IMMUNOCHEMICAL PROPERTIES OF COLD HEMAGGLUTININS OF RABBITS

N. P. Mesropyan and A. Ya. Kul'berg

UDC 612.118.221.2.017.1

Cold hemagglutinins of rabbits, obtained after repeated intravenous immunization of the animals with stroma of sheep's erythrocytes, contain antibodies of the 19S and 7S globulin type. Two proteins, differing in their antigenic structure, were detected in the 7S antibodies, one of them being identified as an immunoglobulin of the IgG subclass.

Cold hemagglutinins (CH) are the only type of antibodies whose affinity for antigen is substantially modified by elevation of the temperature. Since the reversible loss by CH of their antigen-binding power at 37° may be the result of conformational transformations in the molecule, directly or indirectly modifying the active center, investigation of these antibodies may be of great importance in the structural analysis of active areas of the polypeptide chains of immunoglobulins. In man, CH have been detected in some cases under normal conditions, and also in certain pathological states. They have been found to contain immunoglobulins belonging to subclasses IgM, IgA, and IgG* [3, 4, 7-9, 11, 12]. Only in one case has the obtaining of CH experimentally been described, as the result of immunization of rabbits with a culture of Listeria monocytogenes isolated from patients with hemolytic anemia [6].

The object of this investigation was to obtain CH in experimental animals and isolate them in a pure form, and then to analyze them immunochemically.

EXPERIMENTAL METHOD

The stroma was obtained from sheep's erythrocytes by Kabat's method [13].

Antisera. Rabbits were sensitized by intravenous injection of stroma in a dose of 0.1 mg (as N_2). During the next 5 days the animals received daily injections of antigen in a dose of 1 mg (as N_2), and after an interval of 2 days, the cycle of immunization was repeated with daily injections of antigen in a dose of 2 mg (as N_2). On the 5th-6th day after the end of immunization, the animals were bled. The resulting serum was kept in the frozen form.

Isolation of CH. Erythrocyte stroma (40 mg as N_2) was mixed for 18 h at 0° with 40 ml antiserum. The stroma with adsorbed antibodies was separated by centrifugation at 0° for 15 min at 6600 g, and the residue was washed repeatedly with 0.15 M NaCl solution under the same conditions. Completeness of removal of the unbound serum proteins was judged from absorption by the washing liquid at 280 mμ (SF-4A spectrophotometer). The washed stroma was suspended in 10 ml physiological saline and incubated for 1 h at 37°. The suspension was centrifuged at 37° for 30 min at 18,000 g and the supernatant was collected. This procedure was repeated three times. The protein content in each eluate was determined by Folin's method.

*The nomenclature of immunoglobulins and their subunits recommended by the World Health Organization [14] is used in this article.

Laboratory of Immunochemistry, N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR G. V. Vygodchikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 69, No. 1, pp. 64-68, January, 1970. Original article submitted April 4, 1969.

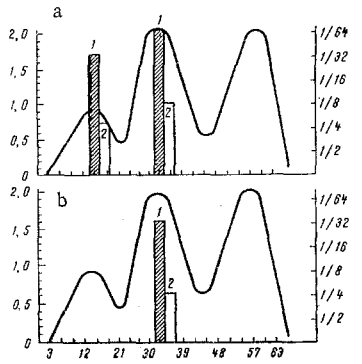

Fig. 1. Titers of cold and warm hemagglutinins of protein fractions obtained by gel filtration on Sephadex G-200. Abscissa: volume of eluate (in ml); ordinate: on the left, extinction at 280 mμ, on the right, titers in hemagglutination test at 4° (1) and at 37° (2). a) Before reduction with 2-mercaptoethanol; b) after reduction with 2-mercaptoethanol.

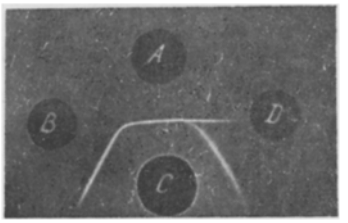

Fig. 2. Precipitation of CH eluate in agar. A) CH eluate; B) control IgG preparation; C) antiserum against rabbit globulins; D) F_{AB} fragment.

Serologic Tests. The hemagglutination test with sheep's erythrocytes was carried out at 4, 20, and 37° by the usual method. The complement fixation test in the cold was performed in the quantitative modification of Konikov and Tarkhanova [2], using stroma of sheep's erythrocytes as the antigen.

