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

I. Experimental Tuberculosis

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In preceding studies (4–6), evidence was obtained that there exist at least three different kinds of antibodies independent of each other in tuberculous serum: antiprotein, antipolysaccharide, and antiphosphatide. In the study reported here, efforts were made to explore their behavior in the course of experimental tuberculous infection. For this purpose, rabbits were infected with avirulent or virulent tubercle bacilli by different routes, and following infection, their sera were periodically tested by the three different hemagglutination tests, that of Middlebrook-Dubos for measuring antipolysaccharide, that of Boyden for measuring antiprotein, and the phosphatide hemagglutination test for measuring antiphosphatide (9, 10). The specificity of these three tests had been established in preceding studies (4, 5). Thus, the influence of the mode of infection and the virulence of bacilli on the behavior of the three different kinds of antibodies was studied. An abstract of the findings has been reported elsewhere (5).

Materials and Methods

Serological Tests.—The procedures for the three serological tests used were the same as described in preceding papers (5, 9, 10), except that in the Boyden test use was made of a 0.5 mg/ml solution of antigen and a 5,000-fold solution of tannic acid for tanning sheep red cells, because the test was found in a preliminary experiment to be most sensitive under these conditions. Test sera were inactivated by heating at 56°C for 30 minutes.

Antigens[1].—The protein antigen, *Nakano TPr* (nitrogen, 11.1 per cent), was a protein fraction isolated from a Sauton culture filtrate of virulent human type tubercle bacilli, strain Nakano. An 8-week-old Sauton culture filtrate was Seitz-filtered, concentrated *in vacuo* to about one-tenth of the original volume, and dialysed in a cellophane bag against running tap

[1] Of several polysaccharide and protein fractions prepared in this laboratory which were tested for their homosensitizing ability, the two fractions to be described, *Nakano TPr* and *Nakano TPs*, were found to be most active.

water for 2 consecutive days. The protein fraction was precipitated from the filtrate by addition of an adequate amount of 10 per cent trichloroacetic acid. After standing overnight in the refrigerator, the precipitate was collected by centrifugation. It was then dissolved in a dilute NaOH solution, freed from impurities by centrifugation, and reprecipitated at pH 4.2 by addition of dilute HCl solution. This procedure was repeated several times and the final precipitate collected by centrifugation, washed with acetone, vacuum-dried, and stored at room temperature until used.

The polysaccharide antigen, *Nakano TPs* (nitrogen, 0.45 per cent), was a fraction precipitated from the deproteinized filtrate by addition of methanol; the deproteinized filtrate was concentrated to about one-tenth of the original volume, dialysed in a cellophane bag against running tap water for 2 consecutive days, and Seitz-filtered. Methanol was added to the filtrate to 40 per cent. The precipitate formed was removed by centrifugation (this fraction is inactive for the Middlebrook-Dubos test), and methanol was again added to the supernate to 80 per cent. The precipitate was collected by centrifugation. It was again dissolved in water, freed from impurities by centrifugation, and reprecipitated by addition of methanol to the same concentration. This procedure was repeated several times and the final product was washed with acetone, vacuum-dried, and stored at room temperature.

The phosphatide antigen, *Pd. ha* (nitrogen, 0.3 per cent; phosphorus, 2.8 per cent), was a purified methanol extract isolated from acetone-killed mixed bacilli of the human strains, H37Rv and Aoyama B, according to the method described in previous papers (5, 10).

The procedures for preparing antigen solutions for sensitizing sheep red cells, normal or tanned, were the same as described in previous papers (5, 10).

When tested against the same rabbit antiserum, the antibody titer of the Middlebrook-Dubos test using *Nakano TPs* was 1/2,560, that of the Boyden test using *Nakano TPr*, 1/320, and that of the phosphatide hemagglutination test using *Pd. ha*, 1/1,280.

Bacillary Cultures.—Use was made of cultures of BCG and of the virulent Nakano strain, human type, 2 weeks of age on Ogawa's egg medium.[2] Bacillary saline suspensions were conventionally prepared in a flask containing glass beads previously sterilized.

