INVESTIGATION OF THE "VALENCY" OF ANTIBODIES BY MEANS OF AZOPROTEINS

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In a previous communication [1] we showed that a mixture of monoazo derivatives, and binary azo derivatives of proteins give rise to the same effect when used as test antigens in immunization and in the ring precipitation test. Under these circumstances complete suppression of the specificity of one hapten by the other was not observed i.e., antibodies to both haptens contained in the antigen were found in the immune sera in all cases. It is of considerable interest to determine the number of specifically reacting groups in these antigens and thereby to pave the way for the investigation of the problem of the "valency" of antibodies.

No unanimity exists in the immunochemical literature on this subject [3, 4, 6-12, 14, 15].

In order to study this problem we used the method of exhaustion of antisera against azoproteins, followed by performance of the cross precipitation reaction both with antibodies remaining in the supernatant portion of the serum and with the antibodies contained in the precipitate, after preliminary separation from antigen.

EXPERIMENTAL METHODS

We obtained mono- and diazo derivatives and used them to immunize rabbits as in the previous work [1]. We investigated an immune serum to horse sulfanylanthranylazoglobulin and an immune serum to a mixture of horse sulfanylazoglobulin and human anthranylazoglobulin. To determine the strength of the serum (the highest dilution of the test antigen) and to ensure complete precipitation of the antibodies (maximum precipitate with the test antigen) the precipitation reaction was performed in a volume of 1 ml (0.5 ml of antigen and 0.5 ml of immune serum). The test antigens were standardized by weight; an 8% stock solution was used and from it were prepared dilutions of 1:2, 1:4, 1:16 and so on.

To exhaust the antibodies, 10 ml of antiserum was mixed with 10 ml of antigen in the appropriate dilution. The mixture was allowed to stand for 2 hours in the incubator and then left at room temperature overnight. The liquid was separated from the precipitate by centrifugation. Completeness of precipitation of the antibodies was tested by the addition of a portion of antigen to 0.5 ml of the centrifugate. In the absence of a precipitate the exhaustion of the serum was considered complete.

EXPERIMENTAL RESULTS

The change in the titer of the precipitating serum after its exhaustion is shown in Table 1.

We can see that the antiserum against a mixture of horse sulfanylazoglobulin and human anthranylazoglobulin (experiments 1-9) precipitated horse globulin, human globulin and theirsulfanyl- and anthranylazo derivatives (experiment 1). In this antiserum were found antibodies whose specificity clearly and fully reflected the structure of the immunizing azoproteins. This can be seen from the fact that human sulfanylazoglobulin in

TABLE 1

Change in the Titer of a Precipitating Serum after its Exhaustion

Antiserum	Experiment No.	Antigen used for exhaustion of the serum	Maximum dilution of test antigen							
			horse globulin	horse azoglobulins			human globulin	human azoglobins		
				sulfanylazoglobulin	anthranylazoglobulin	sulfanylanthranylazoglobulin		sulfanylazoglobulin	anthranylazoglobulin	sulfanylanthranylazoglobulin
Against horse sulfanylazoglubulin + human anthranylazoblobulin	1	Unexhausted serum	16	256	128	—	64	128	256	—
	2	Horse globulin	0	128	64	—	64	128	256	—
	3	Horse sulfanylazoglobulin	0	0	64	—	64	0	256	—
	4	Horse anthranylazoglobulin	0	128	0	—	32	64	64	—
	5	Horse anthranylazoglobulin + human globulin	0	128	0	—	0	64	2	—
	6	Human globulin	16	256	128	—	0	64	128	—
	7	Human sulfanylazoglobulin	8	64	64	—	0	0	64	—
	8	Human anthranylazoglobulin	16	256	32	—	0	32	0	—
	9	Human sulfanylazoglobulin + horse globulin	0	4	32	—	0	0	64	—
Against horse sulfanylanthranylazoglobulin	10	Unexhausted serum	32	512	512	1 024	0	256	128	128
	11	Horse globulin	0	256	256	512	0	128	128	64
	12	Horse sulfanylazoglubulin	0	0	64	256	0	0	16	64
	13	Horse anthranylazoglobulin	0	128	0	256	0	32	0	32
	14	Horse sulfanyl- + anthranylazo glubulin	0	0	0	16	0	0	0	2
	15	Horse sulfanylanthranylazoglobulin	0	0	0	0	0	0	0	0
	16	Human sulfanylazoglobulin	4	64	128	512	0	0	64	32
	17	Human anthranylazoglobulin	4	256	64	512	0	128	0	64
	18	Human sulfanylanthranylazoglobulin	2	32	16	64	0	0	0	0
	19	Horse globulin + human sulfanylanthranylazoglobulin	0	0	0	4	0	0	0	0
	20	Control — normal horse serum	0	0	0	0	0	0	0	0

a solution of horse globulin did not completely extract the antibodies to horse sulfanylazoglobulin (experiment 9), just as exhaustion of the horse anthranylazoglobulin serum in a solution of human globulin did not lead to complete exhaustion of antibodies to human anthranylazoglobulin (experiment No. 5). This fact indicates that the immunological specificity of the azoproteins is determined not by the passive presence of the chemical radical combined with the protein in a certain manner, but by a complex of the chemical compound with the protein. Under the influence of chemical treatment and of the presence of a chemical radical, the protein changes its original structure and its immunological specificity.

