THE USE OF ANTIBODIES AGAINST γ-GLOBULIN IN THE INDIRECT FLUORESCENT ANTIBODY TEST

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During our studies of mouse antigens using the indirect method of fluorescent antibodies [16] we found that the labeled antiserum reacted directly with the sections of mouse organs. In the precipitation test in agar, this antiserum also reacted with an extract of mouse liver. The usual methods of adsorption and exhaustion of cross-reacting antibodies proved of little value, and we therefore attempted to eliminate the crossed reactions by replacing the labeled antiserum by a fraction of antibodies against γ-globulin.

Antibodies against γ-globulin were obtained from the specific antigen–antibody complex by means of an insoluble immunological adsorbent consisting of polystyrene-bound rabbit γ-globulin [5].

EXPERIMENTAL METHOD

The rabbit antiserum used in the experiments was obtained from asses after five cycles of immunization. The first cycle consisted of an intramuscular injection of 500 mg protein of the total serum globulin fraction of rabbits, precipitated by 50% saturation with $(NH_4)_2SO_4$ with Freund's additive [13], and 3 subcutaneous injections, each of 1000-1300 mg protein of the same fraction at intervals of 1 week.

The following cycles consisted of 2 subcutaneous injections at intervals of 1 week: the 2nd and 3rd cycles–each of 1000 mg protein of the same fraction after 1 and 5 months respectively, and the 4th and 5th cycles–injection of a mixture of γ- and β-globulins purified by electrophoresis in agar after 5 and 8 months respectively (400 and 200 mg for the 4th injection, 100 and 170 mg for the 5th injection).

Blood (in a volume of 1.0-1.5 liter) was taken from the jugular vein on the 7th, 9th, and 14th days after the last injection.

The antiserum used gave a positive reaction in the precipitation test in agar with rabbit γ-globulin in a concentration of 50 μg/ml in dilutions up to 1:64.

The rabbit γ-globulin was isolated from blood serum taken from the auricular vein of 8-10 healthy rabbits by 25% saturation with ethanol by Cohn's method [11]; this fraction was further purified by electrophoresis in agar [4].

The immunological adsorbents were obtained on a basis of emulsified nonisotactic polystyrene manufactured by the Kuskovo Chemical Factory [5] and of Norwegian polyaminopolystyrene manufactured by the Norsk Hydroelectric Co. [15], for which we are indebted to A. Ya. Kul'berg.

Nitration, reduction, and diazotization of the Soviet polystyrene were carried out by the first variant of the method described by A. M. Olovinkov [5].

For diazotization we used a 1% solution of rabbit γ-globulin in a veronal buffer at pH 8.6 in a proportion of 150 mg to 1 g sorbent. Diazotization was carried out in the cold on a magnetic mixer for 16-18 h. The uncombined protein was removed by repeated washing with physiological saline, followed by centrifugation until no more protein was present in the washings. The final adsorbent was dried on a Büchner funnel and transferred to a vacuum exsiccator with $CaCl_2$. Some preparations were stored at 4° under physiological saline.

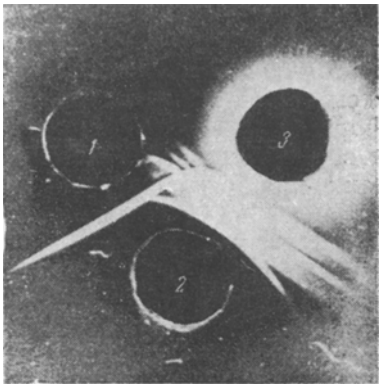

Fig. 1. Precipitation reaction in agar between normal agar rabbit serum and antirabbit serum with antibody fraction against γ-globulin. 1) Eluate of antibodies against rabbit γ-globulin; 2) normal rabbit serum in a dilution of 1: 4; 3) initial antirabbit ass serum.

The treatment of the polyaminopolystyrene–diazotization and formation of an azo compound with γ-globulin–was carried out by basically the same procedure.

