Studies on Immunization with Organ-Specific Haptens.* (28859)

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In performing experiments on the Forssman antigen, Sordelli *et al*(1) and Landsteiner(2) found that the purified Forssman antigen is hardly capable of stimulating antibody formation although it readily combines in serological reactions with an antibody that was formed by immunization with a crude Forssman antigen. Landsteiner(2) [see also Landsteiner and Simms(3)] coined the term hapten to denote substances inactive as immunizing antigens but active as *in vitro* antigens. It was shown(2,3) that Forssman hapten would stimulate the formation of corresponding antibodies if administered in a mixture with a protein originating from a species foreign to the immunized animal. This kind of immunization called by Sachs(4) "combined immunization" would obviously result in formation of antibodies directed against the protein carrier (Schlepper) in addition to those directed against the hapten. In Landsteiner's view the immunizing effectiveness of hapten-protein mixture is due to a loose combination between these two substances.

Recently described thermostable, ethanol-insoluble antigens of adrenal and brain(5,6,7) appeared promising for studies on some basic phenomena related to the immunization with haptens. The organ-specific antigens mentioned are present in organ fractions obtained by extraction at 100°C followed by precipitation at a 72% concentration of ethanol. Organ fractions of this type were referred to as BE fractions for their resistance to boiling and ethanol insolubility.

The adrenal and brain-specific antigens were studied mainly by means of antisera obtained by immunization of rabbits with adrenal or brain BE incorporated in complete Freund adjuvants. The brain and adrenal specific antigens were serologically unrelated. Thus far, the brain-specific antigens were demonstrated in man, ox and pig and adrenal-specific antigens in the same 3 species and also in horse. It was also shown that BE preparations of adrenal and brain, in addition to their respective organ-specific antigens, contain some thermostable antigens which are non-organ but species-specific.

Physicochemical studies have been performed only on the adrenal-specific antigen of bovine origin(8). This antigen was resistant to periodate but it was readily destroyed by chymotrypsin; these and some analytical data enabled tentative classification of the adrenal-specific antigen as a mucoprotein.

Materials and methods. Immunization procedures are outlined in describing particular experiments. All trial and final bleedings were performed 7-10 days after the last immunizing injection. Procedures for obtaining antigenic preparations and performing serological tests have been described(5,6,7).

Results. 1. *Quantitative data on Freund adjuvant immunization.* Six rabbits were subjected to Freund adjuvant immunization with porcine brain BE. The BE preparation was employed at concentrations of 10, 1 and 0.1 mg/ml, respectively, for 3 groups of 2 rabbits each. Four inoculations at weekly intervals were performed. For each inoculation 0.7-1.0 ml of BE-adjuvant mixture was injected intradermally in multiple sites on the footpads and shaved back. Adjuvants used for the first, second and third inoculations were enriched with a mixture of heat-killed *M. butyricum* and *M. smegmatis*; 8, 6, and 4 mg of mycobacteria, respectively, were added per 1 ml of adjuvants. The fourth inoculation was performed using adjuvants without mycobacteria. The antisera obtained after the fourth inoculation were examined by complement fixation test against BE preparations of porcine organs (Table I). Positive results were obtained with sera of

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TABLE I. Complement Fixation Test. Reactions of anti-porcine brain BE sera with BE preparations of porcine organs. Serum dilutions, 1:30; original antigen concentration, 3 mg/ml.

			Highest antigen dilution that gave +++ or ++++ reaction; BE preparations of porcine		
			Brain	Liver	Spleen
Antisera of rabbits immunized with porcine brain BE at a concentration of	10 mg/ml	1205	723	243	81
		2801	243	81	9
	1 "	1206	243	<1	<1
		8563	81	<1	<1
	.1 "	1220	<1	<1	<1
		1221	<1	<1	<1

animals injected with BE at 10 and 1 mg/ml concentrations but negative results were obtained with sera of animals immunized with BE at a concentration of 0.1 mg/ml. Antisera #1205 and #2801 obtained by immunization with brain BE at a concentration of 10 mg/ml combined not only with brain BE but also with BE of other porcine organs even though the titer of reaction with the homologous brain antigen was higher. Cross-reactions of this type have been discussed previously and could be related to the presence of antibodies that are non-organ but species-specific. Interestingly, antisera #1206 and #8563 obtained by immunization with brain BE at a concentration of 1 mg/ml contained only brain-specific antibodies. Apparently the immunizing dose employed was strong enough to stimulate formation of brain-specific antibodies, but too weak to elicit formation of species-specific antibodies.

