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Saprophytic Acidfast Bacilli and Paraffin Oil as Adjuvants in Immunization.*

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It was found recently[1] that horse serum combined with a lanolin-like substance and killed tubercle bacilli suspended in paraffin oil induces more vigorous antibody formation and sensitization than horse serum alone. Antibody formation and sensitization last for a remarkably long time in the animals injected with horse serum and the adjuvants.

The use of these potentiating agents was applied by Landsteiner and Chase[2] and Chase[3] to sensitization and antibody formation using chemicals and protein conjugates. Kopeloff and Kopeloff[4] with the aid of this method succeeded in demonstrating antibody formation against brain extracts in the *macacus rhesus* monkey.

The purpose of the present study was to determine whether *Mycobacterium phlei* can be substituted for tubercle bacilli and what the effect of the lanolin-like substance and paraffin oil is on antibody formation and sensitization. It seemed important to ascertain whether killed timothy bacilli under the conditions of the experiment induce sensitization to tuberculin.

Four groups of guinea pigs weighing from 590 to 1320 g received in the subcutaneous tissue of the back of the neck 0.5 ml of antigen diluted with salt solution or combined with the adjuvants. The materials used are shown in Table I.

The intervals between the injection of antigen and the tests were as follows: titration of antibodies against horse serum 47, test for skin sensitivity to horse serum 49, to tuberculin 62, and to phleolin 133 days.

The collodion agglutination test was done by coating collodion particles with a 1:10 dilution of horse serum. The washed particles were then suspended in 1 ml of guinea pig serum dilutions, the mixtures kept at 4°C overnight and read the next day. Details of the technic may be found in previous publications.[5]

Sensitization to horse serum was tested by intracutaneous injections of 0.1 ml horse serum, either undiluted or diluted 1 in 10. Tuberculin and phleolin tests were made by injecting 0.1 ml of diluted material into the skin.

Table II shows that horse serum in combination with Falba and paraffin oil induced antibody formation more effectively than horse serum alone; the addition of killed acidfast bacilli to the water-in-oil emulsion had an additional augmenting effect on the production of antibodies. Tubercle bacilli were slightly more effective than timothy bacilli.

Table III shows the extent of skin reactions to undiluted and diluted horse serum. Falba and oil seem to have enhanced sensitization only very slightly if at all but the addition of acidfast bacilli to the emulsion intensified the sensitization in a striking manner. Again tubercle bacilli were more effective than timothy bacilli.

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2 Landsteiner, K., and Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 688.

3 Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 238.

4 Kopeloff, L. M., Barrera, S. E., and Kopeloff, N., *Am. J. Psychiat.*, 1942, **98**, 881; Kopeloff, L. M., and Kopeloff, N., *Fed. Proc.*, (Am. Soc. for Exp. Biol.), 1943, **2**, 99; Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1944, **48**, 297.

5 Freund, J., PROC. SOC EXP. BIOL. AND MED., 1930, **28**, 6570; *J. Exp. Med.*, 1932, **55**, 181; *Science*, 1932, **75**, 418.

TABLE I.
Material Injected.

Group	Horse serum, ml	Falba,* ml	Paraffin oil, ml	Killed tubercle bacilli, mg (dry wt)	Killed timothy bacilli, mg (dry wt)	Salt sol. 0.85% ml
A	.125	.125	.25	.1	0	0
B	.125	.125	.25	0	.1	0
C	.125	.125	.25	0	0	0
D	.125	0	0	0	0	.375

* Falba is the trade name of a lanolin-like substance prepared by Pfaltz and Bauer, Inc., New York City.

TABLE II.
Collodion Agglutination Titers.
Number of Sera with Titers Indicated.

	Serum dilutions								
Group	640	320	160	80	40	20	10	5	<5
A	2	3	3						
B		2	2	3					1
C	1		2	1	1	1	2		
D						1	3	2	1

Sera from unimmunized guinea pigs did not react in dilutions higher than 1:5.

TABLE III.
Skin Reactions to Horse Serum.
Number of Guinea Pigs with Reactions Designated.

		Horse serum													
		Undiluted								Diluted 1:10					
Reading	Group	N	P	5	4	3	2	1	±	N	3	2	1	±	0
24 hr	A	3	5	2	5	1				2	1	7			
	B		3		6	2					2	1	3	2	
	C		2	1	3	1	3						1		7
	D				4	1	1							2	4
48 "	A	8		4	4					1			4	1	3
	B	1		4	3	1							1	2	5
	C	1		1	5	1		1							8
	D				2	3	1								6
72 "	A	8		4	1	2		1		3			2	3	3
	B	5		4	1	2	1			1			1	1	5
	C	1			1	3	2	1	1					1	7
	D				2	3	1								6

N Necrosis.
P Purple area.
Edema—diameter in mm:
5 75 or more
4 60-74
3 45-59
2 30-44
1 15-29
± Doubtful
0 Less than 15

Table IV shows that all of the guinea pigs which received killed tubercle bacilli became highly sensitive to tuberculin but only one of them reacted to phleolin. Seven of 8 animals injected with timothy bacilli developed sensitiveness to phleolin and all of them reacted to

TABLE IV.
Skin Reactions to Tuberculin and Phleolin.
Number of Guinea Pigs with Reactions Designated.

