ON THE IMMUNIZATION OF ANIMALS WITH BACTERIAL PROTEOTOXINS (ANAPHYLATOXINS).*

By HANS ZINSSER, M.D., and J. G. DWYER, M.D.

(*From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.*)

The discovery, a few years ago, by Friedberger (1) that powerful poisons were produced when active guinea pig serum was left together with emulsions of various bacteria, has had an important influence upon our conceptions of infectious disease, and, indeed, has furnished cause for reconsideration of the long accepted endotoxin theory of Pfeiffer. It has become at least a reasonable hypothesis that these toxic products of Friedberger, which he has, perhaps somewhat inaptly, named anaphylatoxins,[1] may be the sole factors in the toxemias of such diseases.

Our knowledge of these poisons is, of course, in many features incomplete. Their relation to the actual mechanism of serum anaphylaxis is not positively determined and, since this phase of the subject is quite distinct from the work reported in this paper, we may abstain from discussing it here. In the case of the bacterial anaphylatoxins more particularly, the work of the last few years has opened the important question as to whether or not the bacterial protein actually furnishes the matrix or substrate for the poison. This was the natural assumption of Friedberger and seemed also to follow from the earlier work of Vaughan (2), since there are so many distinct points of similarity between the complement-produced substances of the former and the toxic split products obtained with the aid of purely chemical treatment by the latter. This view is further supported by a number of workers by the apparently autolytic production of such poisons from bacteria in salt solution suspension.

However, the work of Keysser and Wassermann (3), in which anaphylatoxin-like poisons were produced in guinea pig serum when kaolin or barium sulphate was substituted for bacteria, seemed to contradict this conception, and of similar import are certain experiments of Bordet, and the recent work of Jobling and Petersen (4). The last named investigators especially have left little room for doubt that poisons apparently similar to those of Vaughan and

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[1] Although we have long hesitated further to complicate the nomenclature of this involved subject, we believe that much confusion can be avoided by substituting the term proteotoxins for all of the poisons of this class,—viz., poisons produced by the contact of active serum with bacteria, precipitates, kaolin, agar, starch, etc.

of Friedberger (Jobling and Petersen call them serotoxins) can be produced by serum enzymes from the proteins of the serum itself, when these have been exposed by the adsorption of anti-enzymes by the kaolin or by the bacteria.

It is somewhat uncertain, therefore, whether the poison engendered when bacteria and fresh serum are put together at 37.5° C. is a product of bacteriolysis, as at first supposed by Friedberger, or whether it is rather a product of the cleavage of other proteins, a process in which the bacteria play only an indirect part. It is impossible to settle this point at present, although we do not think that the occurrence of one process necessarily excludes the other.

However this may be, it has no direct influence upon the importance of poisons of this description in infectious disease. Whatever the matrix, the fact remains that they are produced when active serum constituents meet with bacteria in suitable quantitative proportions and at body temperature. Their actual occurrence when these elements meet within the animal body has been demonstrated experimentally by Friedberger and Nathan (5). We are, therefore, still justified in attributing to them an important part in bacterial toxemia, in accordance with the theory of Vaughan and the subsequent views of Friedberger.

This being the case, it is of great importance to our general conceptions of immunity to determine whether or not the animal body can develop increased resistance to these poisons. Our own studies were carried on in direct connection with work upon immunity in typhoid fever, a disease in which one of us in a previous paper (6) has attempted to show the great likelihood of participation of the anaphylatoxins.

In spite of a number of investigations bearing directly upon this point, we were unable to find a definite answer to this question, when, incidental to a general study of such poisons, we reviewed the literature.

As early as 1905, V. C. Vaughan, Jr., (7) attacked this problem in connection with the toxic split products obtained by the method of V. C. Vaughan, Sr., from the colon bacillus. His results justified him in concluding that "after the administration of several doses of gradually increasing strength, a point is reached at which the animal is able to withstand the injection of from two to three times the amount which would surely have proven fatal for an untreated control." He interpreted this rather as an evidence of acquired tolerance than as one of immunity in the ordinary sense of the word.

Bessau (8) later studied the same question by a number of different methods, both in relation with serum anaphylaxis and with bacterial anaphylatoxins directly. In one series of experiments he sensitized guinea pigs simultaneously with beef and with horse sera and, after the proper period of incubation, when full susceptibility was developed, he administered sublethal doses of one of these sera. After recovery, animals so treated were found to be less susceptible to reinjection with the other serum than were controls to which the latter serum only was given. These results obviously seem to indicate that the preliminary intoxication with the anaphylactic poison induced a non-specific tolerance.