TABLE 2

Cross Reaction with Antibodies against Horse Sulfanylanthranylazoglobulin Isolated from Precipitates

Type of antibody	Experiment No.	Antigen used for precipitation of antibodies	Test antigens							
				horse azoglobulins				human azoglobulins		
			horse globulin	sulfanyl-azo-globulin	anthra-nylazo-globulin	sulfanyl-anthranyl-azoglobulin	human globulin	sulfanyl-azo-globulin	anthra-nylazo-globulin	sulfanyl-anthranyl-azoglobulin
"Nonprecipitating"	1	Horse globulin	+	++	++	+++	0	++	+	++
	2	Horse sulfanylazoglobulin	±	±	++	++	0	0	±	+
	3	Horse anthranylazoglobulin	±	++	+	+++	0	++	0	++
	4	Horse sulfanylanthranylazoglobulin	0	0	0	±	0	0	0	0
	5	Human sulfanylazoglobulin	+	++	+++	+++	0	±	+	++
	6	Human anthranylazoglobulin	+	++	+	++	0	++	+	++
	7	Human sulfanylanthranylazoglobulin	±	+	+	++	0	±	±	+
"Precipitating"	8	Horse globulin	++++	+++	++	++	0	+	+	+
	9	Horse sulfanylazoglobulin	++	++++	+++	+++	0	+++	++	+++
	10	Horse anthranylazoglobulin	++	+++	+++	+++	0	++	+++	++
	11	Horse sulfanylanthranylazoglobulin	+++	+++	++	++++	0	++	++	++++
	12	Human sulfanylazoglobulin	+	+++	+	++	0	++++	++	+++
	13	Human anthranylazoglobulin	±	++	++++	+++	0	++	++++	+++
	14	Human sulfanylanthranylazoglobulin	±	++	++++	+++	0	++	++++	+++
		Control — normal horse serum	0	0	0	0	0	0	0	0

During the exhaustion of an antiserum with antigens in which the azo components were homologous but the globulins heterologous in relation to the immunizing antigen (experiments 4 and 7), a fall in the titer of antibodies to all the test antigens was observed. These findings suggest the "valency" of the antibodies to monoazoproteins. In fact, if the test antiserum contained mutually independent antibodies to each determinant, the amount of one would not be altered by exhaustion of the others. In experiment 4, horse anthranylazoglobulin (immunizing antigen – human anthranylazoglobulin) completely extracted the antibodies to horse globulin and anthranylazoglobulin and also antibodies not only to human anthranylazoglobulin but also to human sulfanylazoglobulin and globulin. Similar results were observed in experiment 7 during exhaustion of antiserum with horse sulfanylazoglobulin immunizing antigen – human sulfanylazoglobulin.

It did not seem possible to explain this extraction of antibodies by means of nonspecific adsorption, for homologous antigens (experiments 2, 3, 6, and 8) extracted only their specific antibodies. Extraction of a serum with horse sulfanylazoglobulin, for example (experiment 3) did not affect the content of antibodies to human globulin and anthranylazoglobulin.

The results of these experiments thus suggested that a test antiserum against monoazoproteins contained antibodies whose specificity was directed at the same time towards the protein and the determinant group.

Antiserum against horse sulfanylanthranylazoglobulin (experiment 10-19) precipitated horse globulin and also horse and human sulfanyl- and anthranylazoglobulin (experiment 10).

During exhaustion of a serum with horse globulin azo derivatives, complete removal of antibodies precipitating the corresponding human azoglobulins was observed (experiment 12, 13, and 15).

Complete removal of antibodies could only be achieved by the immunizing antigen – horse sulfanylanthranylazoglobulin (experiment 15), and not by mixtures of the corresponding antigens (sulfanyl + horse anthranylazoglobulin –experiment 14 – and horse globulin + human sulfanylanthranylazoglobulin – experiment 19).

In every case in this series of experiments it was found that the antigen used for exhaustion of the serum completely extracted its own antibodies and considerably reduced their titer in relation to other unrelated antigens (experiments 12, 13, 16, 17, and 18). For example, human sulfanylazoglobulin (experiment 16) exhausted not only its own antibodies but also severely reduced the titer of the antiserum to horse globulin, horse anthranylazoglobulin, and human anthranylazoglobulin. From this observation it can be concluded that a single antibody possesses simultaneous specificity to horse globulin and to human sulfanyl- and anthranylazoglobulin.

Our subsequent experiments had the object of determining the valency of the "nonprecipitating" antibodies and the antibodies present in the specific precipitates obtained by exhaustion of sera to horse sulfanylanthranylazoglobulin.

