

THE USE OF ADJUVANTS IN SENSITIZATION OF THE MOUSE

Saul Malkiel, M.D., Ph.D., and Betty J. Hargis, M.A., Boston, Mass.

WE HAVE repeatedly observed¹ in experiments on mice that a consequence of the administration of *Hemophilus pertussis* phase I organisms given in conjunction with a protein antigen is the enhancement of anaphylactic sensitization to the foreign protein antigen. A concomitant increase in the sensitivity of the pertussis-inoculated mouse to serotonin and histamine results also.²⁻⁴ The former effect is probably related to an increase in antibody titer, fixed or sessile, even though neither circulating precipitating nor univalent antibodies have been detected.¹ Since the use of extraneous substances, including bacteria, for the intensification of antibody responses is well known in other species,⁵ it was deemed of interest to investigate the adjuvant effect of a variety of agents in the mouse primarily to correlate anaphylactic sensitization with an increase in antibody titer and a decrease in resistance to the lethal effects of histamine.

MATERIALS

Mice.—Female white mice of the Swiss-Webster strain, each weighing about 20 grams at the time of the experiments, were obtained from Tumblebrook Farm and were used exclusively.

H. pertussis.—A concentrated paste of phase I organisms containing Merthiolate 1:1,000 was supplied by Merck Sharp & Dohme, Inc. This was washed and diluted as required. The semisynthetic, cell-free culture filtrate of Kuwajima and associates⁶ on which *H. pertussis* had been grown was used in certain of the experiments in the place of *H. pertussis* vaccine.* This was dialyzed and concentrated.

Adjuvants.—The following adjuvants were employed: *India ink*, commercial Higgins American India Ink, black; *gelatin* (Bacto-Gelatin, Difco), 5 per cent solution in 0.85 per cent saline; *agar* (Bacto-Agar, Difco), 0.25 per cent solution in 0.85 per cent saline; *kaolin* (5 per cent suspension of Infusorial Earth, B & A), in 0.85 per cent saline; *dextran* (Plavolex, Wyeth), 6 per cent

From the Departments of Medicine and Pathology, Harvard Medical School and the Peter Bent Brigham Hospital.

This investigation was supported by a research grant from the National Institutes of Health, United States Public Health Service.

Received for publication Jan. 28, 1959.

*We are grateful to Miss Wetterlow of the Public Health Department, Institute of Laboratories, Jamaica Plain, Massachusetts, for making this filtrate available to us.

w/v; *polyvinylpyrrolidone* (PVP) (20 per cent solution of Plasdone, General Aniline and Film Corp.), in 0.85 per cent saline, diluted as needed to make a 10 per cent solution; *zymosan*, concentrations ranging from 0.2 to 1.0 per cent suspension in 0.85 per cent saline. The *zymosan* had been either prepared in this laboratory according to the method of Pillemer and Ecker⁷ or purchased from Standard Brands.

METHODS

Sensitization.—Sensitization was effected by a single intraperitoneal injection of a mixture of a given antigen (either 0.03 ml. of horse serum or 1.0 mg. of thrice-recrystallized ovalbumin) and the appropriate "schlepper" or adjuvant agent in a 0.5 ml. volume.

TABLE I. EFFECT OF ADJUVANT ON HISTAMINE AND ANAPHYLACTIC SENSITIZATION

ADJUVANT*	SENSITIZING ANTIGEN†	HISTAMINE PHOSPHATE‡	CHALLENGE ANTIGEN§	
			HORSE SERUM	OVALBUMIN
—	—	1/10	0/11	
—	Ea	0/10		1/10
—	Ea (I.V.)			0/20
—	HS	3/19	5/35	
<i>H. pertussis</i>	Ea	24/25		16/16
6 × 10 ⁹ cells	HS	10/10	52/52	
Filtrate concen- trate	HS	5/6	9/9	
	Ea			9/10
	Ea			10/10
India ink	HS	0/19	19/19	
	Ea			1/10
Gelatin 5%	HS	3/19	3/15	
	Ea			0/10
Agar 0.25%	HS	2/19	8/17	
	Ea			10/10
Kaolin 5%	HS	2/18	13/15	
Kaolin 5%	Ea (I.V.)			12/12
Dextran 6%	HS	1/19	8/18	
PVP 10%	Ea			2/10
20%	HS	3/19	4/16	
Zymosan 1 mg.	HS		9/17	
3 mg.	HS	0/20	27/40	
5 mg.	HS		27/44	
3 mg.	—	1/10	0/10	

*Contained in 0.5 ml. volume.