Tests.—Sixteen albino rabbits weighing from 2 to 3 kg were divided into four groups. The first group (group BCG-skin) received subcutaneously in the abdomen 1 mg of BCG and the second group (group BCG-vein) the same dose through the ear vein. The third group (group Nakano-skin) received likewise subcutaneously in the abdomen 1 mg of Nakano bacilli and the fourth group (group Nakano-vein) the same dose through the ear vein. The viable counts were 1.6×10^7/mg for BCG and 2.5×10^7/mg for Nakano bacilli. At 3, 5, 7, 9, and 12 weeks after infection, the animals were bled through the ear vein and the antibody titers of the sera were measured and recorded. Parallel to these observations, tuberculin skin tests were secured and recorded using 0.1 ml amounts of a tenfold dilution of Sauton tuberculin. At the end of the experiment, all the animals were sacrificed and the gross findings of the visceral tuberculous lesions were recorded. The quantitative culture of the lungs was conducted at the same time.

EXPERIMENTAL RESULTS

The Middlebrook-Dubos Test.—The results of the test are presented in Table I and Fig. 1. The production of antibodies to polysaccharide was found to be prominent in all the animals, and aside from slight individual variations, no

[2] See Obayashi, Y., Dried BCG Vaccine, *World Health Organization*, 1955, 11.

significant difference was found among the four groups. This would indicate that the production of antipolysaccharides takes place almost universally, regardless of the mode of infection and the virulence of infecting bacilli.

The Boyden Test.—As shown in Table II and Fig. 2, the production of antibodies to protein was weaker than that of antipolysaccharides. It is interesting to note that, in the first and second groups which received BCG, the antibody

TABLE I

Results of the Middlebrook-Dubos Test

Material, mode of challenge	Rabbit No.	Antibody titers at				
		3 wks.	5 wks.	7 wks.	9 wks.	12 wks.
BCG (S.C.)	1	640*	320	80	80	160
	2	160	160	40	40	80
	3	160	320	80	80	80
	4	160	80	40	80	40
BCG (I.V.)	5	320	320	320	320	160
	6	80	160	40	40	40
	7	160	160	40	40	40
	8	320	640	160	160	80
Nakano (S.C.)	9	160	320	320	80	80
	10	320	720	160	80	80
	11	80	640	640	320	160
	12	320	160	80	80	80
Nakano (I.V.)	13	320	160	80	160	320
	14	80	320	160	80	40
	15	320	320	160	160	80
	16	160	160	320	160	160

S.C., subcutaneous injection.
I.V., intravenous injection.
* The numerals indicate reciprocals of antibody titers.

titers reached their maxima at 3 weeks after infection and decreased gradually almost at an equal pace towards the end of the experiment, while in the third and fourth group which received virulent Nakano bacilli, the antibody titers were relatively higher than in the two foregoing groups, despite noticeable individual variations. This would indicate that the production of antiproteins is to some extent influenced by the virulence of infecting bacilli. The mode of infection, however, seems not to exert any important influence on the production of

