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Sensitization to Horse Serum by Means of Adjuvants.

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Lewis and Loomis[1] discovered that tuberculous guinea pigs produce more antibodies when injected with various antigens not related to tubercle bacilli than non-tuberculous guinea pigs. Dienes and Schoenheit[2] made the interesting observation that when egg white or horse serum is injected into tuberculous foci sensitization to these antigens is different from that usually seen in guinea pigs free of tuberculosis. Some aspects of the difference are: In tuberculous guinea pigs the cutaneous reactions to animal proteins appear and disappear slower; in tuberculous guinea pigs the reactions are often necrotic but in the non-tuberculous pigs very rarely if ever. This observation was confirmed by Freund[3] and by Hanks.[4] Dienes[5] reported that he was able to reproduce his experiment when killed tubercle bacilli were substituted for living ones. Three different methods of sensitization were partly or wholly successful. The first technic requires repeated injections of killed tubercle bacilli into a lymph node and after a lapse of a few weeks the repeated injections of a mixture of killed tubercle bacilli and egg white into the site of previous injections. The second method consists of two intraäbdominal injections. Twenty mg of killed tubercle bacilli are injected into the peritoneal cavity and 10 days later 20 mg of tubercle bacilli mixed with egg white. Some of the guinea pigs so treated die after the second injection from shock. The third method, which uses the testicles for the site of injections, does not yield consistent results.

In view of the advantages of employing killed tubercle bacilli instead of living ones we attempted to sensitize guinea pigs with killed tubercle bacilli and egg white. We made a single injection of killed tubercle bacilli into the subcutaneous tissue of the groin and 2 days later we injected egg white into the same site. The sensitization to egg white was not of the tuberculin type.

1 Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, **40**, 503; 1925, **41**, 327; 1926, **43**, 263.

2 Dienes, L., and Schoenheit, E. W., *Am. Rev. Tub.*, 1929, **20**, 92; *J. Immunol.*, 1930, **19**, 41.

3 Freund, J., *J. Exp. Med.*, 1934, **60**, 669.

4 Hanks, J. H., *J. Immunol.*, 1935, **28**, 105.

5 Dienes, L., *J. Immunol.*, 1928, **15**, 153; 1929, **18**, 531.

Since the addition of paraffin oil to killed tubercle bacilli enhances both the lesion and sensitization to tuberculin produced by killed tubercle bacilli[6] we[7] injected egg white into lesions produced by killed tubercle bacilli suspended in paraffin oil. Sensitization to egg white was not different from that observed in control guinea pigs. Landsteiner and Chase,[8] however, found that "sensitization to a simple chemical, picryl chloride, can be satisfactorily attained by means of intraperitoneal injections of the compound when killed tubercle bacilli suspended in paraffin oil were used as an adjuvant." These authors[9] later, using the same adjuvant, produced sensitization of the contact-type to conjugates made with homologous erythrocytic stromata. In the experiments just mentioned Landsteiner and Chase injected into the peritoneal cavity killed tubercle bacilli suspended in oil once or twice and soon afterwards the sensitizing substance.

It occurred to us that the difficulty of influencing sensitization to proteins (like egg white) by means of killed tubercle bacilli suspended in paraffin oil might be overcome by combining the antigen in the aqueous phase with a lanolin-like substance and in turn suspending the combination in paraffin oil containing tubercle bacilli. We used a material sold under the name Aquaphor* which can be combined with larger amounts of water than lanolin.

Aquaphor and heavy paraffin oil were sterilized in the autoclave. Tubercle bacilli of human type, a strain designated Jamaica No. 22, were grown on glycerol broth and heated in the Arnold sterilizer at 100°C for half an hour. They were dried *in vacuo* over phosphorus pentoxide, weighed and suspended in paraffin oil.

Horse serum was chosen for these experiments and was combined with the adjuvants in the following way. Ten ml of horse serum were added in small amounts to 10 ml of Aquaphor in a mortar

6 Freund, J., Casals-Ariet, J., and Hosmer, E. P., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 509; Freund, J., Casals-Ariet, J., and Genghof, D. S., *J. Immunol.*, 1940, **38**, 67.

7 Freund, J., and Casals-Ariet, J., unpublished experiment, 1937.

8 Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1940, **71**, 237.

