COMPLEMENT FIXATION TO A 50% HEMOLYSIS END POINT WITH PROTEIN ANTIGEN ADSORBED ON PAPER, AND ITS ACTION ON ANTI-CANCER SERA

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(Received June 21, 1957. Presented by Active Member of the Acad. Med. Sci. USSR N. N. Zhukov-Verezhnikov)

The quantitative complement fixation test (CFT) to 50% hemolysis end point for soluble antigens was developed in Heidelberger's laboratory [5], and later modified by Konikov [3]. We first used this method in our laboratory in 1955 to determine the serological activity of azoantigens and native proteins firmly fixed on chromatography paper [1, 2].

In the present paper we described the quantitative CFT in terms of the 50% hemolytic unit, using "paper" antigens, as suggested by V. S. Gostev who directed the investigation.

EXPERIMENTAL METHODS

Use of the 50% unit in CFT demands a strict control over all reagents and experimental conditions. In the antigen, the protein nitrogen is found by Conway's method, and the erythrocyte weight for the hemolysis is standardized with a photoelectric colorimeter. The complement is first titrated, and enough is then taken so that an excess remains both in the control and in the experimental tests.

In each case the excess complement is back titrated. From the difference, the number of combined complement units is found. A complement unit is taken as the amount of complement (in 0.5 ml) which will cause 50% hemolysis in 30 minutes at 37°C of 0.5 ml of the standard suspension of erythrocytes.

The reaction consists of two stages. Stage I, which consists of the actual combination of the complement with the specific antigen-antibody complex, takes place in the cold. A definite weight (15-20 mg) of antigen adsorbed on paper is placed in 0.3 ml of physiological saline, and 0.2 ml of antiserum diluted 1:10, and 0.1 ml of complement (either undiluted or diluted 1:2, according to its strength), are added. The total volume of the mixture in Stage I is 0.6 ml. Control of the antigen, antiserum and complement is essential.

Stage II consists of the back titration of the uncombined complement. On the following day a definite volume (usually from 0.01 to 0.1 ml) is taken from each experimental and control tube, it is made up to 0.5 ml with physiological saline, and an equal volume (0.5 ml) of a suspension of red cells is added. The mixtures are then placed for 30 minutes in an incubator at 37°C. The tubes are then cooled, centrifuged, and the coefficient of extinction of the supernatant fluid is determined using a photoelectric colorimeter. The percentage hemolysis is read from a graph of percentage hemolysis against the coefficient of extinction. In order to find the number of units of free complement in the sample, an amount of the mixture must be taken which results in a hemolysis of between 20 and 80% of the cells.

The amount of complement giving 50% hemolysis cannot usually be determined directly. It can, however, be calculated from Krogh's formula from the amount of complement causing a known amount of hemolysis:

$$\log x_2 = \log x_1 - n\log \frac{y}{100-y}.$$

where x_2 is the amount of complement required to give 50% hemolysis; x_1 is the amount of complement taken in the experiment; n is the coefficient of extinction, which for the particular experimental conditions was equal to 0.2; and y is the percentage of hemolysis observed.

The number of units of complement in the sample are found from the 50% end point titration value. The amount of complement fixed is found by subtracting the number of units of free complement remaining in the mixture from the number in the control tube. The anticomplementary action of the antigen or antiserum may cause the number of free units of complement in the control tube to be less than the number used in the experimental tube. The calculation is based on the control tube showing the strongest anticomplementary effect. In cases where the anticomplementary effect of antigen or antiserum is very high it is best to increase the amount of complement somewhat, though excessively large amounts may reduce the accuracy of the test.

We used the method described above to test the serologic activity of several groups of "paper" antigens.

Preparation of "paper" azoantigen. Tissue extracts of a human stomach cancer and a normal stomach and spleen were made by triturating with ten times the volume of physiological saline, centrifuging, and then adding the azo dye diazodianizide.

The resulting azoprotein was adsorbed on strips of chromatographic paper, dried at room temperature, washed in water for 5-6 hours, and again dried at room temperature. The incorporation of diazodianizide into the protein molecule causes it to be fixed firmly to the paper.

Preparation of "native" antigen adsorbed onto paper. Strips of chromatography paper are immersed for 10-15 minutes in the saline extracts of human tissue. The paper impregnated with protein is dried in air at room temperature, then washed in tap water for 3-4 hours and again dried at room temperature.