Though indirectly of much interest, the importance of these experiments for the solution of the problem we are discussing rests upon the acceptation of the identity of the so called anaphylatoxins (proteotoxins) with the toxic sub-

stances involved in serum anaphylaxis,—an assumption which is indeed rendered likely by many observations, but which, after all, is not yet a matter of certainty. Of more direct bearing, therefore, are the other experiments of Bessau in which animals that had recovered from anaphylactic shock (both actively and passively prepared), were subsequently injected with typhoid anaphylatoxin (proteotoxin). Here, too, it was found that the animals receiving the preliminary treatment showed but slight symptoms and recovered from doses of the poison which killed the untreated controls. Bessau concludes from his experiments that anti-anaphylaxis is a condition based entirely upon this acquired and non-specific tolerance. We will discuss this view in our conclusions.

Ritz and Sachs (9) later claim to have observed that guinea pigs were protected against *B. prodigiosus* anaphylatoxin after they had been injected with a sublethal dose of the same substance, 20 to 45 minutes before.

Subsequently Friedberger and Lurà (10) repeated and contradicted the experiments of Bessau. In their conclusions they state that animals treated with sublethal doses of anaphylatoxin are just as susceptible as normal animals if reinjected after 24 hours with anaphylatoxin prepared either from homologous or from heterologous protein. They likewise obtained negative results when they treated serum sensitized animals with anaphylatoxin and subsequently reinjected the protein used for sensitization. The animals so treated remained as sensitive as the controls.

The available literature, though on the whole favoring the occurrence of such acquired tolerance to poisons of this description, is contradictory. Moreover, the few experiments cited by the investigators mentioned above, in which repeated injections of bacterial proteotoxin were given, are not sufficiently extensive to be convincing either affirmatively or negatively. For, as our protocols will show, there is frequently observed a marked normal difference in susceptibility to these substances by guinea pigs of the same weight and age,—a confusing factor which necessitates the study of large series before a conclusion can be reached.

METHODS.

The plan of our experiments was a simple one, aimed solely at determining whether guinea pigs treated with sublethal doses of anaphylatoxin, produced by putting together bacteria and fresh guinea pig serum, could be thereby rendered more resistant to subsequent injections of this poison.

The toxic substance was invariably prepared with typhoid bacilli in the following way: Typhoid bacillus, laboratory strain "J," was grown on agar slants for twenty-four hours and the growth was

taken up with one cubic centimeter of salt solution for each slant. This emulsion was then mixed with fresh guinea pig serum in proportions of one half agar slant to each four cubic centimeters of serum. The mixture was incubated for six hours and then centrifugalized for one to two hours. It should be stated that centrifugalization, however long continued, never completely freed the serum of bacteria. We tried subsequently to accomplish this by the addition of small amounts of inactivated, strongly agglutinating serum to the mixtures before centrifugalization, but even then a few microörganisms invariably remained in suspension.

Filtration through Berkefeld candles removed the bacteria but, strangely enough, also rendered the serum non-toxic. This is an observation which greatly surprised us, but which, as we later found, had also been made by Moreschi and Golgi (11). For these reasons it was not possible to work with an absolutely bacteria-free preparation. However, since our experiments deal with resistance to the acute effects of the poison itself, this fact does not in any way complicate our results.

Both the immunizing doses and the test injections were given intravenously, preliminary experiments with intraperitoneal immunization having been entirely negative. As will be seen, it appeared to us necessary to carry out a very large series of experiments before we ventured to draw conclusions. Every now and then guinea pigs are met with which show unusual susceptibility or unusual resistance to the poisons. One or two such animals in a series tend to upset confidence in the results and necessitate repetition. For these reasons also it was necessary to use almost as many controls as test animals. The controls were always heavier than the test guinea pigs, and controls were made both before and after the test guinea pigs were injected, in order to allow for possible alterations in toxicity during the period of incubation, which often exceeded one half hour.

EXPERIMENTS.

In tabulating the experiments we have purposely omitted recording the preliminary observations by which the toxicity of each particular poison, used for the first or immunizing dose, was determined. In giving the first injections we aimed to employ a dose

TABLE I.

Experiment 1.

