ON THE RÔLE OF CARBOHYDRATE HAPTENS IN BACTERIAL ANAPHYLAXIS.

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Though the existence of bacterial anaphylaxis was demonstrated by Rosenau and Anderson (1) as early as 1907, subsequent investigators could not diminish the difficulties in producing a clear-cut anaphylactic shock with the majority of microorganisms. The main difficulty in the study of bacterial anaphylaxis is the primary toxicity of the bacterial protein. According to Doerr (2) the amount of bacterial antigen which produces shock in sensitized animals is negligibly smaller than the lethal dose for normal animals. More recently Zinsser and Parker (3) found that in guinea pigs sensitized passively with typhoid immune serum at least a minimal fatal dose of the typhoid extract is required to elicit in sensitized animals acute shock with death.

In the study of bacterial anaphylaxis many investigators have used suspensions of organisms, not extract, so the *in vivo* tests are complicated by the fact that these suspensions may produce symptoms even in untreated animals. This complication can be obviated by using the Schultz-Dale method. Sherwood and Stoland (4) working with typhoid bacilli found that in some cases the sensitized uterus contracted after the addition of 0.25 cc. of bacterial extract, whereas the minimum toxic dose was 1 cc. Since the uteri of the sensitized animals failed to react in many cases, they conclude, on the basis of comparative examinations, that more positive results can be obtained with the *in vivo* method and that the Dale test is not a necessary criterion of sensitization. Zinsser and Mallory (5) do not share this unfavorable opinion. They find that uterine reactions, like those obtained after sensitization with proteins, can be demonstrated in guinea pigs after both active and passive sensitization against alkaline extracts of pneumococci, but "The margin between the minimum doses which contract the normal and those which contract the sensitized uteri is incomparably smaller than that obtained in analogous experiments with . . . . egg albumen." With regard to the difficulty of demonstrating bacterial anaphylaxis Zinsser and Mallory state: "it would seem to us that active sensitization is so difficult because the bacteria contain relatively little coagulable protein, and that in passive sensitization there is an apparently slower union of antibodies with the animal tissues than in the case of protein antibodies." If this assumption is right we have little hope of improving our methods for the study of bacterial anaphylaxis, the difficulties being inherent in the nature of this phenomenon. It is conceivable however, that the primary toxic substance

of the bacteria is not necessarily identical with the specific antigenic part and that by suitable chemical separation it may be possible to isolate an atoxic specific constituent, responsible for the production of anaphylactic shock. With the discovery of specifically reacting polysaccharides in certain microorganisms a new field was opened for investigation. These substances, immunologically haptens, give powerful precipitation and complement fixation reactions with immune serum, but they do not lead to the formation of antibodies, when injected into animals. According to Doerr the anaphylactic antibodies against bacterial proteins are probably identical with the precipitins, since the power of an immune serum to confer passive sensitization is in proportion to its precipitin content. If this is true we should be able to produce anaphylactic shock with a carbohydrate hapten, since it has a remarkably high activity in precipitin reactions.

As far as we are aware no experiments have been reported which show a difference between the sensitizing and shock-producing part of the antigen in typical anaphylaxis. However in the classical investigations of Zinsser and his associates it appears that such a difference exists in the related field of bacterial allergy. Zinsser and Parker (6) produced typical skin reactions with residue antigen in guinea pigs infected with tuberculosis. In later work Zinsser and Mueller (7), then Zinsser and Tamiya (8) succeeded in isolating from *Bacillus tuberculosis* and the pneumobacillus an active material which produced a positive skin test and yet appeared to be different from the soluble specific substance. Both of these substances lack true antigenic activity, the substance producing skin reactions being a protein-free nitrogenous material, while the soluble specific substance is a carbohydrate. This work was not extended to the study of typical anaphylaxis, probably because it was a generally accepted opinion that true protein anaphylaxis plays an unimportant rôle in the occurrence of spontaneous infections.