2. *Intravenous immunization.* Two rabbits were injected intravenously with porcine brain BE preparation at a concentration of 10 mg/ml. They received a total of 15 injections, 1 ml each, over a period of 13 weeks. None of the sera of these animals showed any serological activity against porcine brain BE preparation.

3. *Combined immunization.* A. *Porcine serum as carrier protein.* Two rabbits were injected intravenously with a porcine brain BE preparation mixed with porcine serum. The method of immunization was similar to that described above in 2 except that before injection, the BE preparation was mixed with 1 ml of 1:2 diluted porcine serum. Two milliliters of BE-serum mixture were administered for each inoculation. The animals received a total of 13 intravenous injections over a period of 9 weeks. Here again sera of both animals gave negative results in serological tests against porcine BE preparations.

B. *Rabbit immune serum as carrier protein.* Three rabbits were injected with a mixture consisting of 0.5 ml of an undiluted rabbit anti-porcine brain BE serum and of 0.5 ml of porcine brain BE at a concentration of 5 mg/ml. For this purpose a potent rabbit antiserum was selected that had been obtained by Freund adjuvant immunization with porcine brain BE. The composition of the antiserum-BE mixture was established on the basis of a test tube titration for optimal precipitation. Before injection the mixture was incubated for 2 hours at room temperature after which time a rich precipitate was visible macroscopically. Thirteen intravenous injections of such a precipitate-containing preparation were administered over a period of 9 weeks. Sera of 2 animals were completely negative and one serum showed only negligible traces of a reaction with brain BE.

4. *Intravenous immunization of rabbits pre-stimulated by Freund adjuvant immunization.* A. For this experiment, rabbits #1220 and #1221 described above in 1 were selected. These animals had been previously exposed to Freund adjuvant immunization with porcine brain BE at a concentration of 0.1 mg/ml and failed to form antibodies. After completing the immunization with adjuvants, the animals were given a rest period of 5 weeks; then the intravenous course of injections was begun using the procedure described above in 2. Both rabbits formed strong antibodies.

TABLE II. Complement Fixation Test. Reactions of various serum samples from rabbit #1220 immunized with porcine brain BE preparation. Serum dilutions, 1/30; original antigen concentration, 3 mg/ml.

		Serum obtained before immunization	Serum obtained after concluding Freund adjuvant immunization		Serum obtained after intravenous immunization	
			1 wk	5 wk	3 inj	8 inj
Antigen dilution, 1:	1	—	—	—	—	++++
	3	—	—	—	++	++++
	9	—	—	—	++++	++++
	27	—	—	—	++++	++++
	81	—	—	—	++++	++++
	243	—	—	—	++++	++++
	729	—	—	—	+++	+++
	2187	—	—	—	—	—

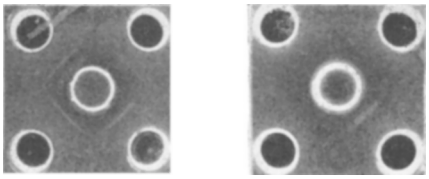
Table II gives results obtained by complement fixation test with serum specimens from one animal. As can be seen, antibodies were already formed after the third intravenous injection. The zonal inhibition phenomenon observed in the reaction with the serum obtained after the third injection did not appear in testing the serum obtained after 8 injections. This would indicate a stronger antibody response on prolonged immunization.

