is true of human tuberculosis, sera from patients with pulmonary tuberculosis and tuberculin-positive healthy persons were subjected to the same serological tests as used in the preceding study: the Middlebrook-Dubos test, the Boyden test, and the phosphatide hemagglutination test. The findings confirmed that the antiphosphatide test is a better index of tuberculous activity than are the antipolysaccharide and antiprotein tests. It was also confirmed that the degree of tuberculin skin hypersensitivity is not directly related to the amounts of the circulating antibodies.

Materials and Methods

Test Serum.—Patients with pulmonary tuberculosis of varying forms in the Second National Sanatorium, Hokkaido, numbering 201, and 50 healthy employees in the same sanatorium were used for the tests. The employees were all tuberculin-positive, but their chest X-ray photographs showed nothing unusual.

Test sera were separated from the fasting blood samples drawn early in the morning while the subject was in the fasting state. Prior to the testing, they were inactivated at 56°C for 30 minutes and removed from normal agglutinins for sheep blood cells according to the conventional technique. (Add 0.1 ml of packed sheep red cell suspension to 1.0 ml of serum. Leave to stand at room temperature for 10 minutes. Add again 0.1 ml of cell suspension. Centrifugation after 10 minutes). Serial twofold dilutions of serum were tested and antibody titers higher than 1:4 were taken as positive.

Antigens.—The polysaccharide and protein antigens used throughout the present study were extracted with urea from cultures of the human tubercule bacillus, strain H37Rv, 8 weeks old on Sauton medium. The cultures were killed by immersion in acetone for 2 consecu-

tive days during which time the acetone was renewed two times, dried and defatted in a Soxhlet apparatus by successive treatment with acetone, methanol, and chloroform, each for 2 to 3 consecutive days. The defatted bacilli were then extracted with 10 per cent urea solution for 2 consecutive days under constant agitation by means of a magnetic stirrer (10 ml of urea solution for 1 gm of bacilli at dry weight). The bacilli were removed from the extract by high speed centrifugation. The supernate was dialysed in a cellophane bag against running tap water for 2 days, Seitz-filtered, and condensed *in vacuo* to about one-tenth of the original volume. A proper amount of trichloroacetic acid was added to the condensed solution to give a slightly brownish white precipitate which was collected by centrifugation, after having been placed overnight in the refrigerator. The precipitate was washed three times with water, followed by three washings with acetone, dried, and stored at room temperature. This fraction was used as the protein antigen (nitrogen, 11.7 per cent).

TABLE I

Results of the Different Hemagglutination Tests

Groups	Serum tests	No. of sera	Total tests		Per cent of tests		Antibody titers	
			Positive	Negative	Positive	Negative	Lowest	Highest
Patients	M.D.	201	201	0	100	0	4*	1,024*
	B.	201	109	92	54.2	45.8	0	1,024
	Pd.	201	159	42	79.1	20.9	0	256
Healthy persons‡	M.D.	50	50	0	100	0	4	128
	B.	50	22	28	44	66	0	128
	Pd.	50	0	50	0	100	0	0

M.D., Middlebrook-Dubos test; B, Boyden test; Pd., Phosphatide hemagglutination test.

* Reciprocals of antibody titers.

‡ All persons are tuberculin-positive.

The deproteinized supernate was again dialysed for 1 day against running tap water. Five volumes of methanol were added to the dialysed solution to give a white precipitate which was collected by centrifugation, washed three times with acetone, dried, and stored at room temperature. This fraction was used as the polysaccharide antigen (nitrogen, 0.72 per cent).

The phosphatide antigen was the same as that used in the preceding experiment, *Pd.ha* (nitrogen, 0.35 per cent; phosphorus, 2.8 per cent) (3).

Against one and the same rabbit immune serum, the protein, polysaccharide, and phosphatide antigens gave antibody titers of 1/2,560, 1/2,560, and 1/640, respectively (Boyden test, Middlebrook-Dubos test, and phosphatide hemagglutination test).