†0.03 ml. horse serum (HS) or 1 mg. ovalbumin (Ea) given intraperitoneally except where otherwise indicated.

‡0.03 mg. per gram mouse of 0.2 per cent solution given intraperitoneally.

§0.1 ml. HS or 1 mg. Ea in 0.1 ml. 0.85 per cent saline given intravenously.

||Number of animals dead per number of animals challenged.

Histamine Sensitivity.—On the fourth day after sensitization the mice received 0.03 mg. of histamine phosphate per gram of animal intraperitoneally as a 0.2 per cent solution. This is approximately the LD₁₀₀ dose for pertussis-inoculated mice. The results of the challenge are recorded as number of animals dead per number of animals injected.

Anaphylactic Sensitivity.—On the tenth day after sensitization mice were challenged by the intravenous injection of the homologous antigen, 0.1 ml. of horse serum or 1 mg. of ovalbumin in 0.1 ml. saline. The results are recorded as number of animals dead per number of animals challenged.

RESULTS

As can be seen from Table I, only *H. pertussis* was found capable of decreasing the resistance of the white mouse to histamine. On the other hand, all of the adjuvants were found capable of enhancing sensitization to some varying extent. The particulate substances—India ink, kaolin, and zymosan—engendered the highest degree of sensitization, and the more soluble agents produced the least. These findings are quite compatible with the concept of enhancing antibody formation by particulate matter onto which soluble antigen has been adsorbed.

TABLE II. TIME RELATIONSHIP BETWEEN ZYMOBAN AND ANTIGEN ADMINISTRATION AND ANAPHYLACTIC SENSITIZATION

DAY OF ADMINISTRATION											
-6	-4	-2	0	2	4	6	8	10	12	14	16
HS*			Z†		1/20‡			1/16			
	HS		Z			0/20		0/19			
		HS	Z				1/20	0/20			
			Z					24/40			
			HS					7/20	7/20		
			Z	HS				4/16		6/17	
			Z			HS		0/18			8/16
			Z I.V.					6/18			
			HS I.P.								
			Z		Z	HS I.P.		Day 11 0/20		5/20	
			Z					0/10			

*Sensitization by 0.03 ml. horse serum (HS) given intraperitoneally.

†3 mg. zymosan given intraperitoneally in 0.5 ml. volume.

‡Intravenous challenge by 0.1 ml. horse serum; number of animals dead per number of animals challenged.

The optimal time relationship for the administration of antigen and adjuvant resulting in maximal sensitization was studied by means of the zymosan system. Antigen (horse serum) was given intraperitoneally to mice at various time intervals before and after the intraperitoneal inoculation of 3 mg. of zymosan as well as concurrently. About half of the mice of each time group were challenged by the intravenous injection of 0.1 ml. of antigen on the tenth day following the initial inoculation of the sensitizing antigen. The other half of the group of mice in each series were challenged on the tenth day following the inoculation with zymosan. Table II shows the results, given as number of animals dead from anaphylactic shock per number of mice challenged. Sensitization is seen to be best accomplished by administering the antigen together with the zymosan. Antigen given prior to zymosan does not enhance sensitization; no increase in the number of anaphylactic deaths

is noted with administration of the challenge dose either ten days after the sensitizing dose or ten days after the zymosan, even when the sensitizing dose precedes the zymosan by only two days. Antigen given up to six days after the administration of zymosan results in a relatively high degree of sensitization when the challenge dose is given ten days subsequently. As the interval between the sensitizing and challenge dose is decreased, the number of anaphylactic deaths decreases. Within the limits of the experiment, it can be said that the minimal time interval between the sensitizing and challenge doses for maximal anaphylactic response is ten days. The relationship between the time of zymosan administration and sensitizing antigen administration does not appear to be critical, provided that the latter is given concurrently or following the adjuvant. The studies further revealed that the intravenous administration of zymosan does not engender a higher degree of sensitivity and that repeated inoculations of zymosan have no greater effect in enhancing sensitization.