	Tuberculin						Phleolin					
	1:100			1:1000			1:100			1:1000		
Group	N	3	2	2	1	0	2	1	0	2	1	0
A	3	6	2	8				1	7			8
B			8		1	7	7		1	3	1	4

Reactions read 48 hours after test injections:
N Necrosis.
Induration—diameter in mm:
3 20-29
2 10-19
1 5-9
0 Less than 5

tuberculin. It may be added that the animals injected with horse serum incorporated in Falba and oil did not react to either tuberculin or phleolin.

At the site of the injections of mixtures containing tubercle or timothy bacilli palpation did not reveal the presence of nodes and suppuration through the skin did not occur.

Discussion. In a previous study[6] it was found that the incorporation of killed typhoid bacilli suspended in salt solution into lanolin-like substances (Aquaphor or Falba) and paraffin oil enhances and prolongs the antigenic effect of typhoid bacilli as judged by the formation of agglutinins. The data presented above concerning antibody formation against horse serum as measured by the collodion agglutination test are in harmony with our previous observations. It is interesting to note that Falba and paraffin oil alone had little if any effect on sensitization to horse serum.

In the preceding paper[6] observations were made as to the effect of the addition of killed tubercle bacilli to a water-in-oil emulsion containing typhoid bacilli. It was found that tubercle bacilli had an additional potentiating effect. In the present experiment a similar result was seen in relation to antibody formation against horse serum. Furthermore it was found that the timothy bacilli employed in this experiment were almost as effective as the pathogenic acidfast bacilli.

[6] Freund, J., and Bonanto, M. V., *J. Immunol.*, 1944, **48**, 325.

Not only tubercle bacilli but also timothy bacilli sensitized the guinea pigs to tuberculin although to a lesser degree. Sensitization to phleolin, however, was demonstrable in only 1 of 8 animals receiving tubercle bacilli; 7 of 8 pigs injected with timothy bacilli reacted to phleolin. It is possible that more of the guinea pigs with tubercle bacilli might have reacted to a higher concentration of phleolin than the one used in this experiment. It may be added that the potency of tuberculin and that of phleolin can not be compared. From the standpoint of human immunization cross-sensitization is important, particularly the observation that timothy bacilli sensitized to tuberculin. The synergistic agents studied may be applicable to the immunization of experimental and domestic animals. In man killed tubercle or even timothy bacilli when suspended in paraffin oil may cause suppuration particularly in persons sensitive to tuberculin. Search is in progress to find potentiating agents to replace the acidfast bacilli employed in the present experiment.

Paraffin oil may cause persistent nodules. Vegetable oil might be less objectionable in immunization of man. It was shown in a preceding paper[6] that peanut oil is effective though not as much so as paraffin oil in enhancing and sustaining antibody formation. A comparative study of various vegetable oils is in progress.

Conclusions. 1. A lanolin-like substance and paraffin oil when combined with horse serum enhance the formation of antibodies but have little if any effect on sensitization to

horse serum. 2. Killed timothy bacilli added to the water-in-oil emulsion promote both antibody formation and sensitization almost as effectively as killed tubercle bacilli. 3. Under the conditions of this experiment killed timothy bacilli sensitize to tuberculin.

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Agglutination of Circulating Leukocytes by Antileukocytic Sera.

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Several investigators had produced antisera against animal and human leukocytes,[1-6] but no successful attempt has been made to test and titrate the antileukocytic serum by agglutination. Some of the workers stated that the cytotoxic qualities of serum prevent a correct evaluation by agglutination.[2,4,7,8] Hueper and Russell[4] expressed the view that tissue culture is the only satisfactory method for correct titration of antileukocytic serum. In our experiments herein reported we were more successful and are able to present a method of agglutinating circulatory leukocytes by antileukocytic sera and of titrating the antisera.

Leukocytic agglutinins were produced in Chinchilla rabbits by intravenous injections of leukocytes secured from normal and leukemic individuals. Normal leukocytes were obtained from blood bank material used for preparation of plasma. The blood was centrifuged, the buff layer removed and washed several times. The animals received a total of 12 to 14 injections of 11,700,000 to 20,100,000 cells suspended in one cc of normal salt solution, twice weekly. Testing was done on the serum pooled from at least 3 animals. Blood was obtained by cardiac puncture in volumes of 10 to 15 cc. When agglutinins were demonstrated in a dilution of 1:2560 the antiserum was considered satisfactory for titration. Antisera were developed against lymphocytes and against cells of the granulocytic series of mature and immature types by the use of blood from normal individuals and from those with lymphoid and myeloid leukemia. Normal rabbit serum and normal salt solution were used as controls for the antisera.

Titrations were set up in a series of 10 x 75 mm test tubes. Blood was collected and mixed with a dry anticoagulant of ammonium and potassium oxalate, centrifuged and the buff layer pipetted off. The leukocytes were suspended in and the antisera diluted with normal salt solution. The necessity for the presence of electrolytes for the visible phase of agglutination has been demonstrated.[9,10] To ascertain the optimum number of leukocytes for an agglutination test, the cell concentration was varied and the volume of antiserum was kept constant. It was determined that 0.05 cc of normal salt solution containing 27,000 cells per cu mm constituted a satisfactory suspension. The leukocyte suspension was added to 0.5 cc volumes of inactivated antiserum diluted with normal salt solution in dilutions of from

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