Serological Tests.—The procedures of the three hemagglutination tests used, the Middlebrook-Dubos test, the Boyden test, and the phosphatide hemagglutination test, were the same as described in preceding papers (5, 6). The specimens of serum were collected by nurses and tested by technical assistants who remained unaware of the diagnoses of the subjects from whom the sera had been obtained.

EXPERIMENTAL RESULTS

Results of the Three Hemagglutination Tests.—As seen in Table I, the anti-polysaccharide test was positive in all patient sera as well as in the sera of

healthy persons; the antiprotein test was about 55 per cent positive for the patient sera and 44 per cent positive for the healthy sera, although the antibody mean values were lower in the healthy sera than in the patient sera. In contrast, the antiphosphatide test was about 80 per cent positive for the patient sera, while it was negative for the healthy sera.

Tuberculin Skin Tests and Antibody Titers.—

Tuberculin skin tests were secured and recorded in 152 arbitrarily selected patients by injecting intradermally 0.1 ml of a 2,000-fold saline dilution of standard Sauton old tuberculin

TABLE II

Relationship Between Tuberculin Skin Tests and Antibody Titers

Serum tests	Skin tests	Antibody titers										Total tests	Positive tests	Per cent of positive tests	A.M.V.
		0	4	8	16	32	64	128	256	516	1,024				
M.D.	+++*	0	0	1	5	12	7	8	2	3	0	38	38	100	169.4
	++	0	0	1	9	8	13	16	9	4	0	60	60	100	180.9
	+	0	1	3	5	17	16	8	4	0	0	54	54	100	94.0
B.	+++	15	0	3	3	2	6	3	5	1	0	38	23	60.3	132.6
	++	24	3	4	3	3	2	4	6	9	2	60	36	60.3	274.9
	+	27	2	2	5	5	1	5	2	3	2	54	27	50.0	239.9
Pd.	+++	8	4	10	2	4	3	5	2	0	0	38	30	78.9	77.8
	++	9	4	5	7	13	10	7	5	0	0	60	51	85.0	91.1
	+	14	4	7	9	5	6	4	1	0	0	54	40	74.2	55.0

A.M.V., Antibody mean values (logarithmic mean values).

* For meaning of these signs, see the text. For other symbols, see the footnotes of Table I.

manufactured at the National Institute for Public Health, Tokyo. Readings were recorded 24 hours after injection according to the following key: positive (+), reactions showing only erythema; strongly positive (++), reactions showing erythema and induration; very strongly positive (+++), reactions showing halo and vesicles beside erythema and induration.

The comparison between the readings of the tuberculin skin test and the antibody titers for the three serological tests is presented in Table II.

Close examination of the table discloses that there is no direct relationship between the degree of tuberculin skin hypersensitivity and the amount of any one of the three different circulating antibodies.

Extent of Tuberculous Lesions and Antibody Titers.—

According to the classification of the National Tuberculosis Association, New York, the patients from which the sera were obtained were divided into three groups: far advanced active cases, moderately advanced active cases, and minimally active cases. The antibody

titers of the three different kinds of antibodies in each of these three groups are compared in Table III.

In the case of the antipolysaccharide and antiprotein, no significant correlation in the distribution of the antibody titers was noticed among the three groups, the antipolysaccharide mean value being rather higher in the moderately active cases than in the far advanced active cases (174.4:209.7) and the antiprotein mean value being higher in the minimally active cases than in the moderately active cases (154.0:175.2). On the contrary, a distinct correlation

TABLE III

Relationship Between Extent of Lesions and Antibody Titers

Serum tests	Extent of lesions	Antibody titers										Total tests	Positive tests	Per cent of positive tests	A.M.V.
		0	4	8	16	32	64	128	256	512	1,024				
M.D.	F.A.	0	1	2	7	11	17	17	11	4	0	70	70	100	174.4
	Md.A.	0	0	5	6	24	20	18	6	4	2	85	85	100	209.7
	Mn.A.	0	1	3	12	10	12	3	3	2	0	46	46	100	134.6
B.	F.A.	26	2	7	3	3	4	7	10	4	4	70	44	63.0	293.5
	Md.A.	36	4	6	7	7	6	5	9	5	0	85	49	57.7	154.0
	Mn.A.	30	2	0	4	2	0	2	1	5	0	46	16	34.9	175.0
Pd.	F.A.	5	2	10	7	16	13	12	5	0	0	70	65	92.9	92.3
	Md.A.	12	10	19	16	13	7	5	3	0	0	85	73	85.9	61.9
	Mn.A.	25	8	4	5	3	0	1	0	0	0	46	20	46.0	21.3

