THE RELATIONSHIP BETWEEN SITE OF ANTIBODY ACTION WITHIN THE ORGANISM AND THE SITE OF ANTIBODY FORMATION (USING TETANUS ANATOXIN IMMUNIZATION AS A MODEL)

COMMUNICATION I. A NEW MODIFICATION OF THE BACHER-KRAUS METHOD AND ITS APPLICATION IN DETERMINING THE TISSUE CONTENT OF TETANUS ANTIGEN

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The goal of the present series of studies of which this is the first communication was an investigation of the topography of immunogenesis and an exploration of the laws associated with the time and space relationships governing the action of the antigen upon those structures of the entire organism sensitive to it. The necessity for such a coordinated approach arises from the circumstance that while the problems associated with the site of antibody formation (the fate of the antigen in the organism and its place of action, the dependence of the effectiveness of the immunization on the site of introduction of the antigen) are all the subjects of much study by immunologists throughout the world, these problems are being approached, as a rule, individually, without taking into due account their mutual interdependence. For this reason we took as a goal the investigation of the entire complex of the pending problems by the utilization of a single immunization model.

Immunization with tetanus anatoxin represents in this connection a rather convenient model in view of the circumstance that the rabbits, which act as experimental animals, do not possess natural antibodies and register very clearly the revaccination effects in the absence of anaphylactoid reactions. A negative feature of this model is the difficulty of determining small amounts of the antigen without which it is impossible to follow the resorptive processes within the organism.

As is well known, tetanus anatoxin can be demonstrated with difficulty even by the use of methods as sensitive as ring precipitation, complement binding and anaphylactic testing; it is difficult to employ radioactive tracer and luminescence methods because the usual anatoxin preparations suffer from the presence of considerable amounts of impurities. This, probably, was the reason why we were unsuccessful in attempting the use of the method of hemagglutination inhibition [8].

With the aim of being able to detect minute quantities of tetanus anatoxin, we developed a new modification of the Bacher-Kraus method, the basic principle of which consists in a binding of the anatoxin by the antitoxin which is tested by subsequent addition of more toxin and injection of the mixture into mice [4, 5].

*As a basis for the presentation of the entire problem complex we employed the fact that the lymphoidal apparatus sensitizes the entire system in the process of primary immunization (the relevant material was presented at the conference of the Institute of Normal and Pathological Physiology on March 4, 1957 and will be detailed in later communications).

The changes introduced by us consist in the following.

1) The dose of the antitoxin was decreased to 0.004 AE/cc instead of the 0.2 AE/cc as usually used. The corresponding dose of the toxin was also decreased but still was kept at 2–2¼ times the lethal level. The proportional decrease in the concentrations of the reacting components led to a corresponding decrease in the excess of the antitoxin which under the presenting conditions was bound by much lesser quantities of the antitoxin. It is important to note that the decrease of the concentrations apparently caused a partial dissociation of the toxin–antitoxin complex as a result of which the mixture ceased being hyper-becoming hyponeutral and so caused in mice localized tetanus and even in individual cases generalized tetanus leading to death by the 5th to 7th day with symptoms of generalized tetanus. This circumstance makes imperative the setting up of corresponding controls. When the mixture does not contain any antitoxin, all the animals perish by the 3rd to 5th day with symptoms of generalized tetanus.

2) In the usual scheme, death of the majority of mice by the 4th day is accepted as a positive result. In the modification of the Bacher–Kraus method we propose that the period of observation be lengthened to 7 days and, along with the fatalities, consideration be given to the severity of the illness of the survivors. A very essential part of the evaluation of the results obtained is a statistical treatment of the material obtained.

The experiment was performed as follows. To a single volume (0.3–1 cc) of standard antitoxin serum, diluted with physiological saline to a titer of 0.004 AE/cc, there was added an equal volume of the fluid being examined (containing known or unknown anatoxin) and one volume of physiological saline. The mixture was incubated for 45 minutes at 37° after which there was added to it one volume of tetanus toxin (we used toxin series No. 580 obtained from the Gamaleya Institute of Epidemiology and Microbiology), diluted with physiological saline to a titer of 1 : 50 L_t (for 0.1 AE) per 1 cc. After carefully shaking the mixture, it was again incubated at 37° for 45 minutes and then injected into the mice, each animal receiving 0.4 cc under the skin over the internal medial surface of the thigh. The experiments were performed, as a rule, with several anatoxin dilutions always using a control (the liquid volume being tested being replaced with physiological saline). The infected mice were watched for 7 days; daily and at the same hour the dead were counted and at least three separate times the clinical picture was recorded (usually on the 2nd, 4th and 7th days). Below are recorded the average results of several such experiments in which the sensitivity of the usual Bacher–Kraus method is compared with our proposed modification (in this as in all subsequent experiments we used tetanus anatoxin No. 336-4 obtained from the Gamaleya Institute of Epidemiology and Microbiology).

