TREATMENT OF LIVING CELLS WITH FERRITIN ANTIBODIES

N. A. Dorfman

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Two methods of treating living cells with ferritin antibodies in order to prevent their denaturation in physiological solutions are suggested. In the first method a medium which preserves the ferritin antibodies in a soluble form and the cells in a living state is used. In the second method ferritin antibodies which are not denatured in physiological saline are obtained.

One of the most important factors in electron-microscopic immunocytochemical research is ensuring optimal interaction between the test antigens and the corresponding antibodies and preservation, as far as possible, of the ultrafine organization of biological structures. To stabilize the material and maintain its antigenicity, most workers use mild fixation with 4% formalin or 1-2% glutaraldehyde solution [2]. However, the antigenicity of a test substance in a fixed object is evidently closely connected with its nature and, in particular, with the number of antigenic determinants on the molecule and the presence of radicals (especially amino-groups) in them which could combine with molecules of the fixative, thereby giving rise to blocking of the antigenic determinants. A decrease in the antigenicity of structures as a result of their partial denaturation by the action of the fixative must evidently accompany even the mildest fixation. If the density of distribution of the test antigen is low, even very slight denaturation may have a significant effect on analysis of the results. Treatment of unfixed material provides optimal conditions for the reaction between antigens and antibodies, but technical difficulties arise during the investigation of animal cells, and the object of the investigation described below was to overcome them.

In the investigation of surface antigens on mouse leukemic cells the main obstacle was the virtually complete insolubility of ferritin antibodies in physiological saline, so that it was impossible to treat living cells. To obtain ferritin antibodies, xylidene-metadi-isocyanate [6], toluene-2,4-di-isocyanate [7], and difluorodinitrodiphenylsulfone [5] were used as binding agents. The complexes obtained with the aid of these compounds could be kept for a long time in solutions of weak electrolytes (veronal-medinal or tris buffer), but the presence of salts with high dissociation (for example, the NaCl in physiological saline) caused their rapid precipitation, only to dissolve again when transferred into veronal-medinal buffer. Either no antibody activity was found in the supernatant, or it was very weak and transient. This behavior of the ferritin antibodies is connected with the well-marked hydrophobic nature of the conjugating reagents used and with competition of the salts added to the solution for the protein-bound water. To reduce the hydrophobic character of the resulting protein complex, reducing the quantity of binding agent to one-tenth of that suggested by the authors of the original method was tested. As a result the solubility of the ferritin antibodies in physiological saline was appreciably increased, and their total yield in the conjugation reaction reduced. However, the soluble complexes were very unstable and could not be kept for a long time. An attempt was then made to select a medium in which the cells could remain alive for a time, and the ferritin antibodies would not be precipitated, and parallel with this a method was developed for obtaining complexes readily soluble in physiological saline. Both these problems were solved, and this made it possible to obtain highly satisfactory results of antigen analysis of mouse leukemia and hepatoma cells.

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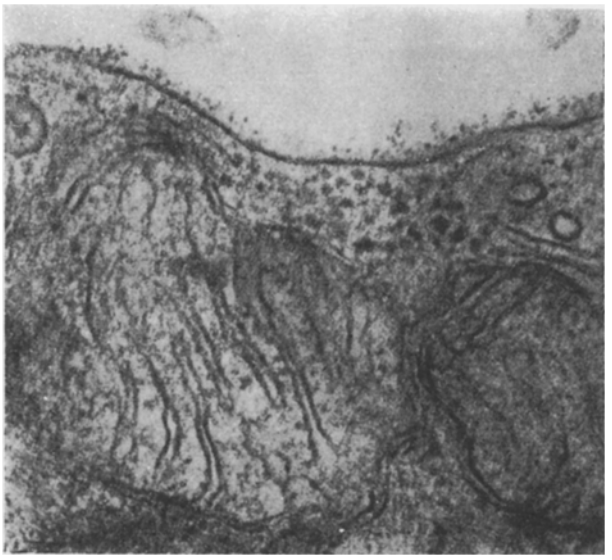

Fig. 1. Part of a Rauscher leukemia cell treated with anti-H-2d serum and with ferritin conjugated with rabbit antimouse γ-globulin. Conjugation carried out with toluene-di-isocyanate. Cells treated in medium described in the text.

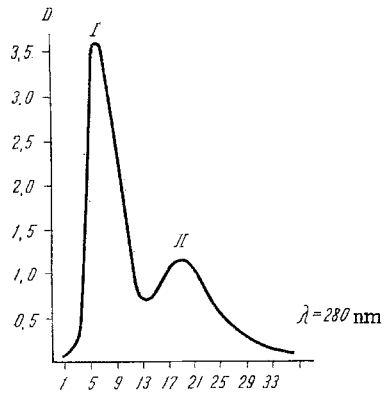

Fig. 2. Gel-filtration of reaction products of conjugation between ferritin and rabbit γ-globulin on 5% granulated agarose. Abscissa, fraction No.; ordinate, optical density.