F.A., Far advanced active cases; Md.A., moderately advanced active cases; Mn.A., minimally active cases. For other symbols, see the footnotes of Table I.

is seen to exist between the antiphosphatide titers and the extent of lesions, both the positive percentage and the antibody mean titer being highest in the far advanced cases, followed by the moderately advanced cases, and lowest in the minimally active cases (92.9:85.9:46.0 per cent, and 92.3:61.9:21.3).

Types of Disease and Antibody Titers.—

According to the classification of the Japan Committee for Classification of Pulmonary Tuberculosis sponsored by the Ministry of Education of Japan, the patients were classified into three groups: type F, serious mixed type; type B, caseoinfiltrative type; and type C, fibrocaseous type. The relationships between the disease types and the antibody titers are presented in Table IV.

It is clear from the table that, of the three different antibodies, only the antiphosphatide runs parallel with the severity of tuberculosis disease. It was worthy of notice that the antiphosphatide was 100 per cent positive in type F,

while the percentage decreased regularly toward type C (100:86.0:72.5 per cent). Likewise, the antiphosphatide mean titer was highest in type F, followed by type B, and lowest in type C (104.2:74.3:56.5).

TABLE IV

Relationship Between Disease Types and Antibody Titers

Serum tests	Disease types	Antibody titers										Total tests	Positive tests	Per cent of positive tests	A.M.V.
		0	4	8	16	32	64	128	256	512	1,024				
M.D.	F	0	1	1	0	2	9	6	3	2	0	24	24	100	189.1
	B	0	0	2	11	12	10	7	7	1	0	50	50	100	151.8
	C	0	1	7	14	31	30	25	10	7	2	127	127	100	201.5
B.	F	6	1	2	2	1	1	5	2	2	2	24	18	75.2	344.0
	B	23	3	5	2	3	0	4	5	4	1	50	27	54.0	223.4
	C	63	4	6	10	8	9	5	13	8	1	127	64	50.4	179.2
Pd.	F	0	0	1	4	5	6	6	2	0	0	24	24	100	104.2
	B	7	6	9	10	7	5	3	3	0	0	50	43	86.0	74.3
	C	35	14	23	14	20	9	9	3	0	0	127	92	72.5	56.5

F, serious mixed type; B, caseoinfiltrative type; C, fibrocaseous type. For other symbols, see the footnotes of Table I.

TABLE V

Relationship Between Existence of Cavities and Antibody Titers

Serum tests	Cavity	Antibody titers										Total tests	Positive tests	Per cent of positive tests	A.M.V.
		0	4	8	16	32	64	128	256	516	1,024				
M.D.	Positive	0	1	6	12	36	38	31	17	8	0	149	149	100	149.7
	Negative	0	1	4	13	9	11	7	3	2	2	52	52	100	251.9
B.	Positive	58	6	10	9	10	9	12	19	12	4	149	89	61.1	243.5
	Negative	34	2	3	5	2	1	2	1	2	0	52	18	34.6	109.8
Pd.	Positive	11	13	30	22	30	20	16	7	0	0	149	138	92.7	74.7
	Negative	31	7	3	6	2	0	2	1	0	0	52	21	40.0	44.3

For symbols, see the footnotes of Table I.

Existence of Cavities and Antibody Titer.—

The patients were divided into two groups, cavity-positive and cavity-negative. The antibody titers in the cavity-positive patients are compared in Table V with those in the cavity-negative patients.