Comparison of the Generally Accepted Method of Titrating the Tetanus Anatoxin by the Bacher–Kraus Procedure and our Original Modification (1 L_t toxin plus 0.2 AE Antitoxin)

Titratable concentrations of anatoxin		number of mice	Results of observations by the 4th day of infection		% dying ($M \pm 2_m$)
in physiological saline dilutions	in ACE		died	survived	
1:50	1,5	12	9	3	$75 \frac{+25}{-26}$
1:75	1,0	16	8	8	50 ± 25
1:100	0,8	12	1	11	$8 \frac{+16}{-8}$
Control		12	-	12	$0 \frac{+15^{**}}{-0}$

* and ** see next page.

An analysis of the table permits the following observations. In the first place, the modified method is at least 7 times more sensitive than the generally accepted procedure. This method is quite specific as neither the boiled tetanus nor the diphtheritic anatoxin even when used in considerably larger doses produced an effect characteristic for natural tetanus anatoxin. Also quite noticeable is the direct correlation between the dose of the anatoxin and the effect produced: mice receiving the larger dose of the antigen perished more quickly (dilutions of 1 : 300 and 1 : 500). The same correlation holds in the transition to lower doses of the anatoxin (1 : 1000) manifesting itself in the greater survival of the animals. Finally, when comparing the smaller doses (1 : 1000) with the controls, there is observed a severity of the clinical picture in the surviving animals. This makes clear the inadequacy of using only one indicator for an evaluation of the results being obtained.

Modified Method ($^1/_{20}$ L_t toxin + 0.004 AE antitoxin)

Titrable concentrations of anatoxin		Number of mice	Results of observations									Evaluation of the indicator (M ± 2 m)		
			Days after being infected in which animals died					Survived with symptoms of tetanus						
in physiological saline solution	in AGE •		2–3	3–4	4–5	5–6	6–7	+++	++	+	+	% dying	Prolongation of life (in days) •••	Index of intoxication •••
1 : 300	0 25	16	10	5	1	—	—	—	—	—	—	$\frac{100+0}{-12}$ ••	2 9±0.3	7.6±0.3
1 : 500	0.15	10	1	6	2	1	—	—	—	—	—	$\frac{100+0}{-18}$ ••	3 8±0.6	6.7±0.6
1 : 1000	0.08	15	—	2	—	2	1	9	1	—	—	27±25	4.9±1.1	3.8±0.8
Control	Physiological saline	16	—	—	1	2	1	1	5	6	—	25±22	5.5±0.9	2.4±0.9
	Boiled anatoxin 1:100	14	—	—	—	1	1	4	1	4	3	$\frac{14+20}{-14}$	6 0±1.2	1.9±0.9
	Diphtheria anatoxin 1: 100	14	—	—	1	1	1	3	1	4	3	$\frac{21+23}{-21}$	5.5±1.2	2.1±1.0

• Antitoxin binding units

•• Calculated according to formula $m = \frac{100}{N+1}$ (Pomorsky, 1955)

••• Calculated with corrections for the step groupings

It is also evident that, if instead of an 1 : 500 dilution of anatoxin we had an unknown solution, we would be able to conclude that it contained tetanus anatoxin within limits of 0.08–0.25 ACE/cc (ACE, see footnote). In such fashion, the method being proposed permits a scale of controls (understanding by this term not only a "pure" control but also certain control anatoxin concentrations) which allows a determination of quite small concentrations not only qualitatively but also some approximately quantitative values.