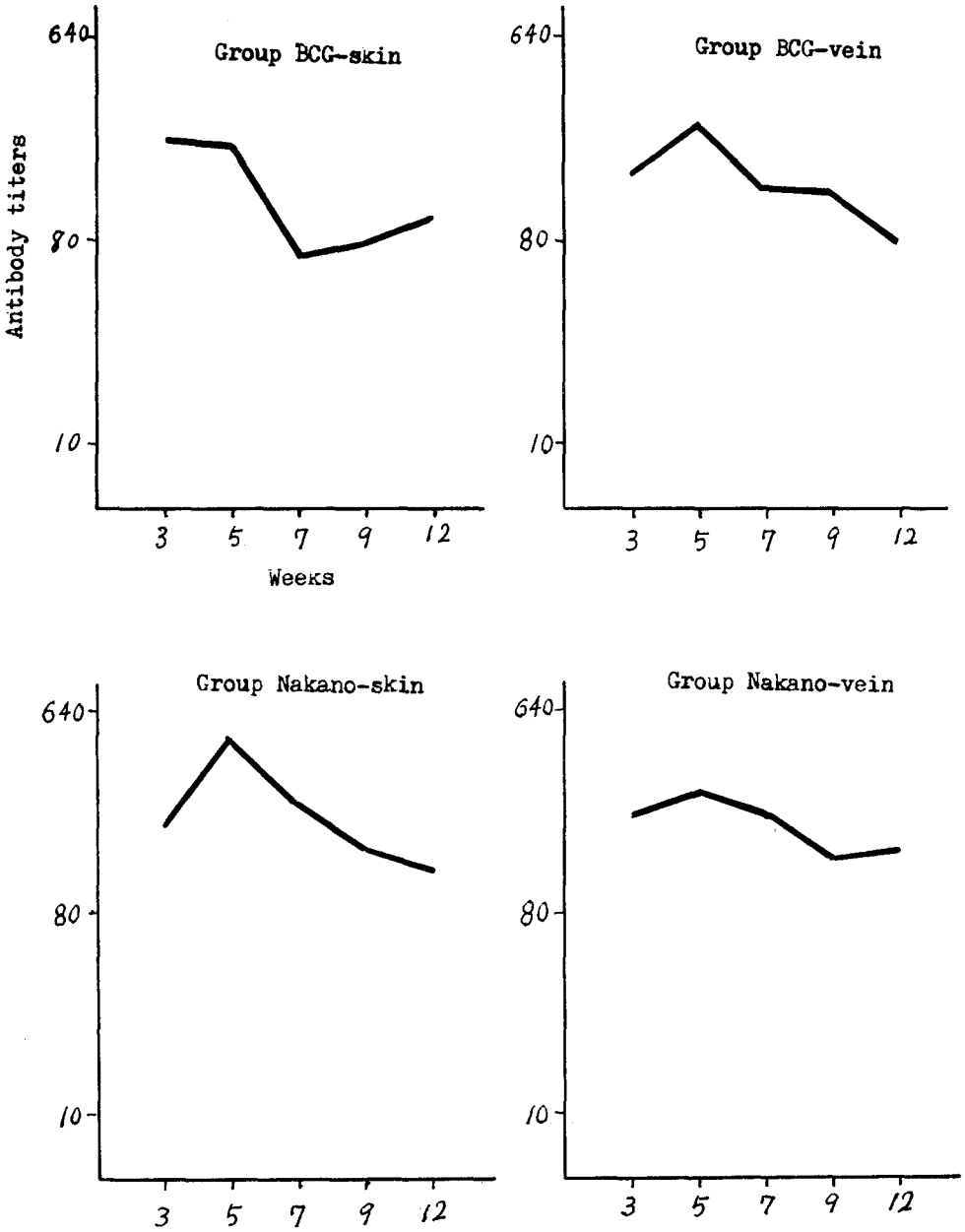

FIG. 1. Results of the Middlebrook-Dubos test. Plots of mean antibody titers (logarithmic mean values) measured periodically.

antiprotein, inasmuch as no significant difference in the antibody titer was observed between the subcutaneous groups (first and third groups) and the intravenous groups (second and fourth groups).

The Phosphatide Hemagglutination Test.—The production of antibodies to phosphatide was almost at the same level as that of antiprotein, as may be seen in Table III and Fig. 3. However, it is worth noticing that significant difference

TABLE II

Results of the Boyden Test

Material, mode of challenge	Rabbit No.	Antibody titers at				
		3 wks.	5 wks.	7 wks.	9 wks.	12 wks.
BCC (S.C.)	1	40	20	10	0	0
	2	40	10	10	0	0
	3	40	20	40	0	0
	4	20	10	0	0	0
BCG (I.V.)	5	40	20	20	0	0
	6	20	10	20	0	0
	7	20	20	20	0	0
	8	20	20	40	0	0
Nakano (S.C.)	9	20	10	20	10	0
	10	40	20	10	0	0
	11	40	80	160	40	160
	12	20	20	10	0	0
Nakano (I.V.)	13	40	20	20	40	160
	14	40	10	0	0	0
	15	20	40	10	0	0
	16	40	20	80	40	160

For signs and symbols, see the footnotes of Table I.

in the mode of antibody production was observed between the BCG groups (first and second groups) and the Nakano groups (third and fourth groups) as well as between the subcutaneous (first and third groups) and the intravenous groups (second and fourth groups). In the first group which received subcutaneously BCG bacilli, no significant production of antiphosphatide was noticed, while in the second group which received BCG intravenously, the antibody production was significant, but it tended to decrease gradually towards the end of the experiment. A similar tendency was observed in the groups which received virulent Nakano bacilli but to a higher extent than in the former groups. It is particularly interesting to note that in the fourth group which was