Of the three antibodies, the antiprotein and antiphosphatide are seen to have positive correlation with the existence and non-existence of cavities. However, it must not be overlooked that the positive percentages were far lower and the

TABLE VI

Relationship Between Sputum Findings and Antibody Titers

Serum tests	Sputum	Antibody titers										Total tests	Positive tests	Per cent of positive tests	A.M.V.
		0	4	8	16	32	64	128	256	521	1,024				
M.D.	Positive	0	1	6	12	25	33	26	16	6	0	125	125	100	160.2
	Negative	0	1	4	13	19	15	11	4	4	2	73	73	100	224.2
B.	Positive	48	4	8	8	8	8	12	16	9	4	125	77	61.6	250.4
	Negative	42	4	5	5	4	1	2	4	5	0	73	31	41.1	149.5
Pd.	Positive	10	9	23	14	27	19	16	7	0	0	125	115	92.0	81.5
	Negative	32	10	9	13	5	1	2	1	0	0	73	41	56.2	39.1

For symbols, see the footnotes of Table I.

TABLE VII

Relationship Between Disease Activity and Antibody Titers

Serum tests	Categories	Antibody titers										Total tests	Positive tests	Per cent of positive tests	A.M.V.
		0	4	8	16	32	64	128	256	512	1,024				
M.D.	Active	0	1	6	16	36	41	33	17	7	2	159	159	100	192.5
	Inactive	0	1	4	9	9	8	5	3	3	0	42	42	100	162.5
	Healthy	0	1	7	15	18	8	1	0	0	0	50	50	100	37.7
B.	Active	62	7	12	11	10	10	13	19	11	4	159	97	61.1	232.4
	Inactive	30	1	1	3	2	0	1	1	3	0	42	12	28.7	144.3
	Healthy	28	2	8	4	6	1	1	0	0	0	50	22	44.0	23.7
Pd.	Active	15	13	31	24	31	20	17	8	0	0	159	144	90.6	76.3
	Inactive	27	7	2	4	1	0	1	0	0	0	42	15	35.8	21.8
	Healthy	50	0	0	0	0	0	0	0	0	0	50	0	0	0

For symbols, see the footnotes of Table I.

difference was smaller in the antiprotein (61.1:34.6 per cent) than in the antiphosphatide (92.7:40.0 per cent).

Sputum Findings and Antibody Titers.—

Again the patients were divided into two groups, sputum-positive and sputum-negative. The patients who showed no bacilli at sputum culture at least during the past 1 year were taken as sputum-negative.