The object of the present work was to determine whether animals could be sensitized to carbohydrate haptens, either actively by the injection of bacteria, or passively by immune serum. Sensitization has been determined by the injection of the hapten intravenously and by the reaction of the uterus according to the Schultz-Dale technic.

Bacillus lactis aerogenes was chosen for the major part of our study. The isolation of the specific substance from this bacterium and its chemical nature has been described by one of us previously (9). It consists chiefly of carbohydrates containing, after hydrolysis, 66 per cent reducing substance, counted as glucose. It is protein-free, but in spite of several attempts at further purification, its nitrogen content could not be reduced below 0.9 per cent. It gave specific precipitation reaction, when diluted in 1:500,000 and complement fixation reaction when diluted as high as 1:64,000,000.

EXPERIMENTAL.

In Vivo Tests in Actively Sensitized Animals.

Attempts were made at the beginning of our work to produce active sensitization against the *aerogenes* specific substance.

Holobut's method (10) was adopted in sensitizing the animals, using massive intraperitoneal inoculations of living or killed organisms on 10 consecutive days and testing the sensitivity 3 weeks after the last injection. The difficulty we encountered was the high toxicity of the bacillus for guinea pigs. In the first lot of 12 guinea pigs, 6 were inoculated with living bacteria and 6 with bacteria killed at 60°C. Each animal died after the second or the third injection, when the single dose was 1/20th of the growth from a 24 hour agar culture. In inoculating the second lot of guinea pigs, smaller doses were used, beginning with 1/200th and slowly increasing to 1/60th part of a 24 hour agar culture. 6 guinea pigs were inoculated with living and 6 with killed bacteria and daily injections were given on 9 consecutive days. At the end of this treatment 3 animals survived from each group. These were tested for sensitivity 3 weeks after the last injection, in part by intravenous inoculation of 1 mg. aerogenes specific substance, in part by testing the isolated uterus. No reaction was observed in any of these tests.

Passive Sensitization toward the Aerogenes Specific Substance.

Our experiments in regard to active sensitization were not extensive enough for the formulation of any conclusion. In view of the difficulty of obtaining sensitization in this way we limited ourselves in further work to the study of passive anaphylaxis. One part of these experiments was reported by one of us in a preliminary paper (11).

The immune serum we have used for the passive sensitization was prepared in rabbits by six to eight intravenous injections of the killed suspension of *Bacillus lactis aerogenes*. The interval between the injections was either 3 or 4 days. The rabbits were bled 8 days after the last injection.

In performing the sensitization, guinea pigs weighing 250 to 380 gm. were inoculated intraperitoneally with from 1 to 4 cc. immune serum. After 16 to 24 hours, 1 cc. of the various dilutions of the specific substance in saline was injected in the saphenous vein. Each of the 18 sensitized guinea pigs tested intravenously with 1 to 0.033 mg. of the specific substance died, showing typical anaphylactic symptoms. Death usually occurred within 2 to 3 minutes after the injection and never later than 5 minutes. The lungs were markedly distended and pale. 1 guinea pig receiving 0.02 mg. specific substance showed symptoms but survived.

Control tests were made on 11 untreated guinea pigs weighing 250 to 300 gm. None of these animals showed any immediate symptoms. When the dose was as

high as 1 to 2 mg. some of the animals died on the 2nd or 3rd day, without showing any symptoms other than drowsiness and emaciation. At the autopsy no changes were observed. Smaller amounts than 1 mg. never caused any symptoms in untreated animals. The slight toxicity of the aerogenes specific substance in normal animals can be explained probably by the presence of a small amount of nitrogenous material. The margin between the primary toxic dose (2 mg.) and the amount which produced a fatal shock (0.033 mg.) was much wider than that found previously with bacterial extracts.

It should be noted that there was not a single experiment in which we failed to produce a fatal shock in sensitized animals, provided the amount of the extract inoculated intravenously was not less than 0.033 mg.