To isolate antibodies against rabbit γ-globulin, the ass antiserum was incubated for 1 h mixed with the immunological adsorbent. The serum was then removed by centrifugation for 10 min at 5000 rpm and the residue was washed free from nonbound serum with physiological saline 4-5 times, followed by centrifugation. The antibodies bound by the adsorbent were eluted by the repeated addition of citrate-phosphase buffer at pH 2.2. The eluates were neutralized to pH 7.0-7.4 by means of a concentrated solution of Na_2CO_3 or 1 N NaOH, a 1: 10,000 solution of merthiolate was added, and the precipitation test in agar was performed. The active eluates of antibodies were pooled and concentrated by precipitation at 50% saturation with $(NH_4)_2SO_4$. The precipitation test in agar was performed as its micromodification [3].

Completeness of adsorption of the ass antiserum and the activity of the isolated antibodies were determined by titration by Fainberg's method using the precipitation test in agar [12] with a standard solution of rabbit γ-globulin containing 50 μg/ml [6]. Protein in the various preparations was estimated by the biuret reaction [14].

For use in the indirect fluorescent antibody method, the ass antirabbit serum or antibodies against rabbit γ-globulin were bound with fluorescein isothiocyanate by the usual method [7, 11]. The technique of detection of tissue antigens by the method of fluorescent antibodies using monospecific antibodies to individual antigens was described previously [7].

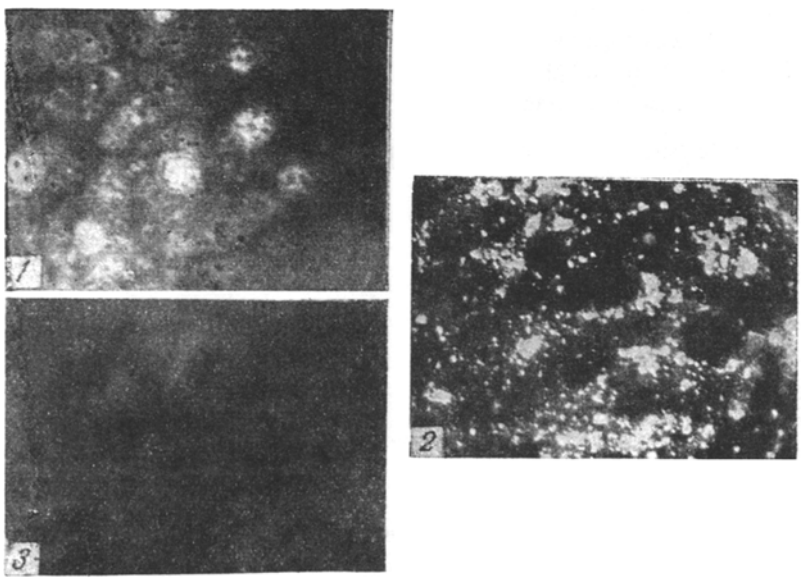

Fig. 2. Sections of the liver of a mouse, treated with: 1 and 2) monospecific antibodies against two different (V and IV') organ-specific liver antigens; 3) nonimmune rabbit γ-globulin, followed by antibodies against rabbit γ-globulin, labeled with fluorescein isothiocyanate. Ocular ×10, objective ×90.

EXPERIMENTAL RESULTS

The immonological adsorbents prepared from Soviet polystyrene contained about 10 mg γ-globulin per gram of sorbent. At the first adsorption they exhausted the antibodies from 5 ml of crude antiserum.

The main difficulties arose during elution of the antibodies. Citrate–phosphate buffers with pH values of 2.2 and 3.2 and a glycine–sulfate buffer at pH 2.7 were used for the elutions. The best results were obtained with citrate–phosphate buffer at pH 2.2, but in this case a very small proportion of the adsorbed antibodies passed into the eluate after the first adsorption of the serum. At the second adsorption the volume of sorbent was reduced to 75-50% of its initial value, but elution of the antibodies, although increased, nevertheless remained low and not above 12% of the adsorbed activity.