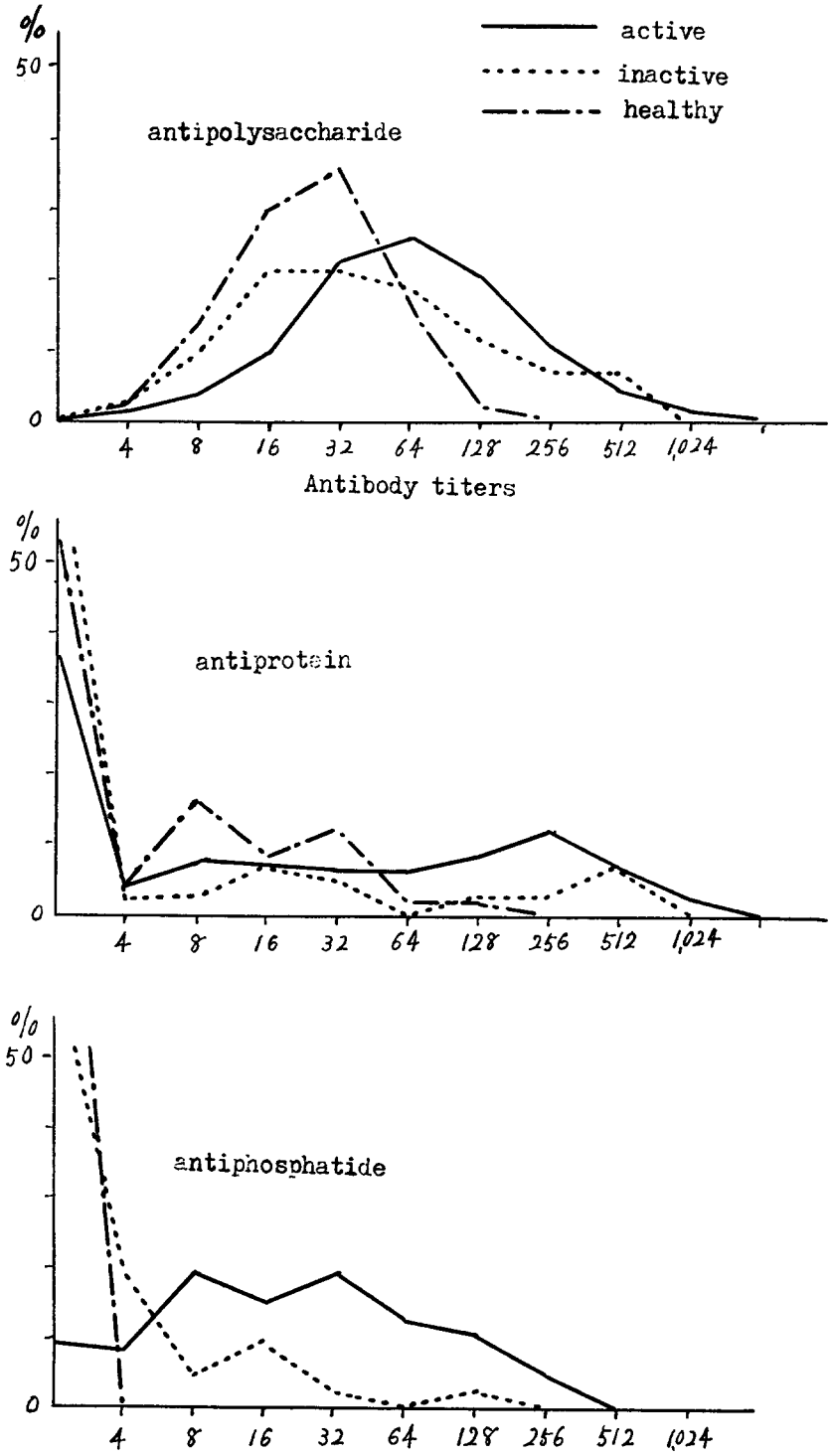

FIG. 1. Distributions of the three different antibody titers as compared with disease activity.