An interval of 2 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	240	3.0 c.c.	Severe shock	235	3.5 c.c.	Death in 3½ min.
2	230	3.0 c.c.	Moderate shock	230	3.5 c.c.	Death in 2½ min.
3	235	3.0 c.c.	Moderate shock	230	3.4 c.c.	Death in 4 min.
4	225	3.25 c.c.	Moderate shock	220	2.5 c.c.	Death in 5 min.
Controls before experiment						
1				240	2.5 c.c.	Severe shock. Recovered.
2				230	3.5 c.c.	Death in 3 min.
Controls after experiment						
1				230	3.5 c.c.	Death in 3½ min.
2				225	3.5 c.c.	Very severe shock. Recovered.

In this experiment there is no evidence of increased resistance in the animals, if tested two days after the first administration of the poison.

TABLE II.

Experiment 2.

An interval of 4 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	225	3.25 c.c.	Very severe shock Recovery	225	3.5 c.c.	Death in 3½ min.
2	225	3.25 c.c.	Severe shock	225	3.5 c.c.	Death in 5 min.
3	200	3.25 c.c.	Moderate shock	200	3.5 c.c.	Death in 2½ min.
4	200	3.25 c.c.	Severe shock	200	2.5 c.c.	Death in 2¾ min.
Controls before experiment						
1				235	3.0 c.c.	Severe shock. Recovery.
2				240	3.5 c.c.	Death in 3½ min.
Controls after experiment						
1				225	3.5 c.c.	Death in 4 min.
2				190	3.5 c.c.	Severe shock. Recovery.

Here there is no evidence of increased tolerance if reinjection is practised after an interval of 4 days.

TABLE III.

Experiment 3.

An interval of 7 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	195	2.0 c.c.	Severe shock	200	3.5 c.c.	No shock.
2	210	2.5 c.c.	Severe shock	205	3.5 c.c.	No shock.
3	210	2.5 c.c.	Slight shock	185	3.0 c.c.	Very slight shock.
4	205	2.0 c.c.	Slight shock	200	3.0 c.c.	No shock.
5	195	2.0 c.c.	Slight shock	195	3.0 c.c.	Moderate shock. Recovery.
6	195	2.0 c.c.	Moderate shock	200	3.5 c.c.	No shock.
Controls before experiment						
1				210	3.0 c.c.	Death in 3½ min.
2				200	2.0 c.c.	Death in 5 min.
Controls after experiment						
1				235	2.5 c.c.	Death in 2¼ min.
2				240	2.5 c.c.	Death in 4 min.

In this experiment we obtained strong evidence that after 7 days a considerable degree of tolerance to the poison was established. The poison here used was exceptionally powerful and killed the heavier controls without exception in doses of 2 to 3 c.c., whereas the test animals, all of them lighter than the controls, lived, some of them showing no shock whatever.

TABLE IV.

Experiment 4.

An interval of 14 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result
1	220	3.0 c.c.	Severe shock	220	5.0 c.c.	Death in 3 min.
2	260	3.5 c.c.	Severe shock	217	4.0 c.c.	Slight shock. Lived.
3	250	3.5 c.c.	Severe shock	240	4.5 c.c.	Slight shock. Lived.
4	250	3.5 c.c.	Severe shock	230	4.0 c.c.	Slight shock. Lived.
Controls before experiment						
1				255	4.0 c.c.	Death in 2 min.
2				245	3.0 c.c.	Death in 2½ min.
3				235	2.5 c.c.	Death in 4 min.
Controls after experiment						
1				260	4.0 c.c.	Death in 3 min.
2				240	3.0 c.c.	Death in 3½ min.

Again we have evidence of the development of tolerance, as in the preceding experiment.

which would give moderate shock without killing the guinea pig. The controls mentioned in the tables are always those carried out with the poison used for the second injection, by which the resistance of the previously injected guinea pigs was tested.

TABLE V.

Experiment 5.

An interval of 15 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	185	2.5 c.c.	Severe shock		4.5 c.c.	Slight shock. Lived.
2	220	3.5 c.c.	Slight shock		4.5 c.c.	No shock. Lived.
3	225	3.0 c.c.	Moderate shock	Not	5.0 c.c.	No shock.
4	180	2.5 c.c.	Slight shock	taken for	4.5 c.c.	No shock.
5	185	2.5 c.c.	Slight shock	these	4.5 c.c.	Death in 4 min.
6	180	2.5 c.c.	Slight shock	seven	4.0 c.c.	No shock.
7	225	4.0 c.c.	Very severe shock	animals	5.0 c.c.	No shock.
Controls before experiment						
1				195	3.5 c.c.	Death in 2 min.
2				255	4.0 c.c.	Severe shock. Lived.
3				300	4.5 c.c.	Severe shock. Lived.
Controls after experiment						
4				235	2.5 c.c.	Death in 2 min.
5				240	2.5 c.c.	Death in 2½ min.
6				240	2.5 c.c.	Death in 3½ min.
7				210	2.5 c.c.	Death in 3 min.