TABLE III. ANTIBODY TITRATION OF MOUSE ANTISERUM BY BOYDEN TECHNIQUE

SERUM DILUTION	DAY ON WHICH HORSE SERUM GIVEN IN RELATION TO ZYMOSAN							ZYMOSAN I.V. HORSE SERUM I.P. BOTH ON DAY 0
	-6	-4	-2	0	2	4	6	
1:5	-	-	-	++	++	-	-	++
1:10	-	-	-	++	++	-	-	++
1:20	-	-	-	++	++	-	-	++
1:40	-	-	-	++	++	-	-	++
1:80	-	-	-	++	++	-	-	++
1:160	-	-	-	++	++	-	-	±
1:320	-	-	-	-	++	-	-	±
1:640	-	-	-	-	±	-	-	±
1:1,280	-	-	-	-	±	-	-	-
1:2,560	-	-	-	-	±	-	-	-
Control, un- coated cells	-	-	-	-	-	-	-	-
Anaphylactic deaths	1/16	0/19	0/20	10/20	7/20	4/16	0/18	6/18

Sera withdrawn on tenth day after intraperitoneal zymosan, prior to challenge.
Reactions: ++, positive; ±, questionable; -, negative.

The specific effect of zymosan on the titer of antibody was further studied because of the relationship of zymosan to the immune system⁸ and the above observations that it possesses the marked ability to enhance sensitization. Blood was withdrawn from the ophthalmic sinus ten days after zymosan administration, just prior to challenge. Blood samples from mice within each group were pooled and allowed to clot, and the expressed serum was removed. Antibody studies were carried out by the Boyden tanned-red-cell technique,⁹ using human red blood cells of type A in the titrations. The results of these studies are seen in Table III. A direct correlation is observed between maximal anaphylactic sensitization and highest antibody titer. However, anaphylactic deaths (25 per cent) did occur in one group in the absence of any titrable antibody.

DISCUSSION

Anaphylactic sensitization by the use of *H. pertussis* may be attributed to the adjuvant capabilities of bacteria in general. They have this capacity in common with a variety of extraneous and unrelated substances as foreign proteins, lipids, carbohydrates, metallic salts, inert adsorbents, etc. One of the criteria of a classical adjuvant is demonstrated by *H. pertussis* in that adsorption of a soluble antigen may take place on its cellular surface, thus allowing for increased retention of antigen in the tissues and providing for a slower and more gradual release of antigen for prolonged stimulation of antibody-forming sites. In addition, there is the possibility of wider dissemination of the antigen in the body with its inevitable greater accessibility to the antibody-forming sites as a result of phagocytosis of particulate matter by the cells of the reticuloendothelial system.

Most of the experimental data, however, seem to mitigate against the acceptance of the classical definition of adjuvant as applied to the mode of *H. pertussis* action. Previous studies¹ utilizing Freund's adjuvant in the white mouse have shown that although anaphylactic sensitization does occur, it neither appears as rapidly nor reaches the high degree of sensitization as it does with *H. pertussis*. Adler¹⁰ has shown appreciable titers of antibody to antigens in the mouse, using an incomplete Freund's adjuvant at a time (twenty-eight days) beyond which maximum anaphylactic sensitization is known to occur (ten to fifteen days). Freund's adjuvant does not enhance histamine sensitivity.¹¹

In addition, there are the following facts: (1) *H. pertussis* also increases susceptibility to histamine² and serotonin^{3, 4} in the mouse. (2) *H. pertussis* in various stages of growth other than phase I does not have the ability to sensitize.¹² (3) Heating *H. pertussis* causes a marked decrease in the capacity of the organism to sensitize.^{13, 14} (4) Other serologically related organisms, such as *H. influenzae*, fail to enhance either anaphylactic sensitization or susceptibility to histamine.¹⁵