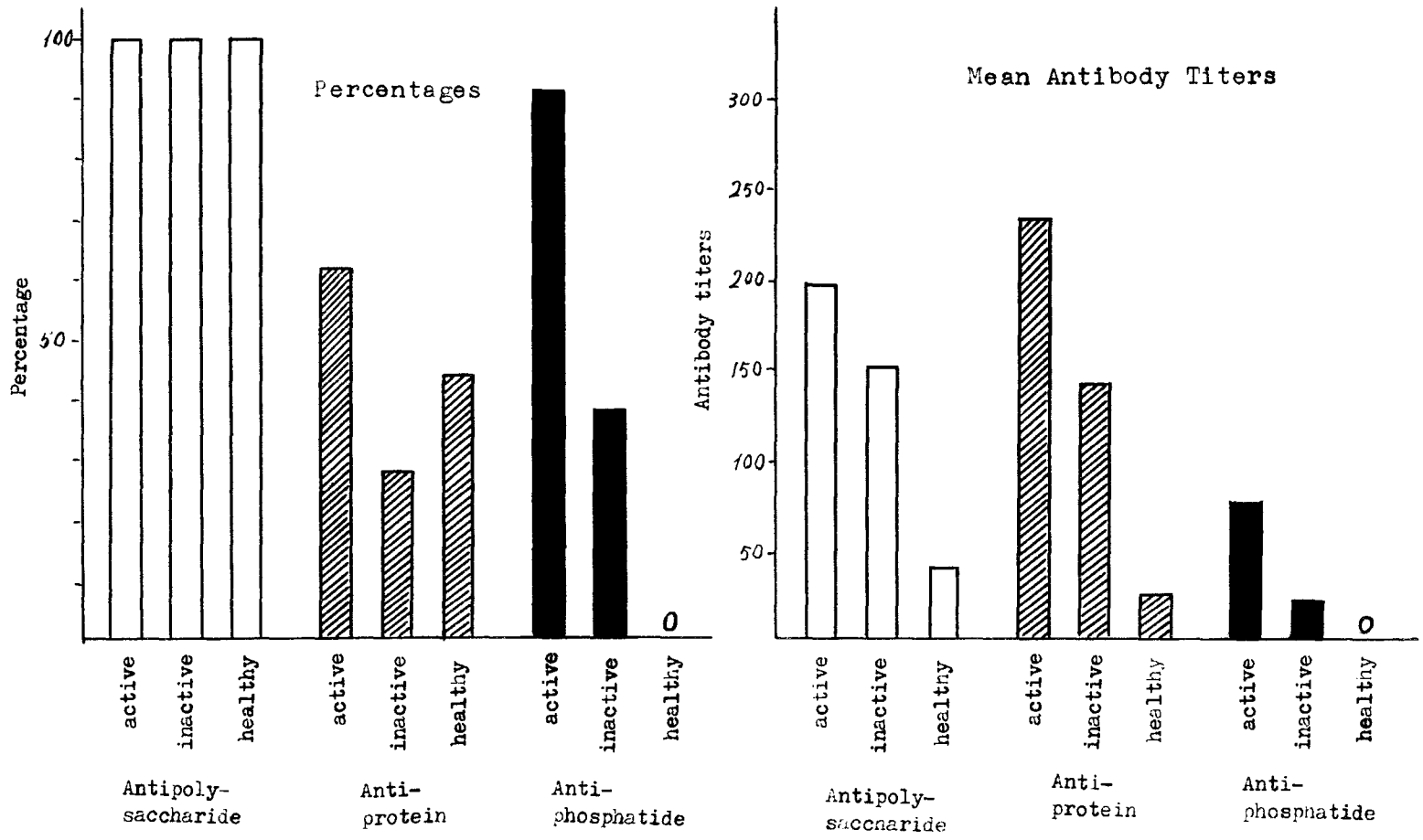

FIG. 2. Positive percentages and mean antibody titers as compared with disease activity.

As shown in Table VI, from the antibody mean titers, the antiprotein and the antiphosphatide tests were well correlated with the existence and non-existence of bacilli in the sputums (250.4:149.5 and 81.5:39.1). However, in the case of the antiprotein, the percentages were low and only a slight difference was seen between the two groups (61.6:41.1 per cent). In contrast, in the case of the antiphosphatide, the sputum-positive showed as high a rate as 92.0 per cent, while the sputum-negative as low a rate as 55.7 per cent.

Disease Activity and Antibody Titers.—

Finally, the data were reviewed on the basis of the degree of disease activity. The patients who, having no active lesion, remained radiologically stationary and sputum-negative at least during the past 1 year were taken as inactive cases. They numbered 42 (29.0 per cent). The other patients not belonging to this category were all taken as having active lesions. They numbered 159 (71.0 per cent). Table VII and Figs. 1 and 2 compare the distributions, percentages, and mean values of the antibody titers of the three kinds of antibodies among the two categories of patients, active and inactive, and the tuberculin-positive, healthy persons.

It is seen that a significant difference existed in the antipolysaccharide antibody mean value between the patients and the healthy persons, but no significant difference between the active and the inactive cases (192.5:162.5). In the case of the antiprotein, the antibody mean value was highest in the active cases, and it decreased with significant differences in order of the inactive cases and the healthy persons (232.4:144.3:23.7), but the percentages were irregular in the three categories, the healthy persons showing a percentage higher than the inactive cases (61.1:28.7:44.0 per cent). In contrast, in the case of the antiphosphatide, both percentages and antibody mean values were higher in the active cases than in the inactive cases (90.6:35.8 per cent and 76.3:21.8); none of the healthy persons gave a positive test. This clearly indicates that of the three different kinds of antibodies, only the antiphosphatide as measured by the hemagglutination test furnishes exact information about the degree of disease activity.