Electrophoresis and immunoelectrophoresis were carried out in 1% agar gel (Difco) in veronal buffer, pH 8.6 and μ 0.1, in a potential gradient of 6 V/cm. In the electrophoretic experiments the agar plate was cut in the equatorial plane into strips 0.5 cm wide, and protein was eluted from them after disintegration of the gel by freezing and thawing.

The double diffusion reaction was carried out in 1% agar gel made up in 0.15 M NaCl in the micro-modification of Gusev and Tsvetkov [1]. Both in electrophoretic and double diffusion in agar methods, ass serum against rabbit globulins was used for development.

Fractionation of Antiserum on Sephadex G-200. Rabbit antiserum was added in a volume of 5 ml to a column of Sephadex G-200 in 0.15 M NaCl solution buffered with 0.01 M phosphate buffer to pH 7.3, and protein was eluted with the same solution. Fractions of 4 ml each were collected with an automatic collector and analyzed on the spectrophotometer at 280 mμ.

The 7S IgG globulins were isolated in two stages. To begin with, the globulin fraction was extracted from the serum by precipitation with ammonium sulfate at 40% saturation. The globulin fraction was dialyzed exhaustively against 0.01 M phosphate buffer, pH 6.6, and filtered through a column with DÉAÉ A-50 in the same buffer.

Reduction of the hemagglutinins with 2-mercaptoethanol was carried out at 37° for 1 h at pH 7.3. The concentration of 2-mercaptoethanol in every case was 0.1 M.

EXPERIMENTAL RESULTS

Antiserum obtained after immunization of the animals with stroma from sheep's erythrocytes had a titer of 1:640 at 37° and 1:10,240 at 4° in the hemagglutination test. After incubation of the antiserum with 0.1 M 2-mercaptoethanol solution its titer was 1:2560 at 4° and 1:320 at 37°. The above ratio between hemagglutination titers at 4° and 37° was observed regularly, before and after treatment with 0.1 M 2-mercaptoethanol solution, in all ten batches of antiserum tested. The antisera did not agglutinate keterologous hen's erythrocytes in the cold or at 37°.

The hemagglutinating serum was fractionated on a column with Sephadex G-200, and the titer of cold and warm hemagglutinins in each protein peak was determined before and after treatment with 2-mercaptoethanol (Fig. 1).

The 19S and 7S fractions of the serum proteins contain both warm and cold hemagglutinins (Fig. 1a). The 19S-hemagglutinins lost their activity after treatment with 2-mercaptoethanol. A very slight decrease in titer of the 7S-hemagglutinins could take place after reduction on account of slight overlapping of the peaks of the 7S- and 19S-immunoglobulins (Fig. 1b). The 7S IgG globulins isolated from the antiserum on

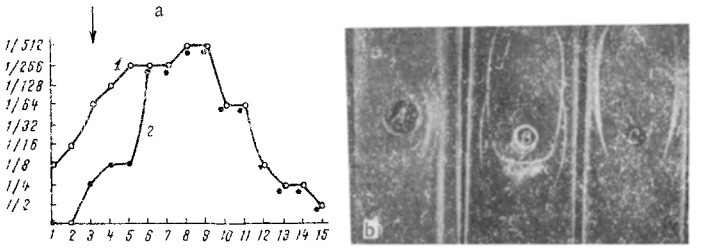

Fig. 3. Immunochemical characteristics of CH fractions obtained by preparative electrophoresis: a) CH titers in hemagglutination test; abscissa, fraction Nos.; ordinate, hemagglutination titers. 1) Before reduction with 2-mercaptoethanol; 2) after reduction with 2-mercaptoethanol; b) immunoelectrophoresis of CH fractions; A) fraction 2-5; B) fraction 6-11; C) control IgG preparation; gutters contain antiserum agains rabbit globulins.

DÉAÉ-Sephadex and, according to the results of antigen analysis, not contaminated by other serum proteins, in a concentration of 2 mg/ml gave a hemagglutination reaction at 4° in a dilution of 1 : 8180 and at 37° in a dilution of 1 : 1024.

CH eluted from the erythrocyte stroma at 37°, in a concentration of 0.5 mg/ml, had a titer of 1 : 512 in the hemagglutination test at 4°, 1 : 128 at 20°, and 1 : 4 at 37°. After treatment with 2-mercaptoethanol the titer at 4° fell to 1 : 128.