The preliminary washing of the sorbent with buffer at pH 2.2 had the same effect as a single adsorption of the serum: the adsorption capacity was approximately halved, although the percentage of elutable antibodies increased. Best elution of the antibodies was observed when the sorbent was incubated with an excess of antiserum. However, when working with sorbents prepared from polystyrene obtained from the Kuskovo factory, the activity of the eluted antibodies did not exceed 12-14% of the adsorbed activity.

Better results were obtained when polyaminopolystyrene was used as the original material. In this case 1 g of sorbent contained about 70 mg of γ-globulin and adsorbed antibodies from 15 ml of original antiserum. About 20% of adsorbed antibodies could be eluted from these sorbents, and after repeated adsorption the capacity of the sorbent was reduced by a smaller amount than in the first case.

Sorbents of both types were used in the preparation of antibodies against γ-globulin. Antibodies to rabbit γ-globulin were isolated both from antiserum labeled with fluorescein isothiocyanate, and from crude antiserum subsequently labeled by the usual method. In every case identical results were obtained: antibodies to γ-globulin were specifically adsorbed from the sera, while the titer of antibodies against albumin remained unchanged. During precipitation in agar the isolated antibodies formed only one precipitation line with normal rabbit serum, corresponding to γ-globulin (Fig. 1).

Some preparations of antibodies isolated from labeled sera completely failed to give a nonspecific staining reaction in sections of mouse organs and were used in the experiments without preliminary adsorption. In other cases, and in the case of antibodies bound with the dye after isolation from the crude antiserum, the nonspecific staining of the sections was readily removed by adsorption in the ordinary way with an acetone liver powder [10], after which the background luminescence practically disappeared.

The use of monospecific labeled antibodies against rabbit γ-globulin in conjunction with monospecific antibodies against individual antigens could be used to study the tissue and intracellular localization of certain organ-specific mouse liver antigens in sections of liver, of normal organs, and of transplantable mouse hepatomas [7, 8], by means of theindirect method of fluorescent antibodies. Organ-specific antigens (V-IV') were found only in the cells of the liver parenchyma, the first in both nucleus and cytoplasm, the second only in the cytoplasmic granules (Fig. 2, 1, 2).

Fluorescence was not present in the control sections treated with nonimmune rabbit γ-globulin, followed by labeled antibodies against rabbit γ-globulin, and not with monospecific antibodies, so that specific fluorescence could be detected even when in very low intensity. Good results also were obtained when labeled antibodies against rabbit γ-globulin were used to study polyoma virus in a culture of embryonic mouse tissue by means of the indirect method of fluorescent antibodies.

When working with the indirect method of fluorescent antibodies, replacement of the "serum" antiserum by antibodies against γ-globulin prevents the possibility of cross reactions between the labeled antiserum and the test preparations inherent in the direct method. The nonspecific staining of the sections is also less marked: a monospecific labeled antiserum contains practically no ballast proteins, so that the activity of the antibodies is equal to the activity of the antibodies in an ordinary antiserum although its total protein concentration is much lower–about 1 mg/ml. The nonspecific binding between an antiserum of this sort and the sections is easily prevented by ordinary adsorption methods, and even when the intensity of specific fluorescence is low, reliable results may be obtained.

Different immunological adsorbents may be used to isolate monospecific antibodies against γ-globulins.

Our antibody yields were inferior to those described in the literature, both for polystyrene adsorbents [5, 15] and for adsorbents using other carriers [1, 9]. It is apparent that quantiatively better results may be obtained by the use of the techniques and carriers recently described by A. E. Gurvich and co-workers [1, 2].

SUMMARY

The antibodies against rabbit γ-globulin were obtained both from native and labeled antirabbit serum with the aid of polystyrene immunosorbent. These antibodies when used in the indirect fluorescent antibodies method gave the opportunity to exclude all the cross-reactions of the labeled serum with the sections; nonspecific staining of the sections also essentially decreased.

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