In tests to confirm the results obtained *in vivo*, the smooth muscle reaction was used in further work. 20 guinea pigs weighing 210 to 260 gm. were sensitized by the intraperitoneal injection of 1 cc. aerogenes immune serum and the responses of their uteri tested, using the Schultz-Dale method. The uteri of all these animals reacted to the specific substance, when tested 2 hours to 12 days after the injection of serum. According to earlier authors the optimal incubation time for the development of passive anaphylaxis is about 1 day. Zinsser and Parker (3) in their experiments with typhoid bacilli never succeeded in finding the animals sensitized in less than 3 to 5 days, the highest degree of sensitization being developed in 1 week. Using the aerogenes specific substance we did not observe appreciable difference in the sensitivity even when the incubation time varied between 2 hours and 12 days. In establishing accurately the smallest amount of specific substance which causes a contraction in the sensitized uterus, one difficulty was observed, apart from the individual variations of the uteri. The two horns of a uterus were suspended approximately at the same time in the two baths. To the first horn 0.5 cc. of a 1:80,000 dilution of the specific substance was added, without causing any contraction, but 1:50,000 dilution caused a distinct reaction of the other horn. After changing the bath 1:80,000 dilution added to the second horn caused a reaction with a curve similar to that following the previous larger dose. That is a slighter preliminary reaction increased the sensitivity of the uterus. This observation has been previously made by Weil (12) in his studies in protein anaphylaxis.

The smallest amount of the specific substance which caused a distinct, but not maximal contraction, was 0.00625 mg. added to a bath of 125 cc. The final concentration of the substance therefore was 1:20,000,000.

Desensitization of the uterus was demonstrated after the contraction due to one addition of 5 mg. of the substance to the bath. If, however, 1 mg. of the specific substance was used, three subsequent doses were required, when the bath was changed after each contraction, to obtain a complete desensitization.

As controls the uteri from 6 normal guinea pigs were tested and not one gave a reaction following the addition of as much as 10 mg. of the specific substance to the bath. When tested for sensitivity to specific substances obtained from other types of aerogenes, there was no response indicating overlapping specificity so that the results correspond to those obtained with the precipitin reaction.

These experiments seem to justify the conclusion that passive anaphylaxis toward a non-protein bacterial extract can be demonstrated with the same degree of sensitiveness and specificity as observed in protein anaphylaxis.

It might be mentioned briefly that in a few cases the skin test was tried in passively sensitized animals, though these experiments do not warrant any definite conclusion.

11 guinea pigs were sensitized by the intraperitoneal injection of 1 to 5 cc. immune serum. After 24 hours 0.1 cc. of 1:500 or 1:1000 dilution of the specific substance was injected into the abdominal skin. In most of these animals a moderate swelling developed in 3 to 5 hours, the largest being 2 cm. in diameter, with an inflammatory red area in the middle. The reaction disappeared in 12 to 18 hours. In 7 unsensitized animals no changes were observed in the injected area.

The type of skin reaction we observed in the sensitized animals can be classified neither as an immediate, evanescent urticarial reaction, characteristic of protein anaphylaxis, nor as a late allergic reaction, since it disappears in 24 hours. We feel that additional work is needed to establish the nature of this reaction.

Passive Sensitization to the Carbohydrate Hapten of the Pneumobacillus.

In this further work, our object was to confirm the results obtained with the aerogenes specific substance, using the carbohydrate haptens

of other microorganisms which can be purified more highly. Accordingly the specific substance was isolated from a strain of pneumobacillus, originally obtained from a normal throat.