The second method of preparing the paper and antigen is simpler and causes less denaturing of the protein.

The antisera used in the above experiments were obtained by immunizing rabbits by injecting macerated human stomach cancer intraperitoneally.

EXPERIMENTAL RESULTS

Table 1 shows a typical example, which is one of the 40 experiments using paper azoantigen and the 50% end point.

TABLE 1

"Paper" azoantigen	Control antigen	Rabbit serum No. 1831		Normal rabbit serum		Control	
		Units of complement					
		free	combined	free	combined		
Human stomach tissue	30	5,5	24,5	30	—	Diazodianizide treated paper	30
Human splenic tissue	30	6	24	30	—		
Human serum globulin	30	—	30	30	—	Pure chromatography paper	30
						Rabbit serum No. 1831	30
						Normal rabbit serum	30
						Complement	30

Note: Serum No. 1831 is antihuman gastric carcinoma. The figures indicate the number of complement units.

These results show the marked serological activity of the "paper" azoantigens of tissues from the human stomach and spleen and the serum globulin; these combine specifically with a considerable amount of complement in the presence of the corresponding antiserum. In this particular case, anticarcinoma serum was used. The specific combination of the complement with normal tissue proteins in the presence of anticarcinoma antiserum is due to a common antigen present in normal and cancerous human subjects.

Many experiments were also carried out to determine the serological activity of "native" "paper" antigens.

The results of one of these experiments are shown in Table 2.

TABLE 2

Antiserum		"Paper" tissue antigen					
		of gastric carcinoma		of normal stomach		of spleen	
		units of complement					
		free	combined	free	combined	free	combined
Serum No. 3804		4.1	28.9	4.1	28.9	4.1	28.9
" No. 2124		3.8	29.2	4.1	28.9	3.8	29.2
" not containing Antibody		33	—	33	—	33	—
Control	Antigen	33	—	33	—	33	—
	Serum No. 3804	33	—	—	—	—	—
	Serum No. 2124	33	—	—	—	—	—
	Serum of normal rabbit	33	—	—	—	—	—
	Complement	33	—	—	—	—	—

Note: Sera Nos. 3804 and 2124 are rabbit antihuman gastric carcinoma sera. The figures indicate the number of complement units.

It can be seen from Table 2 that the "native" antigens, fixed by drying on chromatography paper, show a high activity in their action on polyvalent antihuman gastric carcinoma antiserum.

Comparative experiments showed that "paper" azoantigens are not superior to "native" "paper" antigens. On the contrary, "native" "paper" antigens are not only highly active serologically but they are less easily denatured, and this enables them to be preserved unchanged.

The antigens adsorbed onto paper preserve their power to react with serum for several months.

Our results show that complement fixation tests to 50% end point can be made on antigens adsorbed on paper. Fixation of complement to 50% end point with the paper antigen not only gives an accurate quantitative result, but has the further important advantage that the antigen adsorbed on paper may be readily removed from the mixture and replaced by a different antigen of known immunological specificity as required by the particular investigation.

V. S. Gostev suggested that we should use this method to attempt a specific extraction of antibodies so as to obtain antisera with a narrow range of specificity.

We give here the preliminary results which we have obtained. For this extraction we took immune sera obtained from rabbits by immunizing with ground up human gastric carcinoma given intraperitoneally. "Native" "paper" antigens from normal and cancerous human stomachs were used as antigens.

TABLE 3

Extraction of Antihuman Gastric Carcinoma Sera by Normal Stomach Protein Adsorbed on Paper (Experiment No. 12 of 2/4/1957. Total number of experiments equals 18)