9 Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1941, **73**, 431.

* "Eucerin or Aquaphor, is an ointment base obtained from wool fat, possessing a much greater water-absorbing capacity than lanolin; it being capable of taking up 7 to 8 times its own weight in water. The saponifiable fatty acids are removed from the crude suint, from the cholesterin mixture (or soft fat) consisting of the esters of isocholesterol and oxycholesterol with oleic, myristinic and carnaubic acids. To this soft fat, petrolatum hydrocarbons are added as diluents and the product is marketed under the trade name." Arny, H. V., and Fischelis, R. P., *Principles of Pharmacy*, W. B. Saunders Co., Philadelphia and London, 1927, page 140.

and the two blended thoroughly. Twenty ml paraffin oil containing 40 mg killed tubercle bacilli were mixed in the mortar with the combination of horse serum and Aquaphor. The whole was shaken in a test tube and 0.5 ml injected into guinea pigs. One dose contained 0.125 ml horse serum, 0.125 ml Aquaphor, 0.5 mg killed tubercle bacilli and 0.25 mg paraffin oil. The guinea pigs weighed from 690 to 920 g. Five animals were injected intramuscularly into the back of the neck about 1 cm laterally from the midline† and 5 intramuscularly into the thigh. They were tested by intracutaneous injection of various concentrations of horse serum 7, 13, and 19 days later and again 6 and 12 months after injection. Another 10 guinea pigs were injected with 0.125 ml of horse serum diluted with saline solution into the back of the neck. They were tested with horse serum in exactly the same way as the animals sensitized with horse serum and the adjuvant 1, 2, 3 weeks and one year after sensitization. The cutaneous reactions were observed 3 to 4 hours after the injection and then every day until they disappeared. The experiment was repeated; 6 guinea pigs were injected with horse serum and the adjuvants into the neck and 6 with horse serum alone.

The adjuvants modified sensitization in several respects. (1) The rate of appearance of the reactions to undiluted horse serum was similar in all groups of pigs, *i. e.*, conspicuous swelling was present in 3 hours; but in the first experiment with the higher dilutions of horse serum there was a difference in the rate of appearance of reactions. In the group with tubercle bacilli the swelling was noticeable only one day after the injection except in the test one year after the sensitiza-

TABLE I.
Necrotic Reactions to Horse Serum.

Time between sensitizing and tests	Guinea pigs sensitized					
	with horse serum + adjuvants			with horse serum alone		
	1:1*	1:10	1:100	1:1	1:10	1:100
1 wk.	—	2/10	0/10	0/8	0/10	0/10
2 "	8/10†	4/10	0/10	0/10	0/10	0/10
3 "	5/8	0/8	0/8	0/10	0/10	0/10
6 mo.	4/5	1/5	—	—	—	—
12 "	2/3‡	0/3	—	0/2	0/2	—

*Horse-serum dilution.
†8 of 10 pigs with necrosis.
‡Hemorrhage within 3 hours followed by necrosis.

† The advantages of the neck as a site of injection are: the distance from the site of skin-tests and the ample space for the lesion caused by the adjuvants. The pigs injected into the neck became slightly more sensitive than those injected into the thigh.

TABLE II.
Cutaneous Reactions One Year After Sensitization.

After intracutaneous inj., hrs	Guinea pigs sensitized									
	with horse serum and adjuvants						with horse serum alone			
	No. 1		No. 2		No. 3		No. 4		No. 5	
	1:1*	1:10	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10
3	35x35x3† H 8x4	35x35x3	40x40x2 H 15x8	30x15x2 H 8x4	30x15x4 H 10x5	30x30x3	0	0	0	0
24	50x45x3 H 8x4	40x40x4 H 6x4	50x50x10 H 8x5	20x20x1	75x50x5 C		15x15x1 I.d.	0	25x25x1 I.d.	0
48	75x55x4 N 5x4	C	80x60x10 N 9x9	C	65x60x4	C	0	0	0	0
72	80x60x4 N 5x4	C	50x45x5 N 5x5	scaling 4x4	scaling 5x5	0 C	0	0	0	0

*Serum dilution.
†Diameter of redness and swelling in mm.
H—hemorrhage.
N—Necrosis.
C—Confluent.
I.d.—Ill defined.