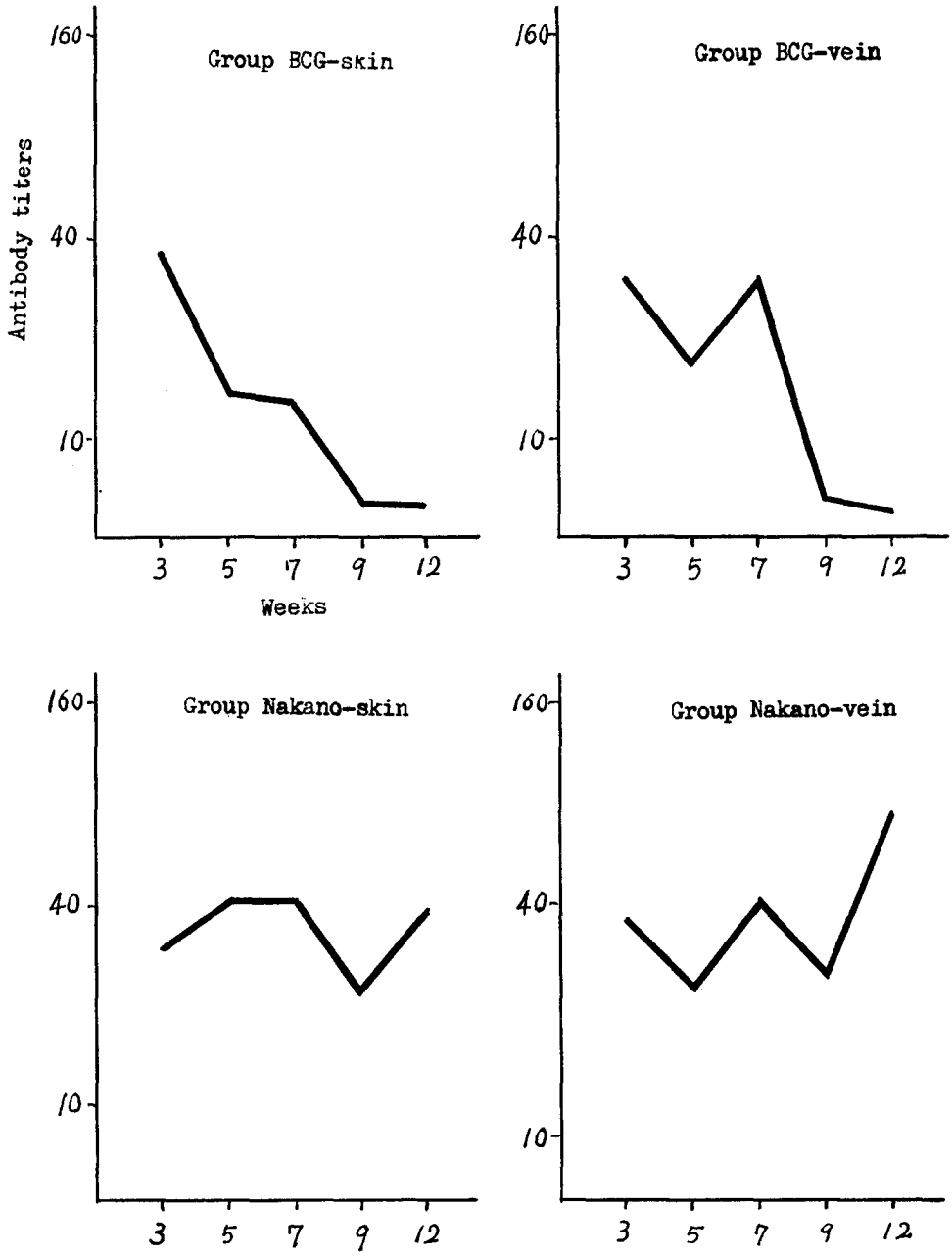

FIG. 2. Results of the Boyden test. Plots of mean antibody titers measured periodically.

intravenously infected with Nakano bacilli, the antibody titer increased towards the end of the experiment, parallel to the possibly rapid formation of tuberculous lesions in the animals.

