QUANTITATIVE REACTION OF SPECIFIC NITROGEN FIXATION BY PROTEIN ANTIGENS ADSORBED ON DERMATOL AND PAPER

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Of much importance to serology is the use of quantitative methods of study, which express the results of a specific reaction not by plus signs but by exact, readily reproducible figures. Among such quantitative methods one of the most important is the determination of immunological affinity between antigen and antibody with respect to the amount of protein nitrogen stably bound in the products of a specific reaction [3, 4, 7, 9, 10, 11, 12]. Dermatol* in particular has been proposed [1, 2, 6] as adsorbent of antigenic substances.

Attempts were made in our laboratory to develop a quantitative method for serological study of antigens adsorbed on inert particles. A. K. Saakov (1953) showed that dermatol particles after adsorbing antigens upon reaction with appropriate antiserum give a specific nitrogen increase in the agglutinate form.

The purpose of the present paper is further development of methods for quantitative study of the serological reaction of adsorbed antigens.

In the first part of the study we reproduced data obtained by Saakov in his work on dermatol-antigen in order to evaluate it and to attempt to find more convenient procedures for quantitative serology.

EXPERIMENTAL METHODS

Preparation of antigens adsorbed on dermatol:

a) Preparation of dermatol fractions. 200 mg dermatol was suspended in 30 ml distilled water. The suspension was fractionated by centrifuging in a hand centrifuge. Three fractions of dermatol particles were obtained:

Fraction I (heavy) – precipitate obtained after 10 minutes of centrifuging.
Fraction II (medium)– precipitate obtained after 15 minutes of centrifuging.
Fraction III (light) – precipitate obtained after 15 minutes of centrifuging supernatant fluid from Fraction II.

Fractions I and III were discarded. Fraction II was dried and kept in a weighing bottle. We used the medium dermatol fraction for experiment, since it was found in preliminary experiments that compared to the other fractions it has the greatest adsorptive properties with respect to protein antigens.

b) Preparation of antigen. From an aqueous-saline extract of cancer tissue of human stomach, prepared by grinding with 10 volumes of physiological solution, after centrifuging, the globulin fraction was isolated by

* Russian trade name.

means of 33% saturation with ammonium sulfate. After dialysis the protein content of the globulin solution was determined by Conway's method.

c) Adsorption of protein on dermatol particles. Batches of fraction II of dermatol (60 mg) were placed in centrifuge tubes, to each tube was added 5 ml of a solution of the globulins of cancer tissue of human stomach, containing about 1 mg protein in 1 ml. Tubes were stoppered with rubber stoppers and shaken in the cold every 2-3 minutes for 1 hour, then placed in the refrigerator overnight. On the following day shaking of tubes was repeated every 2-3 minutes in the cold for 1 hour. Washing samples free of nonadsorbed protein was carried out strictly in the cold by repeated centrifuging until a clear centrifugate was obtained.

d) Antiserum was obtained by intraabdominal immunization of rabbits with a brei of cancer tissue of human stomach.

e) Reaction of specific protein increase in dermatol agglutinate. To dermatol-antigen was added 3 ml of antiserum (1 : 10 dilution) in the experimental sample and 3 ml of normal rabbit serum (1 : 10 dilution) in the control. Experimental and control each consisted of three parallel samples. All samples were placed in the thermostat at 37° for 2 hours, shaken every 10 minutes and placed in the refrigerator overnight. The next day all samples were washed in the cold with physiological solution by repeated centrifugation, then transferred quantitatively to Kjeldahl flasks for combustion with concentrated sulfuric acid. The results obtained are given in Table 1.

TABLE 1

Protein Increase (in mg) With Respect to Nitrogen in Dermatol Agglutinate In Presence of Normal and of Immune Serum

Expt. No.	D-A + normal rabbit serum	Antiserum	D-A + antiserum	Specific protein increase
1	1.575	№ 1831	1.6275	0.0525
2	1,575	№ 1672	1.8725	0.2975
3	1.9425	№ 1831	2.7325	0.79
4	1.9425	№ 1672	2.2225	0,28
5	1.8025	№ 1382	2.2175	0.415
6	1.8025	№ 1672	2.3275	0.525
7	1.61	№ 1387	2.24	0.63
8	1.61	№ 1672	1.75	0.14
9	2.1	№ 1387	2.415	0.315
10	2.1	№ 1880	2 607	0.507
11	1.229	№ 1775	1.721	0.492
12	1 229	№ 1951	1.47	0.241
13	1.00625	№ 1231	1.27625	0.27
14	1,00625	№ 1951	1.16375	0.1575
15	1,4	№ 1387	2.131	0.731
16	1,19	№ 1672	1.505	0.315
17	0 69125	№ 1672	0,76125	0.07
18	1.705	№ 1382	1.8025	0.0975
19	1.3475	№ 1672	1.8025	0.455

Note: D-A – dermatol-antigen (globulin fraction of cancer tissue of human stomach); antiserum – serum of rabbit immunized with brei of cancer tissue of human stomach.

