THE DETECTION OF TISSUE ANTIGENS

REPORT NO. III. THE COMPARATIVE SENSITIVITY OF THE ANAPHYLAXIS REACTION AND SEROLOGICAL INVESTIGATION IN THE DETECTION OF SMALL DOSES OF ANTIGEN

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The proposal to use the reaction of anaphylaxis to detect the individual components of the antigenic complex is based, as we know, on the idea that it is more sensitive than serological methods. However, as was shown in previous reports [1, 2] the use of the anaphylacitc reaction for this purpose is beset with serious difficulties and a number of conditions must be rigidly complied with. Further, the great improvements which have taken place in recent years in serological methods of investigation must be borne in mind. The question of comparison of the two methods naturally arose, in order to determine whether and in what conditions the anaphylactic reaction was more sensitive than the serological examination, and whether the level of sensitivity of the latter could be increased to that of the former.

This question could be answered by comparing the minimum dose of antigen to which guinea pigs sensitized passively with varying amounts of immune serum would react with the dose of antigen which could be detected by the same serum in experiments in vitro.

EXPERIMENTAL METHODS AND RESULTS

Guinea pigs were injected intravenously with immune serum to horse albumin in different doses from 0.3 to 0.002 ml per 100 g body weight; the corresponding possible (maximum) content of immune serum in the body of the animals in the different groups was calculated. In order to produce an anaphylactic reaction, antigen (horse serum) was injected (24 hours after injection of the immune serum) also in different quantities, so that by calculation in relation to the weight of the animals its concentration in the body varied from 1 : 500 to 1 : 160,000.

The results of the experiments are given in Table 1. It was shown that with the smallest dilution of immune serum (1 : 320) a clear anaphylactic reaction could be obtained if the antigen was injected in quantity to give a concentration in the body of the animal of 1 : 80,000. As shown in Tables 1 and 2, at such a dilution the antigen is found very clearly also by an in vitro experiment by means of the complement fixation reaction with immune serum diluted 1 : 100 and even 1 : 400. However a clear anaphylactic reaction was also observed when the antigen content of the animal was lowered (to 1 : 6400 and even to 1 : 256,000) if the quantity of antigen injected was increased and its final concentration in the body of the animal reached 1 : 10,000 or over. By the serological method it was not possible to detect the antigen in this dilution even when immune serum was injected up to a concentration of 1 : 1600 or over.

The results obtained showed that the great sensitivity of the anaphylactic reaction was due to the fact that it requires a relatively small quantity of antibody. This is presumably because the antigen-antibody complex formed is inadequate for adsorption of complement but may act as a stimulus to the animal. With an

immune serum of high titer and used in small dilutions, antigen may be detected by means of the serological method in the same minimal quantity as by means of the in vitro experiment. Unfortunately it is not certain that immunization with tumor material produces an immune serum with a high content of antibody to the tumor cells. This gave rise to the question whether it is not possible to increase the sensitivity of the seroreaction at this particular antibody titer. This would obviously require the introduction of a larger amount of antibodies into the reacting mixture, i.e., if the dilution of the serum is unchanged then it would have to be injected in larger volume. In fact, when this same immune serum which was used in the experiments described above was tested by the complement fixation reaction in a dilution of 1: 6400 but in ten times the volume of the antigen, a positive results was obtained with a high dilution of antigen (over 1 ; 64,000). In this case the results of the in vitro experiment were not inferior to those of the passive anaphylaxis experiment in guinea pigs which had been sensitized by the same immune serum in doses of such a size that the concentration of antibody in the animals was equal to that used in the serological experiment (Table 3).

TABLE 1

Detection of Horse Albumin by Means of the Anaphylactic Reaction with Passive Sensitization

Concentration of antigen injected as the assaulting dose into the guinea pigs	Reaction of the animals in which immune serum had been injected to cause sensitization in an amount of:				
	1:320	1:3200	1:6400	1:12 800	1:25 600
1 : 500					+++
1 : 1000					+++
1 : 10 000		Died		++	++
1 : 20 000		+++	++(+++)		++
1 : 40 000		+(++)	+++	+(++)	
1 : 80 000	++(+++)	+			
1 : 160 000	—				

Meaning of symbols: +++ marked anaphylactic shock; ++ clear reaction with marked bronchospasm (spasmodic cough); + weak reaction: sneezing, scratching, repetitive movements, shortness of breath.

In view of these results it was important to discover if it is possible by immunization with a mixture of antigens in different quantitative proportions to obtain antibodies in adequate titer to the "minor" antigen and if antibodies to the "minor" antigen could be retained in the serum after removal of the antibodies to the "major" antigen.