The foregoing facts would indicate that the production of antiphosphatide in the course of experimental tuberculosis is significantly influenced by the mode of infection as well as by the virulence of infecting bacilli.

TABLE III

Results of the Phosphatide Hemagglutination Test

Material, mode of challenge	Rabbit No.	Antibody titers at				
		3 wks.	5 wks.	7 wks.	9 wks.	12 wks.
BCG (S.C.)	1	10	0	10	0	0
	2	20	10	0	0	0
	3	10	0	0	0	0
	4	10	0	0	0	0
BCG (I.V.)	5	40	20	80	20	10
	6	40	40	80	20	40
	7	10	10	10	10	0
	8	10	40	40	10	0
Nakano (S.C.)	9	10	0	0	0	0
	10	10	0	20	10	0
	11	10	10	20	20	0
	12	20	20	10	0	0
Nakano (I.V.)	13	20	20	40	80	320
	14	20	20	20	40	40
	15	10	10	10	10	0
	16	40	80	160	160	40

For signs and symbols, see the footnotes of Table I.

Tuberculin Skin Tests.—As may be seen in Table IV, tuberculin skin tests were almost of the same intensity in all the groups except the second group, in which BCG was injected intravenously. Retardation in the development of tuberculin skin hypersensitivity is often encountered in laboratory animals challenged intravenously with bacilli of BCG but its mechanism is still unknown (1, 2).

For comparison, in the first and second groups, the average values of the tuberculin reactions are plotted in Fig. 4, beside the curves showing the blood levels of the three different kinds of antibodies. The amount of each of them was