This hapten was isolated first by Heidelberger, Goebel, and Avery (13) and after a long and careful process of purification, it was found by them to be free from nitrogen and to consist of a polysaccharide built up from glucose units. For technical reasons we could not follow the method used by these authors, but adopted Toeniessen's method (14). The raw extract thus obtained, which corresponds probably to the capsular material, was subjected to subsequent purification. At the beginning of our work the capsule of the freshly isolated strain was exceptionally abundant; 3 or 4 months later, after repeated transfer on meat infusion agar medium, it became much less. At this stage the yield of the extract was also smaller, but did not differ in reactivity from the first extract. It gave precipitation with a pneumobacillus rabbit immune serum, when diluted in 1:300,000. From the bacterial growth of 100 Kolle flasks 1.2 gm. of extract was obtained, in the form of a white powder easily soluble in water. A weakly alkaline solution of this substance in a dilution of 1:200 was precipitated 6 times in succession with 3 volumes of absolute alcohol. The resulting powder gave a specific precipitin reaction, when diluted 1:500,000 and layered over immune serum. Additional purification was carried out using the uranyl nitrate precipitation method. In this we followed very closely the technic given by Heidelberger, Goebel, and Avery (13). The resulting powder was dissolved in water and dyalized for 2 days in a parchment bag, then filtered through Seitz filter, and precipitated by alcohol. In the final stage it was precipitated by the corresponding immune serum when diluted in 1:2,000,000. It gave no precipitation with any of the pneumococcus type sera. In the presence of 1:20 dilution of the immune serum it gave complement fixation when diluted 1:32,000,000.

After hydrolysis it contained 71.5 per cent reducing substance counted as glucose. When the hydrolysis was made in normal sulfuric acid solution, the optimal time was found to be 9 hours. The nitrogen content of this specific substance was 0.26 per cent, considerably lower than that of the aerogenes extract.

The method for demonstrating anaphylaxis to the pneumobacillus carbohydrate was the same as that described above with the aerogenes specific substance.

Guinea pigs were sensitized by the intraperitoneal injection of 1 cc. pneumobacillus immune serum and 24 hours later injected intravenously with various dilutions of the specific substance. The smallest amount of the carbohydrate extract which produced invariably a typical anaphylactic shock with death was found to be 0.01 mg. 5 untreated guinea pigs were used as controls and failed to show any symptoms after the injection of amounts as large as 2 mg.

These results seem to us, to be especially convincing since the carbohydrate extract did not possess any primary toxicity and yet it produced anaphylactic shock even when given in smaller amounts than had been used previously with proteins. According to Wells (15) the smallest amount of protein capable of causing anaphylactic shock is 0.1 to 0.05 mg.

The results *in vivo* were confirmed by the Schultz-Dale reaction. A 1:12,500,000 dilution of the carbohydrate in the bath was enough to produce contractions in sensitized uteri. It is interesting that a single contraction due to the addition of 1 mg. carbohydrate to the bath was enough to desensitize the muscle completely.

Experiments with Yeast Gum.

Studies in yeast anaphylaxis have been made by Rosenau and Anderson (1) and by Axamit (16). Yeast cells or crude extracts were used in testing the sensitivity of the treated animals. The slight difference between the primary toxic and the shock-producing doses made the interpretation of these results just as difficult as in the case of bacterial anaphylaxis.

Our object was to test passively sensitized guinea pigs with yeast gum, the hapten nature of which has been shown by Mueller and by one of us (17).

The strain of yeast used in this work was obtained from a case of stomatitis. The yeast cells grown on Sabouraud culture medium were collected and freed from the constituents of the culture medium by alcoholic precipitation. The extraction was made by the hot alkaline method. From this extract the gum was obtained by the usual procedure, precipitating with Fehling reagent. The last trace of the copper was taken out by repeated alcoholic precipitation and dyalisis.

In the final stage of purity 83 per cent of the gum consisted of reducing substances counted as glucose. Nitrogen was present in trace, approximately 0.2 per cent.

Passive anaphylaxis toward the yeast gum was studied both *in vivo* and with the uterine reaction. Since the results of these experiments entirely correspond to those obtained with the specific substances of *Bacillus lactis aerogenes* and of the pneumobacillus, their description can be omitted to avoid repetition. The only difference observed was that repeated additions of larger amounts of yeast gum were

necessary to produce desensitization of the uterus, whereas this was easily accomplished with pneumobacillus extract. The injection of the yeast gum into six untreated animals proved to be without effect.