For this purpose rabbits were immunized with a mixture of horse and rat sera, in proportions of 50: 1 and 250: 1. The total quantity of "minor" antigen (rat serum) injected was 0.04 ml per 1 kg body weight of the animals in the first group, and 0.008 ml for the second group; this was divided into 4 intravenous injections given at intervals of 4-5 days. Antibodies to rat serum were obtained in two of the three animals of the first group and in one rabbit out of three in the second group.

The content of antibodies in the blood in two pairs of rabbits which had been immunized with corresponding doses of rat serum alone was found to be higher. It should be mentioned that by using the method of a large volume of antibodies it is possible to increase considerably the sensitivity of the seroreaction. By this method, in experiments with sera obtained from rabbits immunized with a mixture of antigens, the importance was clearly shown of optimum proportions between the reacting components: the reactions were negative with a larger dose of antigen and positive at higher dilutions.

In another series of experiments rabbits were immunized with a mixture of so-called "weak" antigens, i.e., nucleoproteins from organs of the horse and rat in proportion of 50: 1 , so that the calculated quantity

of proteins of the "major" antigens injected was 32 mg and that of the "minor" antigens was 0.64 mg for the whole cycle of immunization (4 intravenous injections at intervals of 4-5 days). In this case antibodies to the "minor" antigen were detected (in dilutions of sera of 1:25 and antigen up to 1:100) in all 3 rabbits in the experiment, but at a lower titer than in animals immunized by the "minor" antigen alone in the same dose (in this case clear positive results were obtained also in serum dilutions up to 1:100 with antigen in dilutions of 1:100 and 1:200).

Investigation of the problem of preservation in the serum of antibodies to the "minor" antigen after removal of antibodies to the "major" antigen was made necessary by the fact that in experiments with soluble antigens we had to resort to a number of additional manipulations (dialysis of the sera and fractionation) which might affect the content of antibodies.

TABLE 2

Detection of Horse Albumin by the Complement Fixation Reaction with Various Dilutions of Immune Serum and Antigen

Dilution of antigen	Dilution of immune serum				
	1:100	1:400	1:800	1:1600	1:3200
1:100	++++	++++	−	−	−
1:5000	++++	++++	++++	−	−
1:10 000	++++	++++	++++	±	−
1:20 000	++++	++++	++++	−	−
1:50 000	++++	++++	+	−	−
1:100 000	+++	+	−	−	−

Meaning of symbols: ++++ total suspension of hemolysis with a double dose of complement; +++ partial suspension of hemolysis with a double dose and total suspension with 1½ doses of complement; ++ total suspension of hemolysis with 1½ doses of complement; + partial suspension of hemolysis with one dose of complement.

In setting up experiments with immune sera obtained by immunization with a mixture of two "potent" antigens, as a first step the dose of the "major" antigen was determined which was sufficient to completely extract the corresponding antibodies. Exhaustion of the sera was brought about by doses which were 4 and 10 times greater. It was found that antibodies to the "major" antigen were completely extracted and they could not be detected by the large volume method. At the same time antibodies to the "minor" antigen were retained in the serum, although in a somewhat reduced amount. In these cases it was possible by using the method of large volumes of immune serum to detect by means of these sera very small quantities of the "minor" antigen.

Less clear results were obtained in experiments with sera from rabbits immunized with a mixture of the so-called weak antigens. In order to achieve complete exhaustion of these sera from the "major" antigen (horse nucleoprotein) relatively larger quantities were required than in the similar experiments with sera against strong antigens (100-200 mg of protein against 20 ml for 1 ml of serum). After such treatment the reactions with the "minor" antigen were weak and not sufficiently clear.

DISCUSSION OF RESULTS

As shown by the results described, the superiority of the anaphylactic reaction over serological investigations lies in the fact that with it it is possible to detect antigen with a low concentration of antibodies, insufficient for giving a positive result in experiments in vitro. It is necessary to make use of the high sensitivity of the living reacting system but to reduce so far as possible the difficulties inherent in experiments in vivo, described in previous reports [1, 2]. For this purpose passive anaphylaxis could be tried, excluding desensitization, i.e., sensitizing guinea pigs with tumour antiserum from which the antibodies to normal tissues had been extracted. It is possible that in the presence of specific (tumor) antibodies they would prove adequate to

produce a reaction in the animal to injection of tumor material. This method would remove many difficulties: total exhaustion of the immune serum could be checked with sufficient accuracy; the animal would not be subjected to repeated and far from indifferent influences before the experiment; all controls would be clear and straightforward. It should be pointed out that pilot experiments to study this method gave positive results. However a detailed quantitative analysis is required, and this must be one of the next tasks to be undertaken in the investigation.