Results of 19 experiments are shown in Table 1, from which is seen the marked increase in protein nitrogen in the dermatol-antigen through specific fixation of antiserum proteins by the adsorbed antigen.

However, treatment of dermatol-antigen with normal serum not containing the antibodies also leads to marked nitrogen increase in the dermatol precipitate through nonspecific fixation of serum proteins. In order to determine the amount of specific fixation of antiserum proteins from the total increase in protein nitrogen it is necessary to deduct the protein nitrogen bound to dermatol-antigen in the control with normal serum.

The quantitative agglutination reaction of dermatol particles which have adsorbed antigen has a number of substantial disadvantages. The method is very laborious, it requires a powerful centrifuge for washing the dermatol precipitate and leads to great expenditure of work and time. The quantitative transfer of the dermatol precipitate to Kjeldahl flasks for combustion is a great inconvenience. Dermatol-antigen cannot be used in the complement-fixation reaction in view of its strong anticomplementary action. These substantial defects of the dermatol agglutination method led us to search for other adsorbents for antigens, permitting readier and more rapid quantitative study of serological reactions.

We selected chromatographic paper, cotton fabric and cotton wool as adsorbents.

These materials fix proteins stably if the latter are coupled with bis-diazotized benzidine or dianisidine. This recommendation was made to us by Professor A. I. Korolev for which we express our sincere gratitude.

Protein antigens, fixed to a paper surface, in the presence of antiserum are able to fix stably the serum proteins, producing specific increase of protein nitrogen.

1. Preparation of azo-antigens fixed to paper. Dianisidine ($C_{14}H_{16}O_2N_2$, molecular weight 244.3) in the amount 61 mg is dissolved in 50 ml distilled water and diazotized with cooling in an acid medium by fractional addition in 0.5 ml amounts of 0.1 N $NaNO_2$ solution. HCl is added in excess, since the diazonium salt formed upon reducing acidity of the medium may react with unreacted amine with formation of secondary products. The diazotization is controlled by testing on starch-iodine paper. A positive reaction on starch-iodine paper, whose stable and distinct blue color from 1-2 drops of sample does not disappear during 6-8 minutes, indicates presence of excess sodium nitrite in the medium and consequently completion of diazotization.

The next step is coupling the diazodianisidine obtained with protein.

Aqueous-saline extracts of human organs – cancerous stomach, normal stomach, spleen and human serum globulins were used in our experiments.

The protein solution was made alkaline by addition of a half volume of 5% Na_2CO_3 solution and the freshly prepared diazodianisidine (diazonium salt) was added with constant stirring in the cold in divided portions of 0.5 ml. A positive reaction with N-acid indicates completion of the coupling reaction. The azo-protein obtained is adsorbed on strips of chromatographic paper, dried at room temperature and after washing with tap water for 5-6 hours again dried at room temperature. The diazodianisidine was introduced into the protein molecule in order to assure stable fixation of protein to the chromatographic paper. Azo-protein can also be fixed to cotton fabric and cotton wool.

2. Reaction of specific protein increase. In order to study specific activity of paper azo-antigen we developed a new serological reaction – the reaction of specific fixation of antiserum protein by azo-protein adsorbed on paper [5, 8].

To a batch of paper azo-antigen (6 mg) on a glass slide with a depression, 0.1 ml of appropriate undiluted antiserum is added. As control 0.1 ml of undiluted serum not containing antibodies to the given antigen is added to a similar batch of antigen and 0.1 ml physiological solution to another. The samples on glass slides in a moist chamber are placed in a thermostat at 37° and after 2-hour incubation are put in the refrigerator overnight. Then the pieces of paper azo-antigen are washed with 3 successive portions of 10 ml of cold physiological solution for 5 minutes each. The washed samples are subjected to combustion with sulfuric acid and their protein content is determined by Conway's method.