The CH eluate possessed complement-fixing activity, for 1 mg of the preparation fixed 57,600 units of complement from a total of 219,600 units in the zone of equivalence. The CH eluate was tested by the method of double diffusion in agar and by electrophoresis with serum against rabbit globulins (Fig. 2). It is clear from Fig. 2 that the eluate contained at least three individual proteins. One of them gave a reaction of complete identity with the rabbit IgG globulin isolated on DÉAÉ-Sephadex.

The experiments with preparative electrophoresis revealed the electrophoretic nonhomogeneity of the CH (Fig. 3a). Fractions located near the starting line (2-5) were sensitive to the action of 2-mercaptoethanol. Immunoelectrophoretic analysis of these fractions revealed two antigenic components with identical immunoelectrophoretic mobility (Fig. 3b). During immunoelectrophoresis of fractions 6-11, resistant to the action of 2-mercaptoethanol, two components with different electrophoretic mobility and different antigenic structure were discovered. One of them had a charge identical with that of rabbit IgG (Fig. 3b).

The results described above demonstrate that CH can be obtained by immunization of rabbits with the stroma of sheep's erythrocytes.

The CH are distributed in fractions 19S and 7S of the serum globulins. The 19S CH are inactivated by reduction with 2-mercaptoethanol, while the 7S CH are resistant to such treatment.

The results of antigen analysis showed that CH eluted from the erythrocyte stroma belong to different subclasses of immunoglobulins. One of the protein components of CH was identified as rabbit 7S IgG. The presence of CH in the composition of IgG was confirmed by investigation of the fraction of this protein isolated by ion-exchange chromatography from hemagglutinating antiserum.

Because of the absence of standard rabbit IgM and IgA preparations it was not possible to identify the other components of CH detected by antigen analysis. It is evident, however, that the CH fractions isolated by preparative electrophoresis and sensitive to 2-mercaptoethanol have properties identical with those of immunoglobulins of the IgM subclass.

It follows from this investigation that in the case of repeated immunization of rabbits with stroma of sheep's erythrocytes, CH belonging to three different subclasses of immunoglobulins are synthesized.

As was mentioned above, CH also belonging to the three main subclasses of immunoglobulins have been found in man in certain diseases, but in each case, as a rule, CH of only one subclass were discovered, and the others were exceptions.

The results of the present investigation show, in particular, that if the character of the serologic behavior of CH is determined by their chemical structure, it must be typical of antibodies belonging to different subclasses of immunoglobulins. In this connection the identity of structure of the light chains composing the immunoglobulins of different subclasses should be borne in mind [5]. It has been shown previously that the conformation of light chains undergoes reversible changes as the temperature is raised, and that the critical point for light chains of human immunoglobulins is 56° [10]. If, because of structural differences, the critical point for conformational changes of the light chains of CH is at 37°, this may entail reversible changes in the configuration of the active center itself at this temperature.

LITERATURE CITED

1. L. A. Zil'ber and G. I. Abelev, The Virology and Immunology of Cancer [in Russian], Moscow (1962), p. 383.
2. I. A. Tarkhanova and A. A. Konnikov, Zh. Gig. Épidemiol. (Prague), 1, 387 (1957).
3. C. D. Angevine, B. K. Andersen, and E. V. Barnett, J. Immunol., 96, 578 (1966).
4. P. A. Charlwood, Brit. J. Haemat., 3, 273 (1957).
5. S. Coen, Nature, 214, 449 (1967).
6. N. Costea, V. Yakulis, and P. Heller, Science, 152, 70 (1966).
7. N. Costea, V. Yakulis, and P. Heller, Blood, 26, 323 (1965).
8. J. J. Costanzi and C. A. Coltman, Clin. Exp. Immunol., 2, 167 (1967).
9. C. C. Curtain, A. Baumgarten, I. Gorman, et al., Brit. J. Haemat., 11, 471 (1965).
10. G. M. Edelman and I. A. Gally, J. Exp. Med., 116, 207 (1962).
11. L. S. Goldberg and E. V. Barnett, J. Immunol., 99, 803 (1967).
12. K. S. Gordon, J. Immunol., 71, 220 (1953).
13. E. Kabat and M. M. Mayer, Experimental Immunochemistry, Springfield (1964), p. 110.
14. Bull. World Health Org., 30, 447 (1964).