B. To amplify the information obtained in the experiment just described, 3 groups of rabbits were selected. All these animals were exposed to perparatory immunization by Freund adjuvant procedure following the method described above in 1. Animals of the first group were inoculated 3 times using Freund adjuvant-saline mixture. The second group received 3 inoculations of porcine adrenal BE preparation incorporated in Freund adjuvants and the third group was inoculated 3 times with porcine brain BE preparation incorporated into Freund adjuvants. The BE preparations of both adrenal and brain were used at a concentration of 0.1 mg/ml. As could be expected on the basis of experiments reported in 1, the immunizing dose was too small to stimulate detectable antibody formation. After concluding preparatory immunization, the animals were given a rest period of 3 weeks. Then all animals were restimulated intravenously with a mixture of BE preparations of porcine brain and porcine adrenal. Each injection contained 10 mg of each of the BE preparations; a total of 9 injections was administered over a period of 8 weeks.

The results obtained in complement fixation test with sera from this experiment are presented in Table III. The animals prepared by Freund adjuvant-saline mixture failed to respond to intravenous restimulation and their sera remained virtually negative. All 3 rabbits prepared by Freund adjuvant immunization with adrenal BE answered to intravenous restimulation by forming anti-

TABLE III. Complement Fixation Test. Reactions of antisera of rabbits exposed to preparatory immunizations by Freund adjuvant procedure and restimulated with BE preparations by intravenous route. Serum dilutions, 1:30; original antigen concentration, 3 mg/ml.

Preparatory immunization		Restimulation	Highest antigen dilution giving +++ or ++++ reaction	
			Brain BE	Adrenal BE
Saline—	1907	Intravenous injections with a mixture of adrenal and brain BE	1	1
Freund adjuvant mixture	1908		1	1
	1909		<1	<1
Adrenal BE—	1913		9	19683
Freund adjuvant mixture	1914		81	2187
	1915		27	729
Brain BE—	1910		729	27
Freund adjuvant mixture	1912		243	<1



A B

FIG. 1. Double diffusion gel precipitation. Central wells: in plate A, antiserum #1914; in plate B, antiserum #1910. Peripheral wells in both plates: BE preparations of porcine organs; upper left well, adrenal, 2 mg/ml, upper right well, spleen, 5 mg/ml, lower left well, kidney, 2 mg/ml, lower right well, brain, 5 mg/ml.

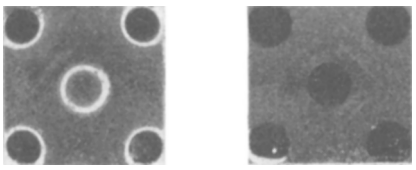
bodies that gave strong reactions with adrenal BE and considerably weaker reactions with brain BE. Similarly, the 2 animals prepared by a mixture of Freund adjuvants with brain BE responded to intravenous restimulation: one formed antibodies combining with brain BE only, whereas the other formed antibodies giving a strong reaction with brain BE and a relatively weak reaction with adrenal BE.

The results obtained by complement fixation test could be further explained by using the double diffusion gel precipitation test. In Fig. 1A, antiserum #1914 which originated from an animal prepared with adrenal BE was tested. The antiserum produced 2 precipitation lines with adrenal BE preparation. The thick line closer to the antiserum well could be recognized as adrenal-specific inasmuch as it did not appear in reactions with BE preparations of other porcine organs. On the other hand, the thinner precipitation line was non-adrenal-specific and was produced with BE preparations of other porcine organs as well. Apparently the weakly positive complement fixation test obtained with this serum and brain BE should be related to antibodies devoid of organ specificity which were represented by the thin line in the precipitation test. In Fig. 1B, antiserum #1910 obtained from a rabbit which was prepared by Freund adjuvant immunization with brain BE was tested. This antiserum precipitated only brain BE. Apparently the non-organ-specific antibodies in this serum were too weak to give a precipitation line even though they were strong enough to produce positive reactions with adrenal BE in complement fixation test.