We know that "nonprecipitating" antibodies are capable of combining with antigens, but they do not form precipitates [8, 13]. In order to obtain a precipitate we used V. A. Engelgardt's method [2]. To 5 ml of serum, completely exhausted with test antigen was added 5 ml of a 1% solution of the same antigen. The mixture was thoroughly mixed and kept overnight at room temperature. After standing, the antigen – antibody complex was precipitated by the careful addition of a saturated solution of $(NH_4)_2SO_4$ until a slight opalescence appeared. After 3-4 hours a floccular precipitate was formed, which was removed by centrifugation; this was used for the isolation and study of the "nonprecipitating" antibodies. As a control we used the same serum exhausted with heterologous azoantigens. The precipitate was considered to be specific if it was formed by a concentration of the salt which did not cause precipitation in the controls.

The antibodies were isolated from the precipitates by the method of Haurowitz and his coworkers [5], with a 5% solution of NaCl in a 0.1N solution of HCl. This solution caused dissociation of the precipitate and precipitates the azoproteins. This method is applicable only to an antibody – azoantigen complex, and so for the isolation of "nonprecipitating" antibodies to horse globulin we used one of its azo compounds which was nonspecific to the test antiserum – nitroaniline-azoglobulin. Before dissociation, the precipitates were preliminarily freed from nonspecific proteins by being twice washed with a 1% solution of NaCl. In every case the antibodies were extracted by the addition of 0.5 ml of 5% NaCl solution in 0.1 N HCl to the precipitate. The mixture was thoroughly homogenized and allowed to stand at room temperature for 2 hours. The supernatant fluid was then separated by centrifugation, neutralized to pH = 7 and diluted 1 : 5 in order to lower the salt concentration, after which it was used in the cross precipitation reaction with the test antigens. For this purpose the fluid thus obtained and the

test antigen, diluted 1:10, were introduced drop by drop into the well of a glass slide. The well was hermetically sealed with a cover slip. After the mixture had been allowed to stand overnight at room temperature, the intensity of the precipitation reaction was observed by means of a hand lens: O – no precipitate, ± – traces of precipitate and +, ++, +++ – increasing amounts of precipitate. The experimental results are shown in Table 2.

"Nonprecipitating" antibodies were found to all the test antigens (experiments 1-7). Both "nonprecipitating" and precipitating antibodies (experiments 7-14) formed a specific precipitate with horse globulin and human sulfanylanthranylazoglobulin, i.e., in this series of experiments they behaved like trivalent compounds. An exception was the "nonprecipitating" antibodies to horse sulfanylanthranylazoglobulin (experiment 4), which reacted only with this particular antigen. Evidently this antigen most completely exhausted all the antibodies of the antiserum.

It should be pointed out also that, after being precipitated and separated, the "nonprecipitating" antibodies became, as it were, more avid and acquired the power of precipitating the antigens used for exhausting them.

It may be concluded from the experimental findings that the antibodies reacted with our test antigens like "multivalent" compounds. The particular "valency" of a chemically modified protein was evidently due to the number of specific groups introduced into the antigen. Thus "bivalent" antibodies appeared against monoazoproteins (see Table 1) and "trivalent" antibodies against diazoproteins (see Table 2).

The presence in an immune serum of a fraction of avid antibodies, strictly specific to the immunizing antigen, alongside "nonprecipitating" antibodies to the exhausting antigen only, indicated the presence of antibodies with varying tendencies to deviate from the mean degree of specificity. Antibodies which reacted more weakly with the corresponding antigen probably reflected its structural peculiarities less perfectly. This finding spoke against the hypothetical plurality of antibodies in a serum against a "polyvalent" antigen. It indicated the formation of a single antibody which reflected more or less clearly the whole complex structure of the antigen used for immunization. The reaction between the antibodies and heterologous antigens must evidently be explained by their structural similarity to the immunizing antigens and not by the presence of particular specific groups in the antibody. This similarity of the immunological specificity of different chemically modified proteins is due to the identical nature of the changes in the protein molecule resulting from denaturation, caused by the treatment itself and by the presence of the corresponding chemical radicals in the protein.

On the basis of the foregoing it can therefore be postulated that during immunization antibodies are formed which show specificity only to the antigen used. This specificity of the antibodies reflects more or less clearly the structure of the antigen. The "valency" displayed by the antibodies is due to the similarity of structure between the heterologous and immunizing antigens and also to the less complete specific correspondence between the individual structural peculiarities of the antigen and its antibodies.

SUMMARY

The "valency" of antibodies was studied by the method of exhaustion of antisera against mono- and diazoproteins, and subsequent cross reactions both with the antibodies left over in the supernatant fluid of the serum and with the "precipitating" and "nonprecipitating" antibodies isolated from the precipitate.

It was proved that the antibodies interact with the antigens as multivalent compounds.

The "valency" determined with regard to the azoproteins is dependent upon the number of groups introduced.

Thus, "bivalent" antibodies correspond to monoazoproteins and "trivalent" ones to diazoproteins.

The "valency" of antibodies is, evidently, determined by the structural similarity of the heterologous and the immunizing antigens as well as by the less complete specific conformity between the individual structural peculiarities of the antigen and its antibody.

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