TABLE I.

Summary of the Reactions Obtained with the Specific Substances of B. lactis aerogenes, Pneumobacillus, and Yeast.

		Specific substances from		
		B. lactis aerogenes	Pneumobacillus	Yeast
Nitrogen		0.9 per cent	0.26 per cent	0.2 per cent
Reducing substances after hydrolysis, counted as glucose		66 per cent	71.5 per cent	83 per cent
Precipitin titer		1:500,000	1:2,000,000	1:1,000,000
Titer in complement* fixation		1:64,000,000	1:32,000,000	1:32,000,000
In vivo tests	Toxic dose for normal guinea pigs	1 mg.	2 mg. non-toxic	2 mg. non-toxic
	Minimal shock-producing dose	0.033 mg.	0.01 mg.	0.02 mg.
In vitro tests with Dale technic	Largest amount failing to cause contraction of normal uterus	10 mg.	10 mg.	10 mg.
	Smallest amount causing contraction of the uterus from sensitized guinea pigs	0.00625 mg.	0.01 mg.	0.025 mg.
	Amount causing desensitization	5 mg.	1 mg.	Not complete after 10 mg.

* Graded amounts of the antigens were tested in the presence of 0.5 cc. 1:20 dilution of immune serum and 2 units of complement. Incubation period was 16 hours at 6° to 8°C. The amount in the last tube which did not show a trace of hemolysis was regarded as the titer.

DISCUSSION.

The experiments described above show that small amounts of protein-free hapten obtained from *B. lactis aerogenes* will produce anaphylactic shock, both *in vivo* and *in vitro*, in guinea pigs which have been passively sensitized with immune serum from rabbits injected

with dead cultures of the same organism. In the same way, haptens from the pneumobacillus and from a yeast caused typical shock in animals sensitized by the appropriate immune sera. When haptens are used to bring out the reaction, the wide margin between the toxic and the minimal shock-producing doses corresponds to that found in protein anaphylaxis. Desensitization is produced in the isolated uterus by the addition of the hapten to the bath. The muscle is of course washed and rested before the desensitization test is made.

Repeated injections of living and killed cultures have not produced a sensitization to the hapten in the few animals that survived the treatment.

Just as haptens fail to cause the production of precipitins and complement-fixing bodies, so do they fail to sensitize guinea pigs when injected in their pure state. That they may play a part in sensitization when combined with protein seems possible.

The haptens used in these experiments gave none of the protein reactions, and while they were not absolutely nitrogen-free, this element was almost negligible in the pneumobacillus and yeast preparations. Since shock was produced by smaller amounts of hapten than will cause a reaction in animals sensitized to purified proteins, it does not seem possible that the small amount of nitrogen present played any part in the reaction. A positive statement is unjustified because of the trace of nitrogen present, but we believe that we have shown in these experiments that anaphylactic shock can be produced by carbohydrates.

Since the serum used for the sensitizations had high contents of precipitin and complement-fixing bodies which react with minute amounts of the appropriate hapten, we feel that the results obtained support the view that precipitins and complement-fixing bodies are closely related to the bodies which have to do with sensitization, if not identical with them.

CONCLUSIONS.

1. In passively sensitized animals bacterial anaphylaxis has been produced, *in vivo* and *in vitro*, with haptens from *B. lactis aerogenes*, the pneumobacillus, and a yeast.

2. The smallest amount of hapten causing fatal anaphylaxis is less

than the minimal amount of protein which will cause death in properly sensitized animals.

3. The haptens used were largely carbohydrates, and gave none of the protein reactions, but since they did contain a small amount of nitrogen we cannot yet assert positively that carbohydrate alone will produce shock.

4. Since haptens will not sensitize animals we must conclude that the anaphylactogenic and shock-producing parts of the antigen are not identical.

5. These experiments provide further evidence of the close relationship of precipitins to anaphylaxis.

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