The results of this study indicate that the typical adjuvants which have been used previously to increase antibody titer in other species are capable of enhancing anaphylactic sensitization in the mouse. The zymosan studies indicate that an increased antibody titer is apparently responsible for this sensitization. However, anaphylactic deaths were noted even though circulating precipitins could not be detected in all the sera by the tanned-red-cell technique of Boyden. It is to be recalled that precipitins have not been detected previously by either the ring technique or the Heidelberger-Kendall quantitative procedure in the sera of mice made highly sensitive.¹ This could mean that only very small, immeasurable quantities of circulating antibody need be available for anaphylaxis to occur. Passive transfer studies^{16, 17} in pertussis-inoculated mice have indicated that about 4 to 8 mg. of antibody nitrogen per kilogram of the mouse's weight would consistently sensitize the animal. If this remained as circulating antibody, it would be well within the limitations of the Boyden titration technique. Another possibility is that the

type of sensitizing antibody produced cannot be measured by the techniques employed. Our previous studies¹⁸ have suggested that *H. pertussis* is capable of enhancing the production of more than one type of antibody, depending upon the antigen with which it is administered. Thus, it was found that there were produced in the mouse a sensitizing antibody against diphtheria toxoid and a protective antibody in response to stimulation by tetanus toxoid.¹⁹ A final proposition is that fixed or tissue antibody is primarily responsible for anaphylaxis. This concept is of considerable interest and will bear extensive investigation.

The mechanism for the enhancement of anaphylactic sensitization by *H. pertussis* is not understood, but there is a tendency to relate this phenomenon to the concomitant sensitization of the pertussis-inoculated mouse to histamine and serotonin,¹² a mechanism which also is not known. None of the adjuvant agents used in these experiments had any effect upon sensitization to histamine. In this the action of *H. pertussis* as a "schlepper" is unique in that both anaphylactic sensitization and sensitivity to histamine and serotonin are enhanced. This speaks for a common mechanism for both phenomena rather than an adjuvant action for anaphylactic sensitization with a distinct mechanism for sensitization to histamine. A recent review¹² discusses the available evidence for a common pathway, but experimental data at present are not sufficient to support any concrete theory.

Studies²⁰ in the mouse comparable with those of Condie and associates^{21, 22} and Johnson and colleagues,²³ who noted that endotoxin given together with bovine serum albumin to the rabbit enhances antibody production against the foreign protein, have given an impetus to the question of mechanism. The ultimate experiment for the elucidation of the "adjuvant" effect of *H. pertussis* probably lies in an understanding of the effect of its endotoxin on the cellular system responsible for antibody production, even as Condie and associates²¹ have conjectured for meningococcal endotoxin. Experiments along these lines are currently in progress and will be the subject of a subsequent publication.

SUMMARY

Particulate adjuvants, such as India ink, kaolin, and zymosan, were capable of enhancing anaphylactic sensitization in the mouse to a foreign protein antigen. Sensitization by the above was as pronounced as with *Hemophilus pertussis*. A lesser degree of sensitization was effected by gelatin, agar, dextran, and polyvinylpyrrolidone. Sensitization attained a maximum about ten days after the intraperitoneal inoculation of the sensitizing antigen, provided it was concurrent with or followed the adjuvant administration. The presence of circulating antibodies, titratable by the tanned-red-cell technique of Boyden at a time when anaphylactic sensitization is at its height, indicates the causal relationship. However, anaphylactic response can occur in the absence of titratable circulating antibody. None of the adjuvants was found capable of a simultaneous enhancement of histamine sensitivity.

The foregoing findings do not necessarily implicate the mode of action of *H. pertussis* as that of a classical "adjuvant."