This experiment, while supplying strong evidence in favor of the development of tolerance, illustrates most clearly the irregularity occasionally encountered in the reactions of normal guinea pigs. Note how five of the seven controls died of relatively small doses, in acute shock, whereas two slightly heavier animals, after severe shock, survived larger amounts. It is to be noted in this connection that small differences of weight often mean considerable differences of resistance, indicating probably that the younger guinea pigs are much more susceptible than the older ones, the difference being greater than could be accounted for by mere difference in weight.

The preceding protocols (tables I to V) sufficiently illustrate the methods by which our experiments were done. A complete tabulation of all our experiments, which include over seventy test animals and sixty controls, would needlessly lengthen our paper without materially adding to clearness. For this reason we will briefly summarize the results in tables VI and VII, adding explanatory re-

marks since it was not possible to include the weights of the individual animals and the dosage employed.

TABLE VI.

Resistance to the Poison of Animals Which Had Received One Previous Injection.

Second injection, after 2 to 5 days.		Controls.	
Total No. of animals.	No. of animals surviving.	Total No. of animals.	No. of animals surviving.
12	2	12	6

The dosage here was practically the same. The weight of the control animals averaged from 10 to 15 gm. more than that of the test animals at the second injection.

The results if analyzed in the individual experiments show that before the fifth day, as a rule, the guinea pig is not only more resistant or tolerant to the poison, but is still suffering from the effects of the first injection and rendered thereby even less resistant than normally.

TABLE VII.

Second injection, after 7 to 60 days.		Controls.	
Total No. of animals.	No. of animals surviving.	Total No. of animals.	No. of animals surviving.
45	24	35	7

The dosage here was often one and one half to twice as high in the test animals as in the controls. The controls in the individual experiments were always heavier than the test animals.

There can be no question, in these experiments, that a definite degree of increased resistance is present in the animals between the seventh and the sixtieth days. It should be noted that, in analyzing the individual protocols, the most uniformly positive results were obtained between the seventh and the fifteenth days.

We have records of a few animals in which the interval between the immunizing and the first injection exceeded sixty days, but in none of these has there been as consistent or reliable evidence of acquired tolerance as in the cases recorded above, in which the intervals were shorter.

SUMMARY.

Our experiments have shown definitely that guinea pigs, once injected with sublethal doses of bacterial proteotoxins (anaphylatoxins), acquire distinct tolerance to these poisons. The degree to which such resistance or tolerance is developed is never very high, in no case in our experiments exceeding the ability to withstand one and one half to twice the fatal dose of the poisons. During the three or four days immediately following the first injection the animals appear to be slightly less resistant than are normal controls, this depending probably upon the injury done by the administered poison. Tolerance begins to be evident after from four to seven days, seems to be most highly developed in about two weeks, but lasts in a diminishing degree for at least as long as sixty days.

Our experience, in this respect, with the poisons resulting from the contact of active serum and bacteria is similar to that of Vaughan with the toxic protein split products obtained by chemical methods.

The development of increased resistance definitely established, the questions immediately arise: (1) Is this tolerance specific? And (2) can it be passively transferred, with the serum, to a normal animal? We have begun to seek answers for these problems but as yet our data are too meager to permit definite conclusions.

The significance of the existence of higher resistance in animals treated with proteotoxins is far reaching both in connection with anaphylaxis and with immunity in general. We are not inclined to attribute to it as predominant a part in anti-anaphylaxis as is assigned to it by Bessau. For, in the first place, tolerance to the poisons is never developed to a very high degree, and, moreover, it does not become evident until three or four days after the first injection, while anti-anaphylaxis develops almost immediately after shock. However, there seems to us to be strong presumptive evidence that such tolerance to the poisons may play an important and, possibly, a non-specific part in anti-anaphylaxis, the chief underlying and specific cause of this phenomenon being the exhaustion of antibodies, or desensitization in the sense of Besredka.

The relation of such tolerance to the resistance of the animal to bacterial infection is, of course, obvious if we accept the possibility

of the production of such poisons in the injected body and their participation in the production of bacterial toxemia. We hope to throw more light on these relations in another paper dealing with the aggressin-like properties of the proteotoxins.

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