DISCUSSION

Quite in accordance with the results obtained on experimental tuberculosis (3), the antipolysaccharide and antiprotein were produced almost universally in persons with tuberculous infection, whether they had currently active disease or not, although the amounts of antibody were smaller in infected but healthy persons than in patients with actual disease (Table I). Moreover, the amounts of these two kinds of antibodies were not correlated with the extent of tuberculous lesions, types of disease, existence and non-existence of cavities, bacteriological findings in sputum, and disease activity. In contrast, while about 80 per cent of the patients with pulmonary tuberculosis possessed antiphosphatides in their sera, none of the tuberculin-positive healthy persons gave a positive phos-

phatide test. It is also worth noticing that a significant correlation was found between the positive percentage and the amount of antiphosphatides on the one hand and the clinical findings mentioned above on the other (Tables II to VII).

The foregoing facts clearly indicate that the antipolysaccharide and antiprotein, especially the former, are produced whenever tuberculous infection takes place and may persist for a long period, even after tuberculous disease is arrested. This is compatible with the fact that these two kinds of antibodies are detectable in tuberculin-positive healthy persons who have never experienced tuberculous disease almost at the same rate as in the patients with actual disease (Table I) and that no significant difference in the antibody mean value is found among far advanced active cases, moderately advanced active cases, and minimally active cases (Table III), nor among serious mixed type, caseoin-filtrative type, and fibrocaseous type (Table IV). In contrast, the production of antiphosphatides is mainly conditioned by the outbreak of disease following infection and influenced by the degree of disease activity (Table VII, Figs. 1 and 2). The possible reason for this has been postulated in the preceding paper (3).

A serologic test, to be of real usefulness in the diagnosis of tuberculosis, must detect only currently active disease but not infection, because tuberculous infection can well be detected by the Mantoux test. It is also to be desired that the test give information about the extent or the activity of the disease. Therefore, judging from the present observations, the diagnostic value of the phosphatide hemagglutination test is expected to be of a very high order, while that of the Middlebrook-Dubos test measuring antipolysaccharides and of the Boyden test measuring antiproteins may be said to be of only a very low order.

One more advantage of the phosphatide hemagglutination test is that the phosphatide antigens isolated and purified according to the method previously described (6) are serologically identical, regardless of the strain used, be it H37Rv, BCG, Nakano, or Aoyama B. The optimum concentration of tubercle phosphatide for sensitizing normal sheep red cells for hemagglutination tests is always 0.25 mg per ml saline, as reported in a previous paper (5).

When test sera are heated at 56°C for 30 minutes for inactivation, the antiphosphatide therein gives only reduced reactions with its corresponding antigen. This disadvantage can be eliminated, however, by replacing heat inactivation by chemical inactivation with EDTA (disodium ethylenediaminetetraacetate) (1, 2). In fact, use of EDTA raises the positive percentage of the phosphatide hemagglutination test in patients with pulmonary tuberculosis to as high as 90 to 95 per cent or more. This fact makes the phosphatide hemagglutination test promising as a quantitative one in the diagnosis of the activity of tuberculous disease. Moreover, it has also been found in this laboratory that suspension of kaolin powder properly prepared can well be sensitized with tubercle phosphatide to give agglutination in the presence of tuberculous serum, and that the

phosphatide kaolin agglutination is serologically of identical value to the phosphatide hemagglutination. Its clinical evaluation is now in progress. The details will be reported shortly.

Finally, as in the case of experimental tuberculosis, no direct correlation was found between the degree of tuberculin skin hypersensitivity and the amount of any of the three different kinds of antibodies, antipolysaccharide, antiprotein, and antiphosphatide.

SUMMARY

In human tuberculosis as in experimental tuberculosis, there exist in the serum of tuberculous patients three different kinds of antibodies completely distinct from each other, antipolysaccharide, antiprotein, and antiphosphatide. The two former antibodies are produced whenever tuberculous infection takes place or exists and they persist for a long period, even though tuberculous disease be arrested. On the contrary, the production of the antiphosphatide seems to be mainly conditioned by the outbreak of tuberculous disease following infection, because none of the tuberculin-positive healthy persons tested gave a positive phosphatide hemagglutination test. The antiphosphatide hemagglutination test furnishes useful information about the extent or the activity of tuberculous disease.

No correlation was noticed between the degree of tuberculin skin hypersensitivity and the amount of any of the three antibodies.

The usefulness of the phosphatide hemagglutination test in the diagnosis of tuberculosis is discussed.

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