In Table 2 is given a typical protocol of one experiment.

It is seen from Table 2 that the paper antigen, an azo-protein from globulins of normal human serum, adsorbed on chromatographic paper, gives a distinct specific increase of protein upon incubation with serum against human tissues. Together with specific adsorption there occurs in this case nonspecific adsorption, which must also be considered in calculating the specific increase.

In spite of a number of advantages of the paper azo-antigens and the simplicity of the method of determining nitrogen increase (the antigen retains activity for several months, can be readily transported and has no appreciable anticomplementary properties) we cannot avoid noting one very substantial factor. In the process of coupling protein with diazodianisidine the protein undergoes quite strong chemical action, which cannot avoid being reflected in its immunological properties. There is no doubt that the protein is to a certain extent denatured.

TABLE 2

Protein Increase (in mg) in Azo-Antigens Adsorbed on Chromatographic Paper

Paper azo-antigen	Amt. of protein in adsorbed antigen	Anti-serum	Normal rabbit serum	Physiological solution	Amt. of protein after incubation	Specific protein increase
weight in mg		in ml			in mg	
6	0.07875	0.1	–	–	0.2625	0.13125
6	0.07875	–	0.1	–	0.13125	–
6	0.07875	–	–	0.1	0.07875	–

Note: Paper azo-antigen – azo-globulins of human serum; antiserum – serum against human tissues (undiluted).

Now we have developed a new procedure for preparing a so-called native antigen fixed to paper which is without the defects of the azo-protein method.

EXPERIMENTAL RESULTS

Into an aqueous-saline extract of human tissue (prepared by grinding tissue in 10 volumes of physiological solution) strips of chromatographic paper were immersed for 10-15 minutes. The paper after saturation with protein solution was dried in air at room temperature, washed with tap water for 3-4 hours and again dried at room temperature. The reaction of protein nitrogen fixation was performed as with the paper azo-antigens described above.

TABLE 3

Protein Increase (in mg) in Native Antigens Fixed to Chromatographic Paper

Paper antigen	Amt. of protein in paper antigen	Anti-serum	Normal rabbit serum	Physiological solution	Amt. of protein after incubation	Specific protein increase
weight in mg		in ml			in mg	
20	0.06125	0.2	–	–	0.21875	0.105
20	–	–	0.2	–	0.11375	–
20	–	–	–	0.2	0.06125	–

Note: Paper antigen – aqueous-saline extract of cancer tissue of human stomach, adsorbed on chromatographic paper; antiserum – serum against brei of operative cancer tissue of human stomach.

In Table 3 are given the results of determination of serological activity by the method of specific increase in protein nitrogen of native proteins fixed to chromatographic paper. The data, in the form of a typical protocol, indicate the pronounced serological activity of the new type of fixed antigens we obtained.

The final form of the method of fixing protein to paper adsorbent proposed in this report is very simple and practicable. Upon saturating paper with protein solution, drying it, washing and again drying, a serologically

active antigen is obtained, which invariably gives the reaction of fixing protein nitrogen of antiserum. In fixing proteins to paper with simple drying at room temperature the protein studied is considerably less denatured than when diazodianisidine is introduced into the molecule.

Paper antigens obtained by the method of coupling with diazo-compounds, as well as native paper antigens, retain serological activity for several months. Paper antigens are being used successfully by us at the present time for extraction of general species antibodies with the purpose of obtaining highly specific serums.

The reaction of protein fixation by antigen adsorbed on paper is a new serological reaction. It cannot be called either agglutination, precipitation or complement-fixation. It is a reaction of a special sort. The antigen adsorbed to the paper or cotton surface is able by binding with antibody to fix stably antiserum proteins. The reaction is specific and expresses quantitatively the degree of immunological affinity in a given antigen-antibody system.

SUMMARY

A specific addition of protein nitrogen was obtained in the agglutinate of dermatol particles, covered by antigen. Protein combined with azo-dye with bis-diazotized dianisidine gives stable sorption on the surface of the paper, cotton material or cotton wool. Such paper azo-antigen gives a pronounced specific addition of protein nitrogen which comes from the proteins of the antiserum. Analogous data were obtained with native paper antigens, secured by simple drying of the protein solution in which chromatographic paper is soaked. Reaction of specific fixation of the protein nitrogen on the surface of the paper antigen represents an easily performed new serological reaction in immunology. This reaction gives a numerical value of quantitative characteristics of the immunological antigen – antibody relation.

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