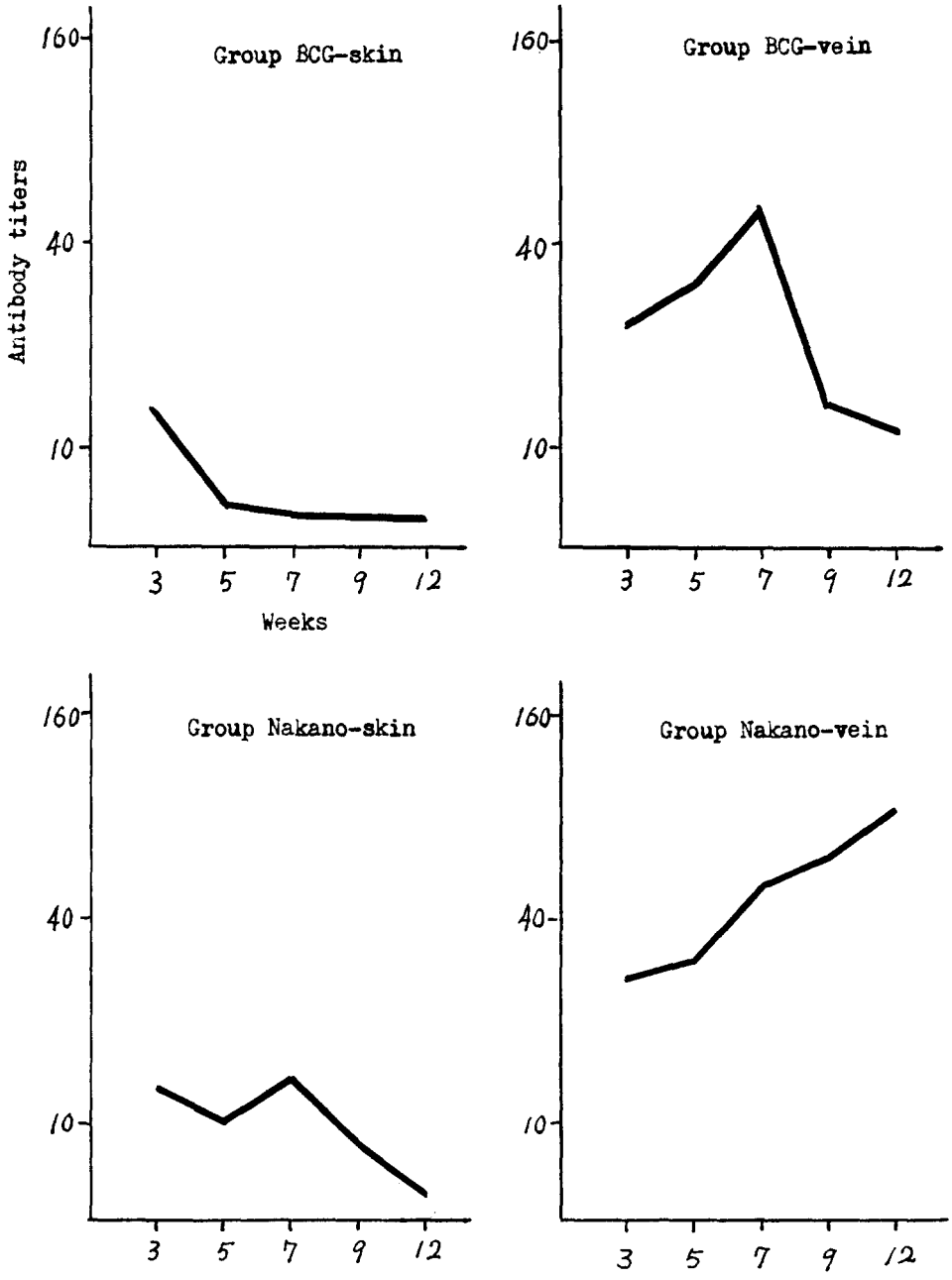

FIG. 3. Results of the phosphatide hemagglutination test. Plots of mean antibody titers measured periodically.

found not to be directly related to the degree of tuberculin skin hypersensitivity.

Relationship Between Antibody Titers and Tuberculous Lesions.—The intensity of the pulmonary tuberculous lesions was arbitrarily graded into the 4 following orders according to the number of tubercles found on the total lungs: ++++ (innumerable confluent tubercles), +++ (innumerable solitary tubercles), ++ (about 100 tubercles), + (less than 50 tubercles), and 0 (no tubercles).

TABLE IV

Results of the Tuberculin Skin Test

Material, mode of challenge	Rabbit No.	Reactions at				
		3 wks.	5 wks.	7 wks.	9 wks.	12 wks.
BCG (S.C.)	1	20 × 16*	24 × 18	17 × 17	15 × 11	0
	2	12 × 20	19 × 19	19 × 20	13 × 13	5 × 5
	3	22 × 21	20 × 19	10 × 10	12 × 10	0
	4	24 × 24	23 × 16	25 × 20	20 × 17	10 × 10
BCG (I.V.)	5	20 × 20	12 × 12	18 × 21	20 × 15	13 × 20
	6	0	0	10 × 8	0	0
	7	0	0	11 × 14	0	0
	8	0	15 × 22	15 × 22	15 × 20	12 × 13
Nakano (S.C.)	9	20 × 20	21 × 20	21 × 25	20 × 18	0
	10	28 × 23	15 × 24	20 × 27	22 × 15	13 × 13
	11	17 × 27	30 × 20	20 × 28	20 × 30	28 × 18
	12	12 × 16	20 × 18	13 × 13	15 × 14	0
Nakano (I.V.)	13	12 × 16	13 × 18	12 × 12	5 × 6	0
	14	13 × 20	18 × 18	22 × 20	18 × 12	0
	15	11 × 14	13 × 13	20 × 22	18 × 20	10 × 15
	16	13 × 27	20 × 20	13 × 14	15 × 16	18 × 23

* The numerals indicate lengths (mm) of long and short diameters of the reactions. For other signs and symbols, see the footnotes of Table I.

At autopsy, the animals of the first and second groups which received BCG presented no tubercle, while the animals which were infected with virulent Nakano bacilli possessed tubercles to various extents. The data are presented in Table V, beside the antibody titers measured at the end of the experiment by the use of the three foregoing different hemagglutination tests. It is seen that, of the three antibodies, the antiphosphatide may be most related to the intensity of tuberculous lesions. In contrast, the antipolysaccharide maintained its titer almost at an equal level in all the animals and the antiprotein was rather inconstant.