REFERENCES

1. Malkiel, S., Hargis, B. J., and Feinberg, S. M.: Anaphylactic Shock in the Mouse Inoculated With *Hemophilus pertussis* Vaccine, *J. Immunol.* **71**: 311-318, 1953.
2. Parfentjev, I. A., and Goodline, M. A.: Histamine Shock in Mice Sensitized With *Hemophilus pertussis* Vaccine, *J. Pharmacol. & Exper. Therap.* **92**: 411-413, 1948.
3. Kind, L. S.: Sensitivity of Pertussis-Inoculated Mice to Serotonin, *Proc. Soc. Exper. Biol. & Med.* **95**: 200-201, 1957.
4. Munoz, J.: Effect of *H. pertussis* on Sensitivity of Mice to Serotonin, *Proc. Soc. Exper. Biol. & Med.* **95**: 328-331, 1957.
5. Raffel, S.: Immunity, New York, 1953, Appleton-Century-Crofts, Inc.
6. Kuwajima, Y., Masui, M., Asano, A., and Matsui, T.: A Soluble Antigen of Phase I *Hemophilus pertussis*. I. Mouse Protection Test of Filtrate or Supernatant of Cultures in a Medium Without Soluble Starch or Carbon Particles, *J. Immunol.* **76**: 175-180, 1956.
7. Pillemer, L., and Ecker, E. E.: Anticomplementary Factor in Fresh Yeast, *J. Biol. Chem.* **137**: 139-142, 1941.
8. Pillemer, L.: The Properdin System, *Tr. New York Acad. Sc., Series II*, **17**: 526-530, 1955.
9. Boyden, S. V.: The Adsorption of Proteins on Erythrocytes Treated With Tannic Acid and Subsequent Hemagglutination by Antiprotein Sera, *J. Exper. Med.* **93**: 107-120, 1951.
10. Adler, F.: Competition of Antigens. In *The Mechanisms of Hypersensitivity*, Boston, 1959, Little, Brown & Company.
11. Malkiel, S.: Unpublished observations.
12. Kind, L. S.: The Altered Reactivity of Mice After Inoculation With *Bordetella pertussis* Vaccine, *Bact. Rev.* **22**: 173-182, 1958.
13. Maitland, H. G., Kohn, R., and MacDonald, A. D.: The Histamine Sensitizing Property of *Hemophilus pertussis*, *J. Hyg.* **53**: 196-211, 1955.
14. Kind, L. S.: Effects of Heat on the Sensitizing and Shocking Properties of *Hemophilus pertussis*, *J. Immunol.* **77**: 115-116, 1956.
15. Malkiel, S., and Hargis, B. J.: Anaphylactic Shock in the Pertussis-Vaccinated Mouse, *J. ALLERGY* **23**: 352-358, 1952.
16. Munoz, J., Schuchardt, L. F., and Verwey, W. F.: Passive Anaphylaxis in *H. pertussis* Treated Mice, *Federation Proc.* **13**: 507, 1954.
17. Pittman, M., and Germuth, F. G., Jr.: Some Quantitative Aspects of Passive Anaphylaxis in Pertussis-Vaccinated Mice, *Proc. Soc. Exper. Biol. & Med.* **87**: 425-428, 1954.
18. Malkiel, S., and Hargis, B. J.: Anaphylactic Shock in the Pertussis Vaccinated Mouse. II. Diphtheria and Tetanus Toxoids as Antigens, *Proc. Soc. Exper. Biol. & Med.* **81**: 109-110, 1952.
19. Malkiel, S.: Active Anaphylaxis in the Pertussis Inoculated White Mouse, *Proc. Vith Internat. Congress Microbiol.* **2**: 265-272, 1953.
20. Malkiel, S., and Hargis, B. J.: Enhancement of Histamine and Anaphylactic Shock in Mice by *Brucella abortus*, *J. ALLERGY* **29**: 524-527, 1958.
21. Condie, R. M., Zak, S. J., and Good, R. A.: Effect of Meningococcal Endotoxin on the Immune Response, *Proc. Soc. Exper. Biol. & Med.* **90**: 355-360, 1955.
22. Condie, R. M., and Good, R. A.: Inhibition of Immunological Enhancement by Endotoxin in Refractory Rabbits; Immunochemical Study, *Proc. Soc. Exper. Biol. & Med.* **91**: 414-418, 1956.
23. Johnson, A. G., Gaines, S., and Landry, M.: Studies on the O Antigen of *Salmonella typhosa*. V. Enhancement of Antibody Response to Protein Antigens by Purified Lipopolysaccharide, *J. Exper. Med.* **103**: 225-246, 1956.