tion, whereas it was seen in the control group within 3 hours. With the former animals all the reactions reached their maximum in 48 hours, in the latter group in 24. However, in the second experiment all the reactions appeared within 3 hours in both groups of animals. (2) The disappearance of the reactions was strikingly different in the two groups. In the group with tubercle bacilli the reactions persisted for 72 hours or longer and in the control group disappeared within 48 hours. (3) There was a sharp difference as to the presence or absence of necrosis. Necrosis occurred frequently at the site of injections of undiluted and occasionally diluted (1:10) horse serum in the group with the adjuvants but never in the group injected with horse serum alone. (Table I.) (4) Reactions to higher dilutions of horse serum occurred more frequently in the group sensitized with the aid of adjuvants. In this group 6 of 10 pigs reacted to a 1:100 dilution of horse serum whereas in the control group only 2 of 10 (test one week after sensitization).

The striking results of skin-tests made one year after sensitization are shown in Table II.

In the second experiment 28 days after the initial injection precipitin-titrations were made using 1 ml horse-serum dilutions and 0.1 ml guinea pig serum. The results are shown in Table III.

The repeated intracutaneous test-injections probably contributed to the sensitization and precipitin-formation.

The mechanism of the effect of the adjuvants used is a complex one. Killed tubercle bacilli probably play the same rôle as living ones, though their rôle has not been elucidated; paraffin oil enhances the cellular reaction caused by tubercle bacilli and protects the bacteria from destruction and sustains sensitization and antibody-formation.[9] Aquaphor may have two effects. It may retard the possible separation of the horse serum, thus delaying destruction and elimination. It

TABLE III.
Precipitin-titers 28 Days After the Injection of

	Horse serum and adjuvants				Horse serum alone			
Guinea pig	1:10	1:100	1:1000	1:5000	1:10	1:100	1:1000	1:5000
1	+++	+	Trace	Faint trace	Trace	+	0	0
2	+++	++	+	0	+	Trace	0	0
3	+++	++	Trace	0	+	Trace	0	0
4	+++	++	+	0	+	Trace	0	0
5	+++	++	+	0	Trace	Trace	0	0
6	—	—	—	—	0	0	0	0

+++ Precipitate on the bottom of the tube.
— Not done.
0 Negative.

may be recalled that Ramon[10] has shown that the addition of lanolin to diphtheric toxin promotes antitoxin production; there are no data in Ramon's papers as to the duration of antitoxin-formation after the injection of toxin combined with lanolin.

Conclusions. When guinea pigs receive an injection of horse serum combined with a lanolin-like substance and killed tubercle bacilli suspended in oil, sensitization to horse serum is similar to that seen in guinea pigs injected with living tubercle bacilli and horse serum insofar as the reactions to the intracutaneous injection of horse serum lasts longer than 48 hours and may be necrotic. The duration of intense sensitization is remarkably long. Precipitin-titers are higher in guinea pigs immunized with the aid of adjuvants. Under the conditions of these experiments, the use of the lanolin-like substance seems to be essential.

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Concentration of Dilute Solutions of Virus of Mouse Encephalomyelitis by Pervaporation and Ultracentrifugation.*

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The finding of the virus of poliomyelitis in sewage emphasizes the importance of methods for its detection in dilute, aqueous solutions.[1, 2] A satisfactory method should be able to concentrate the virus from dilute, non-infectious solutions into small infectious volumes, free from bacteria and chemical and bacterial toxins. To attain this end the experiments to be described represent an attempt to exploit the principles of pervaporation and ultracentrifugation.

Mouse encephalomyelitis virus (Theiler's mouse poliomyelitis virus) was chosen because its properties with respect to filtration, thermal inactivation, and stability to ether, and its capacity of producing an infectious myelitis in mice indicate a close similarity to

10 Ramon, G., Lemetayer, E., and Richou, R., *Rev. d'Immunol.*, 1937, **3**, 202.

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† National Research Council Fellow in the Medical Sciences, 1941-42.

1 Paul, J. R., Trask, J. D., and Gard, S., *J. Exp. Med.*, 1940, **71**, 765.

2 Gard, S., *J. Exp. Med.*, 1940, **71**, 779; Trask, J. D., and Paul, J. R., *J. Exp. Med.*, 1942, **75**, 1.