A medium which satisfied the above requirements contained 5% glucose or 9% sucrose and 10% dimethyl sulfoxide, which were dissolved in 0.05 N veronal-medinal buffer or in 0.02 M tris buffer, pH 7.4. The cells remained alive for several hours in this medium, much longer than the time required for their treatment with ferritin antibodies. The percentage of living cells was determined by staining with a mixture of trypan blue and eosin [8]. Electron-microscopic investigation of cells treated with ferritin antibodies in this medium showed that it does not affect interaction between antibodies and antigens and causes virtually no disturbance of the ultrafine cell structure (Fig. 1). Ferritin antibodies can be preserved in the suggested medium for a year or more at 4°C without appreciable loss of activity or denaturation. An advantageous factor is that veronal-medinal buffer and dimethyl sulfoxide, both of which possess bactericidal properties, prevent bacterial contamination of the antibody solution. This means that one batch of solution can be used several times without special sterilization.

The guiding principle in the development of the method of obtaining ferritin antibodies not undergoing denaturation in physiological saline was the selection of binding agents with hydrophilic properties. The desired result was obtained by the use of diaminodiphenylamine tetrazotate [1] and glutaraldehyde, which have been used previously to conjugate certain proteins [3]. To obtain complexes with the aid of glutaraldehyde, 5 mg γ-globulin of the antiserum was mixed with 15 mg ferritin free from apoferritin in 1 ml 0.1 M phosphate buffer, pH 6.7-6.9. A 1% solution of glutaraldehyde was added drop by drop to the resulting mixture (0.5 mg to 20 mg protein) at room temperature with constant stirring on a magnetic mixer. After 2 h the mixture was dialyzed against tris buffer, pH 7.4, on Sephadex G-25. The ferritin antibodies were purified from unreacting components by gel-filtration on a column of 5% granulated agarose equilibrated with tris buffer, pH 7.4. The mixture of complexes and free molecules was separated into two fractions by chromatography. Complexes of ferritin and γ-globulin of different sizes, containing 60-70% of the protein taking part in the reaction, were contained in the first peak. The second peak consisted of unreacting molecules of ferritin and γ-globulin, which were virtually insep-

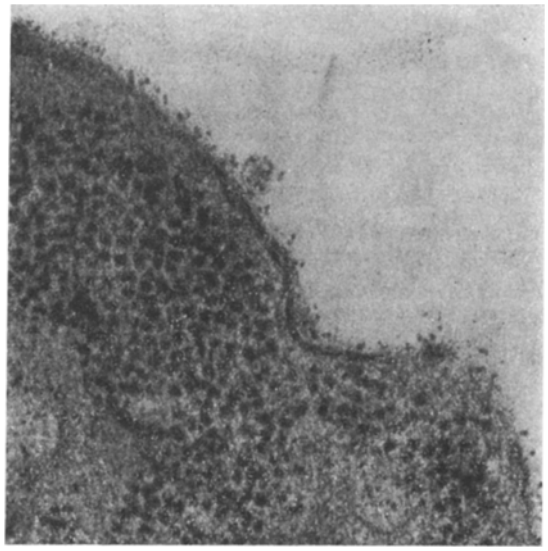

Fig. 3. Part of a Rauscher leukemia cell treated with anti-H-2d serum and ferritin antibodies obtained with the aid of glutaraldehyde. Treatment carried out in Hanks' solution.

arabie (Fig. 2). The activity of the ferritin antibodies, measured by an immunosorbent method, was 85-90% of the activity of the original antibodies, but their precipitating activity was virtually completely lost.

Electron-microscopic investigation of cells treated with ferritin antibodies showed that they were strongly and nonspecifically adsorbed on the surface of the cells, thus causing considerable difficulty when the experimental and control samples were compared. A change in the concentration of antibodies and their absorption by liver powder did not reduce the intensity of the nonspecific reaction to permissible limits. Further purification of the ferritin antibodies was therefore carried out to remove complexes of ferritin with nonimmune γ-globulin, or with γ-globulin which had lost its activity, from the solution. Active complexes were isolated with the aid of immunosorbent from the polycondensed antigen (in this case rat γ-globulin) by Avrameas' method [4]. The antibodies were eluted in glycine-HCl buffer, pH 2.3, neutralized with alkaline, and concentrated to 0.2 mg/ml (estimated as ferritin). The use of antibodies purified in this way in electron microscopy yielded experimental preparations of high quality, with an almost total absence of label in the control (Fig. 3).

With the possibility of treating living cells with ferritin antibodies readily soluble in physiological saline, optimum conditions can now be created for antigen-antibody interaction, leaving the ultrafine structure of the cells intact. As regards the quality of its results this method is not inferior to the method of hybrid antibodies, and if the direct method of treatment is used it may have some advantage because of the much smaller quantity of original antibodies required for the conjugation reaction with ferritin.

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