After completing the described schedule of intravenous immunization, all animals listed in Table III were given a rest period of 3 weeks. Then they received a booster injection of adrenal and brain BE. For this injection 20 mg of each BE preparation was used. One week later, the animals were sacrificed. The sera of animals prepared with Freund adjuvant-saline mixture remained negative. The sera of animals prepared with Freund adjuvant-adrenal BE mixture now gave a different pattern of reaction. In addition to the adrenal-specific line, a brain-specific line appeared as shown in Fig. 2A. The reaction pattern produced by sera of animals prepared with Freund adjuvant-brain BE mixture was unchanged (Fig. 2B).

Discussion. Previous experiments have shown that thermostable, ethanol-insoluble fractions (BE fractions) of mammalian organs stimulate antibody formation when injected into rabbits along with complete Freund adjuvants. In the present experiments, no antibody response was observed when rabbits were injected intravenously with BE preparation of porcine brain, which allows the classification of BE preparations as haptens.

Interestingly, no antibody against BE was formed in the combined immunization experiment performed by the classical procedure of Landsteiner and of Sachs using a protein carrier of foreign species origin. Apparently complete Freund adjuvants have stronger activity in converting BE preparations into im-



A B

FIG. 2. Double diffusion gel precipitation. Central wells: antisera after extended immunization; in plate A, antiserum #1914; in plate B, antiserum #1910. Peripheral wells in both plates: BE preparations of porcine organs; upper left well, adrenal, 2 mg/ml, upper right well, spleen, 5 mg/ml, lower left well, kidney, 2 mg/ml, lower right well, brain, 5 mg/ml.

munizing antigens than the foreign protein carrier; explanation of this observation has not yet been attempted.

In further experiments, rabbits were prepared by Freund adjuvant immunization with a small dose of BE preparation (0.1 mg/ml). This did not result in detectable antibody formation. However, animals prepared by such a sub-threshold immunization responded with strong antibody formation to intravenous injections of BE preparations.†

One might have considered the following explanation of these findings: The animals prepared by sub-threshold immunization (by the Freund adjuvant procedure) contained in their circulation humoral antibodies even though not detectable by the available test tube procedures. The intravenously injected hapten combined with antibodies in the circulation. This resulted in conversion of the hapten into an immunizing antigen. However, this explanation could be rejected on the basis of experiments in which no antibody formation was observed following intravenous injections of complexes which were formed by *in vitro* reaction of BE preparations with their corresponding antibodies. In this connection, one would have to assume that the sub-threshold immunization affects in some way the antibody forming apparatus itself in making it prone to respond to a "naked" hapten.

The question arose whether the antibody response is given only to the very same haptens by which the animal was prepared in the sub-threshold immunization. Experiments were therefore performed which are presented in Table III and Fig. 1 and 2. One can conclude from these experiments that the animals prepared with adrenal BE became capable of responding to the adrenal-specific and species-specific antigens. Similarly, animals prepared with brain BE became capable of responding to the brain-specific and species-specific antigens. In other words, animals were conditioned to respond only to those haptens with which they were prepared. This, however, was only the case within certain limits. It was shown that by extending and increasing the intravenous restimulation the organ-specificity of the response was blurred. Animals prepared with adrenal BE eventually formed also brain-specific antibodies.

Summary. Thermostable, ethanol-insoluble fractions (BE fractions) of porcine brain and adrenal were shown to act as haptens in rabbit experiments. They failed to stimulate antibody response when injected by themselves intravenously. Complete Freund adjuvants, but not a protein carrier of foreign species origin, converted them into immunizing antigens. Animals exposed to preparatory Freund adjuvant immunization with small doses of BE preparations failed to produce detectable antibodies, but they became capable of responding with antibody formation to intravenous injections of BE preparations. Upon intravenous injections of a mixture of brain and adrenal BE, animals prepared with adrenal BE responded only to antigens present in adrenal BE and those prepared with brain BE responded only to antigens present in brain BE preparations. This pattern of an organ specific response was blurred in some animals after the tenth intravenous injection of the BE mixture.

† Somewhat similar observation in relation to capsular polysaccharides of pneumococci was made by MacLeod (personal communication). These substances act as haptens in rabbit experiments. However, antibody formation, once initiated by injecting a rabbit with whole pneumococci, may be restimulated with "boosters" of capsular polysaccharides.

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