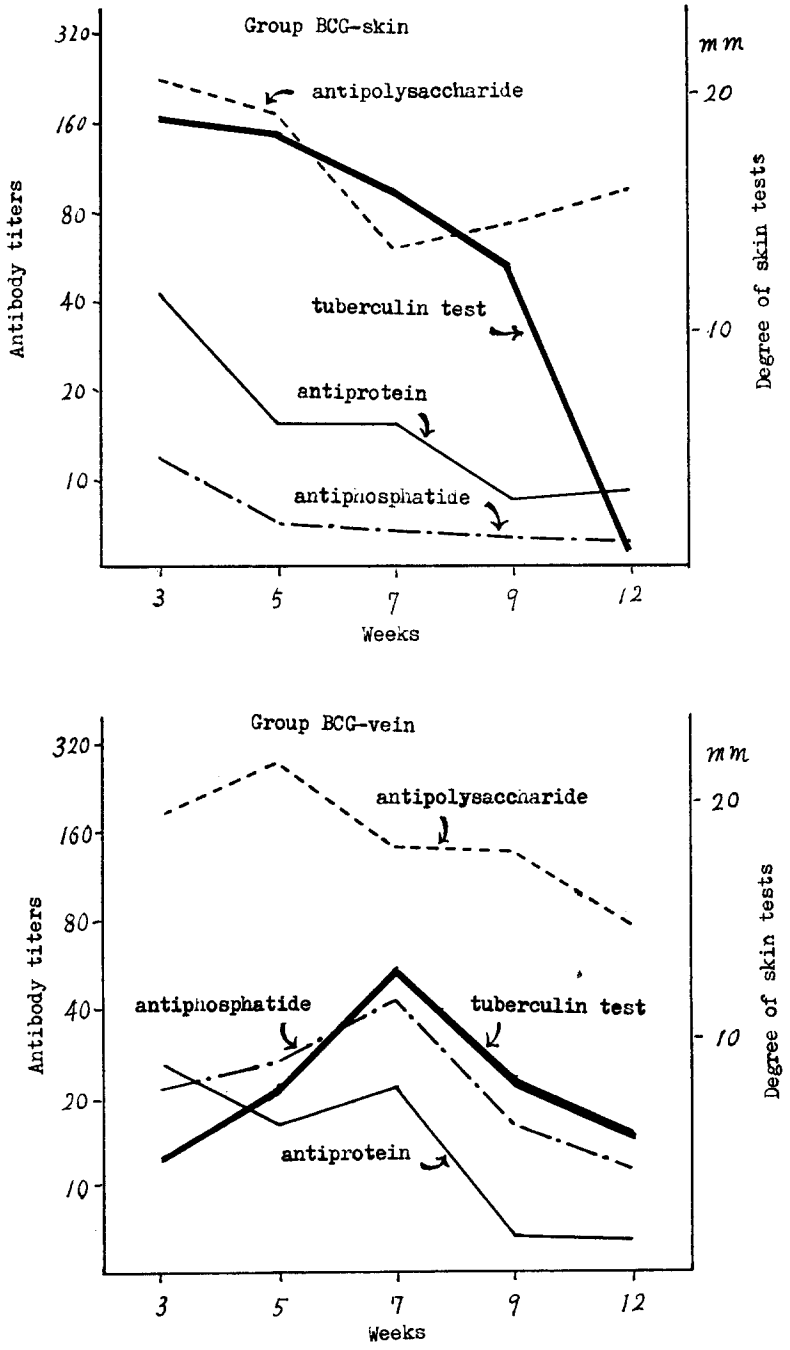

FIG. 4. Tuberculin skin reactions as compared with serum antibody levels.

DISCUSSION

The present study shows that, in the course of experimental tuberculosis, the mode and level of antibody production differ considerably according to the constituents of the tubercle bacillus.