An essential pre-requisite, in addition to the scale of controls, is an adequate number of animals (no less than 6-10 mice per group). In single experiments it is desirable to alter the order of infecting the various groups of the animals. The validity of the obtained data on the mortality and survival rates of the experimental animal groups is checked by the use of universally accepted statistical methods [1, 2]. As far as the indicators of the severity of the intoxication in the surviving animals are concerned (not shown in table), here there can be used along with the average values for the groups the so-called criterion of reconciliation (method x^2).

An essential and adequate basis for forming conclusions as to the variations as to the severity of the intoxication in the experimental and control groups (and thus deciding as to the presence and concentration of the anatoxin in the mixture injected into the experimental animals) lies in a statistically valid difference in any of the above indicators. However, the chosen indicator must characterize a basic portion of the experimental material and the juxtaposition of the other indicators must not contradict the conclusion made.

Naturally, separate use of the three indicators causes inconveniences in the sense that statistical analysis of the material becomes cumbersome and laborious. For this reason it seems desirable to develop some general index which could summarize the severity of intoxication existing in each group of the animals. The literature contains some attempts to solve similar problems [6, 7]. With this in mind, we attempted to devise an index with weighted values: the severity of the intoxication in the surviving animals were valued from 1 to 3, those dying on 7th day were valued as 4, those dying on 6th day were indexed as 5 and so on. The average value for the group was called the index of intoxication and the error was calculated with the usual methods. Our studies demonstrated in practice that the evaluation of the differences between the groups by means of such an index of intoxication was always confirmed when the three indices were taken separately or when other methods of evaluation were used. As, however, our present method cannot be considered generally accepted, we used it only as an auxiliary.

So far, when describing our proposed modification of the Bacher–Kraus method, we have used as a basis the data obtained by titrating the solution of tetanus anatoxin in physiological saline. It was necessary, however, to investigate the applicability of the proposed modification to titrations of fluids containing blood sera or animal tissue homogenates.

Corresponding experiments have shown that the presence in the tested fluid of rabbit blood sera or muscle homogenate did not decrease the sensitivity of the method and did not disturb the above noted correlations between the dose of the anatoxin and the severity of the intoxication of the animals (this served as a basis for the reverse decision as to the concentration of the anatoxin in the fluid being tested).

In the experiments with addition of muscle homogenate, it proved feasible to determine anatoxin concentrations of the order of 0.08 ACE/cc (this was not the limit of the sensitivity of the method), while in experiments with addition of serum anatoxin concentrations of 0.025 ACE/cc could be titrated, this being 40 times more sensitive than the usually accepted procedure. It should be noted that normal rabbit serum had a somewhat potential toxic effect. In connection with this the "control scale", when titrating for the presence of anatoxin in homogenates or sera, must contain equivalent doses of the sera or tissue homogenate of a normal animal. In addition to this, when titrating anatoxin in the presence of sera, at times (if there is found a considerable mortality in the "pure control") it becomes desirable to increase the dose of the anatoxin being added to the mixture up to 0.005 AE/cc. But, if the serum and muscle homogenate of a normal animal does not interfere with the demonstration of small amounts of anatoxin, to what extent is this true for tissue homogenates from immunized animals? In order to answer this question, tetanus anatoxin in various dilutions was added to rabbit muscle homogenate (the animal having received the day before extirpation 0.5 cc of diphtheria anatoxin (series No. 115-B-2, received from I.I. Mechnikov Institute)) after which the mixture was titrated by the method described above. As a control a mixture of normal muscle homogenate and anatoxin was used.

The results obtained demonstrate that the nonspecific or inospecific chemical alterations in the tissues which might have resulted from the addition of the antigen do not affect the titration of the anatoxin by the

proposed method. This in itself again confirms the specificity of the method and its applicability for determining the content of tetanus anatoxin in the tissues under conditions of immunization.

Thus, the more sensitive new method of titrating for tetanus anatoxin is a modification of the Bacher–Kraus procedure which permits qualitative and semi-quantitative determinations of rather small amounts of anatoxin in the presence of blood sera and rabbit muscle homogenate and for this reason this modification may be used for studying the dynamics of the antigen content of the blood and tissues of the immunized organism.

SUMMARY

A very sensitive modification of the Bacher–Kraus procedure for titrating tetanus anatoxin is proposed. This method permits a semi-quantitative titration of very small amounts of anatoxin even in the presence of blood sera and rabbit muscle homogenates. This modification is proposed for use when studying the dynamics of antigen content of the blood and tissues of the immunized organism.

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