Furthermore the results of the experiments show, from the characteristic features of the serological method, that this method should not be abandoned in the study of the antigenic properties of tumors; on the contrary there are grounds for reckoning on increasing its sensitivity and accuracy of reading. Already two points can be mentioned which focus attention in this direction: 1) consideration must be paid to the law of optimum proportions, and the serological examination must be carried out with various doses of test material (the so-called "square" scheme); 2) the results obtained must be checked and supplemented by experiments by the method of large volumes of antibodies.

TABLE 3

Comparative Sensitivity of the Anaphylactic and Complement Fixation Reactions, Using the Method of a Large Volume of Antibodies

Experiment in vivo				Experiment in vitro			
concentration of antigen in the assaulting dose injected into the guinea pigs	content of immune serum in the guinea pigs with passive sensitization			dilution of antigen	dilution of immune serum		
	1:1500	1:1900	1:24000		1:1600	1:6400	1:25600*
1 : 10 000	Died	+++	+	1 : 16 000	++++	++++	++
		++(+++)	+	1 : 64 000	++++	++++	++
1 : 40 000	Died	++	+	1 : 256 000	++++	++	±
	+++	++	+	1 : 1 024 000	++	—	—
				1 : 4 096 000	—	—	—

Meaning of the symbols is the same as in Tables 1 and 2.

However it must be stated that the above-mentioned analysis related to sera which had been obtained by immunization of animals with a mixture of two different antigens. It is extremely important to perform similar experiments with a mixture of related antigens, i.e., possessing common antigenic groups. In the results quoted it was mentioned that exhaustion by a large amount of horse nucleoprotein (and subsequent treatment) extracted a large proportion of the antibodies to rat nucleoprotein from the sera of rabbits which had been immunized with a mixture of these two antigens in a proportion of 50: 1. Attention was directed to the fact that the sera of rabbits immunized against horse nucleoprotein alone, also reacted with antigen obtained from tissues of the rat although, naturally, much more weakly than with the homologous antigen. If this is on account of common components in the preparations used, then a careful study of the conditions in which it is possible to differentiate the individual antigenic components forming part of the common groups is an urgent future task in the investigation. This must first be undertaken in suitable experimental animals.

This careful study in suitable experimental animals also applies to new methods which have been introduced into serological research, such as: 1) replacement of the complement fixation reaction by the agglutination reaction with preliminary adsorption of antigen on the surface of cells or particles of a fine suspension, and 2) the precipitation reaction in a gel with counter diffusion of antigen and antibodies.

Finally we must deal with one aspect of principle, namely the character of tumor antigens and their corresponding antibodies. There would appear to be adequate grounds for considering that they belong to the group of autoantigens. In this case it must be remembered that autoantibodies may belong to the "incomplete" category and consequently require special methods for their detection. Is it not because there is so much that

is difficult, unexplained and even questionable about tumor antigens that we have so far failed to come near to a correct determination of their essential character and to study them ? Investigations in the light of modern ideas on autoantigens and autoantibodies are therefore of urgent importance.

SUMMARY

Sensitivity of the general methods was compared. For that purpose the following determinations were carried out: 1) the minimal dose of the antigen, to which guinea pigs, passively sensitized by various doses of the corresponding immune serum, reacted and 2) the quantity of the antigen which could be determined by the same immune serum in experiments in vitro. Anaphylactic reaction was found to be more advantageous in experiments with low doses of immune serum. Sensitivity of the serological method could be increased in conducting experiments by the so called, "rectangular" scheme, which takes the law of optimal ratios into consideration, as well as by using the method of large volume of antibodies. In order to reveal the components of the complex antigen one should study the method of passive anaphylaxis by sensitization of animals by immune sera, in which the antigens of the normal tissues were previously exhausted in vitro.

The problems which are next to be investigated are, the methods of differentiation of antigenic components which possess common antigenic groups, the characteristics of the new serological methods, the problem of tumor antigens with consideration of conceptions on autoantigens.

LITERATURE CITED

[1] V. I. Ioffe and L. P. Kopytovskaia, Biull. Eksptl. Biol. i Med. 44, No. 7, 82-84, (1957).*

[2] V. I. Ioffe and L. P. Kopytovskaia, Biull. Eksptl. Biol. i Med. 45, No. 1, 74-78 (1958).*

* Original Russian pagination. See C. B. Translation.