It is a well established fact that bacilli of BCG multiply *in vivo* for several weeks and then diminish in number gradually without producing any progres-

TABLE V

Findings at the End of the Experiment (12 Weeks)

Material, mode of challenge	Rabbit No.	M.D.	B.	Pd.	Pulmonary lesions	Viable counts of lungs
BCG (S.C.)	1	160	0	0	0	0
	2	80	0	0	0	0
	3	80	0	0	0	0
	4	40	0	0	0	0
BCG (I.V.)	5	160	0	10	0	0
	6	40	0	40	0	1×10^{2}*
	7	40	0	0	0	0
	8	80	0	0	0	0
Nakano (S.C.)	9	80	0	0	+‡	4.6×10^{4}
	10	80	0	0	+	4×10^{2}
	11	160	160	0	0	0
	12	80	0	0	+++	6×10^{5}
Nakano (I.V.)	13	320	160	320	++++	2.5×10^{6}
	14	40	0	40	++++	2×10^{5}
	15	80	0	0	+	3.3×10^{4}
	16	160	160	40	+++	2.8×10^{6}

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

* Viable counts of bacilli contained in 1 gm of tissue. For other signs and symbols, see the footnotes of Table I.

‡ For meaning of these signs, see the text.

sive lesion. It is also generally accepted that, in experimental tuberculosis, the progression of the disease is more rapid in the case of intravenous challenge than in the case of subcutaneous challenge, because, following subcutaneous challenge, the dissemination of challenged bacilli is hampered by the formation of caseous, encapsulated foci at the site of challenge, while, following intravenous challenge, it takes place rapidly to engender severe systemic disease.

In view of the above facts, the antipolysaccharide and antiprotein, especially the former, may be said to be produced almost universally, if only "infection" takes place, without regard to the severity of the disease engendered by infection, *i.e.*, the mere *in vivo* presence of tubercle bacilli, if any, might be the major

condition for production of the two kinds of antibodies. This is probably due to the water solubility of polysaccharides and proteins of the tubercle bacillus.

It is of great interest that mere infection seems not to stimulate an important production of the antiphosphatide. It is evident from the present data that the production of the antiphosphatide is greatly influenced by the severity of the disease subsequent to infection. The convincing proof thereof lies in the facts that the antiphosphatide production scarcely takes place in the animals given subcutaneous injections of BCG, while it takes place evidently in the animals which received intravenous injections of the same organisms, and that the antiphosphatide tends to be produced to a higher extent in the animals challenged with virulent tubercle bacilli. This peculiar mode of production of the antiphosphatide is probably due to the physicochemical nature of phosphatide. As is well known, tubercle phosphatide, like phosphatides of animal and plant origins, is hygroscopic but not water-soluble. It may be, therefore, that the antiphosphatide production is not stimulated until *in vivo* tubercle bacilli are destroyed to such an extent that an amount of phosphatide sufficient to produce antibodies is released from the bacillary cells. In other words, the antiphosphatide production might take place mainly under the condition where *in vivo* bacilli undergo destruction, *i.e.*, under the condition where "disease" is in progress subsequent to infection.

Be that as it may, the present investigations strongly suggest that, of the three different kinds of antibodies, antipolysaccharide, antiprotein, and antiphosphatide, only the antiphosphatide might deserve to be followed up in the serological diagnosis of tuberculosis. This presumption has been shown to be true in a study in which the three foregoing tests were performed in patients with pulmonary tuberculosis and in tuberculin-positive, clinically healthy persons. The experimental data thereof will be given in the succeeding paper.

Finally, no direct relationship was found between the amount of any of the three kinds of circulating antibodies and the degree of tuberculin skin hypersensitivity. It should be especially borne in mind in this connection that even the amount of circulating antiproteins had no bearing on the degree of hypersensitivity measured by the tuberculin skin test, despite the accepted fact that tuberculin skin hypersensitivity is engendered by sensitization with tubercle proteins (3, 7, 8).

SUMMARY

The mode and the level of production of the three different kinds of antibodies, antipolysaccharide, antiprotein, and antiphosphatide, were found to differ considerably with the mode of infection and the virulence of tubercle bacilli. Evidence is given that production of the antipolysaccharide and antiprotein is stimulated without regard to the mode of infection and the virulence of bacilli, while the antiphosphatide is produced chiefly under conditions where

in vivo bacilli might have undergone destruction. Of the three antibodies, the level of antiphosphatide was shown to reflect most faithfully the progression of experimental tuberculous infection.

The amounts of the three circulating antibodies were found to have no direct relationship to the degree of tuberculin skin hypersensitivity.

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