Sample			1	2	3	4	5	6	7	8	9	10	11
Stage I of the reaction in the cold	1st day	Antiserum in ml	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
		Physiological saline in ml	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0 3
			—	—	20 mg Ns 1 day	20 mg Ns 2 days	20 mg Ns 1 day	20 mg Ns ½ day, 20 mg Ns ½ day	—	20 mg Ns 1 day	20 mg Ns 2 days	20 mg Ns 1 day	20 mg Ns ½ day 20 mg Ns ½ day
	2nd day	'Paper' antigens	—	—	—	—	20 mg Ns 1 day	20 mg Ns 1 day	—	—	—	20 mg Ns ½ day, 20 mg Ns ½ day	20 mg Ns ½ day 20 mg Ns ½ day
	3rd day		—	20 mg Ns	20 mg Ns	20 mg Ns	20 mg Ns	20 mg Ns	20 mg Canc s	20 mg Canc s	20 mg Canc s	20 mg Canc s	20 mg Canc s
		Complement in ml	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Stage II	4th day	Titration of uncombined complement																					
		1st-sample		2nd-sample		3rd-sample		4th-sample		5th-sample		6th-sample		7th-sample		8th-sample		9th-sample		10th-sample		11th-sample	
Units of complement		free	combined	free	combined	free	combined	free	combined	free	combined	free	combined	free	combined	free	combined	free	combined	free	combined	free	combined
Serum No. 2124		51	—	—	51	5.3	45.7	6.4	44.6	51	—	51	—	5.1	45.9	5.5	45.5	5 3	45.7	6	45	7.3	43 7
Serum No. 3017		51	—	5.5	45 5	5.8	45.2	6.6	44.4	51	—	51	—	5.3	45.7	6	45	6	45	6.2	44.8	6.6	44.4
Serum of normal rabbit		51	—	51	—	51	—	51	—	51	—	51	—	51	—	51	—	51	—	51	—	51	—
CSe No. 2124		51	—	CSE of normal rabbit				51	—	Paper alone + serum No. 3017				51	—	Control complement		51	—	—	—	—	—
CSE No. 3017		51	—	Paper alone + serum No. 2124				51	—	Paper alone + serum of normal rabbit				51	—	—	—	—	—	—	—	—	—

Note. Ns — normal stomach; Canc s. — cancer of stomach; CSe — control serum. Numbers in Stage II of reaction indicate number of units of complement.

Extraction of the anticancer serum was carried out in the quantitative CFT as follows.

Eleven samples were taken. To each was added 0.2 ml of antiserum diluted 1:10 and 0.3 ml of physiological saline (Table 3).

Stage I of the reaction lasted up to 3 days: the normal stomach "paper" antigens were replaced, as shown, in samples 1 to 11 for the first 2 days, in order to extract the antibodies to normal stomach antigens. On the third day, in samples 1 to 6, the "paper" antigen of normal stomach was replaced by fresh normal stomach antigen (control to show complete extraction), and in samples 7-11 the normal stomach antigen was replaced by gastric carcinoma antigen, as a control on the specificity of the extraction. No further antigen was added to sample 1, and the second and seventh samples served as controls over the immunological activity of the "paper" antigens. 0.1 ml of whole complement was added together with the last portion of antigen. Next day, after titrating the complement, the uncombined complement was calculated by the method described above.

It can be seen from the results given in Table 3 that complete removal of the antibodies to normal stomach tissue antigens occurs as a result of leaving the mixture in contact with these antigens for two days. In this case, 20 mg of antigen is placed in the test tube on the first day, and is then replaced by more of the same antigen. Sera which have been exhausted in this way do not react with normal gastric tissue antigens (tests 5 and 6), while with gastric carcinoma antigen (tests 10 and 11) they combine with a considerable number of complement units, which demonstrate clearly the specific nature of the antibody depletion.

These results show that protein antigens adsorbed on paper have a specific effect in depleting antisera, and that the effect can be measured quantitatively by CFT.

Thus, the 50% end point quantitative CFT demonstrates a high serological activity in protein antigens fixed on paper either as azocompounds or by simple drying at room temperature. The method allows various antigens to be used one after the other in the course of a single experiment. This permits proteins adsorbed onto paper and the quantitative CFT to be used to determine the optimal conditions for obtaining antisera with a narrow range of specificity.

SUMMARY

Protein antigens fixed on paper in the form of azocompounds or by simple drying at room temperature have a high serological activity and specifically fix the complement in the presence of a corresponding antiserum. This was established by the method of quantitative reaction of complement fixation (50% titer). Complement fixation test (50% titer) for "paper" antigens gives an opportunity of using different antigens in the same test and replacing them during the experiment. This permits the use of antigens adsorbed on paper and the quantitative complement fixation test for investigation of the optimal conditions of obtaining the specific antisera.

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