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COMPARATIVE STUDY OF SOME METHODS OF OBTAINING
FLUORESCENT ANTIBODIES

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In all cases in which the investigator intends to prepare single fluorescent antibodies, he will have to use one of the techniques described in the literature, or devise his own variants. In this note we are describing the studies of comparative techniques as well as an analysis of the results, advantages and disadvantages of some techniques. In this study we have analyzed five points: different variants in the succession of operations carried out; different methods of serum fractionation in order to obtain some purified preparations containing antibodies; different coupling techniques; different methods of removing the uncoupled fluorochrome and inactive immunologic substance; and different methods of uncoupling the non-specific fluorescent material.

Material and Methods:

a) Antibodies. We have utilized horse, goat and rabbit serum hyperimmunized to human globulin of different purity (preparation of the Institute of Hematology, Bucharest, and preparation of the Behring Company). The sera have been kindly supplied to us by the Cantacuzino Institute (Dr. C. Zilisteanu) and Institute of Internal Medicine (Dr. Stoica).

b) Fluorochrome. We have used fluorescein isothiocyanate of the British Medical House.

c) Determination of proteins has been carried out by the Weichselbaum method.

d) The purity of the different fractions has been determined by gel electrophoresis; the intensity of fluorescence was measured spectrophotometrically (excitation at 4200 wavelengths and readout at 5300).

These studies were carried out at the Institute of Internal Medicine by a working group including Dr. Stoica and Marcela Zamfirescu to whom we are herewith expressing our appreciation.

e) Specificity and intensity of immunofluorescence have been studied in order to bring into evidence the antinuclear factor of sera of patients with visceralized lupus erythematosus.

f) We have followed the techniques of Marshall [7], Riggs [9], Chadwick [1], and Niel [8], for the preparation of fluorescent antibodies.

g) Preparation of the globulin solution was through precipitation with a 50% saturated solution of ammonium sulfate [4]; precipitation with a 32% sodium sulfate solution [8], and filtration through DEAE cellulose [6].

h) The elimination of the uncoupled fluorochrome was done through dialysis, agitation with activated carbon [1], filtering through Sephadex G 25 [5]; the isolation of fluorescent-specific antibodies was carried out by adsorption and elution (20% saline solution, 56° and pH 4).

i) The separation of non-specific fluorescent material was through powdered liver in acetone [2], non-water-soluble fraction of the liver¹. The powdered liver in acetone was resuspended in buffered saline solution and incorporated in a column of "Celite" [3].

Results:

1. The succession of tasks was varied in order to obtain various advantages.

The classical method: Preparation of immune globulin, coupling it, separation of the non-coupled fluorochrome and of non-specific antibodies has given us very good results with regard to the intensity of specific fluorescence, but the duration of these tasks is relatively long.

Rinderknecht's Method [10] [11]: Coupling of the total serum and passing it through a Sephadex column, it is quite rapid but about eight hours are used up because the fluorochrome contains a large amount of inactive fluorescent immunologic conjugate (serum albumin).

Use of the Sephadex column according the original column with DEAE cellulose permits obtaining a resulting preparation which contains much fluorescent globulin, but the whole operation becomes much more cumbersome.

Coupling of the total serum with subsequent separation of globulins permits obtaining a product with about 30-40% less fluorescence than preliminary separation of globulins with subsequent coupling.

¹ E.A. Kabanova, personal communication.

Coupling of the total serum and its subsequent purification with adsorption of respective antigens, permits theoretically obtaining for separation non-coupled fluorochrome and inactive immunologic fluorochrome. The results are unsatisfactory in that the yield is extremely low.

2. Fractionation of sera through a DEAE cellulose column seems more advantageous to us, yet necessitates preliminary equilibration for dialysis and subsequent concentration. Defractionation by precipitation with sodium sulfate permits obtaining a preparation of gamma globulin which is purer, but in lower yield than that which may be obtained by ammonium sulfate precipitation. Both of these methods require prolonged dialysis prior to separation of the sera and subsequent concentration.

3. The coupling techniques have given about the same results as the methods investigated [7] [1] [8] [9], but we considered that the most advantageous method is that of Niel with some small modifications as follows: one volume of globulin solution adjusted to the 25-35 mg. of protein/ml. (2.5-3.5 g. protein/100 ml.), cooled to 4°C, is placed in an Erlenmeyer flask of 25-ml. capacity (with mechanical agitator) with one volume of 0.1 M sodium bicarbonate solution², pH 9.2, cooled to 4°C. The separated substance which is obtained is then dissolved in an equal volume of carbonate-bicarbonate solution as above, with 0.5-1.0 mg. of fluorescein isothiocyanate for each ml. of globulin solution (the ratio of fluorescein isothiocyanate to protein goes from 1:30 to 1:40). This solution is then added little by little under continuous agitation to the globulin solution over a period of 20 to 30 minutes. The agitation is continued at 4° C for a period of 18 to 20 hours.

4. Separation of the uncoupled fluorochrome for dialysis is rather long, yet the adsorption of activated carbon may be very good, giving variable results according to the carbon function. If the carbon powder is very fine, the distribution of carbon and prolonged centrifuging at 6000 rpm may be incomplete, with the severe inconvenience that microscopic particles of carbon in the suspension may become fluorescent. Filtration through Sephadex G-25 has on the other hand given us good, constant and reproducible results. Adsorption elution for specific antigen has given us as we have mentioned, unsatisfactory results.

5. Separation of Non-specific Fluorescents. The results are similar with the different techniques which were followed, with rather poor results (the yield is very low) obtained with the Coons technique as well as with the Kabanova technique. One of the disadvantages of the last-mentioned technique is the fact that the liver preparation cannot be preserved except for a very short time. The Hudson technique is more profitable. In one of our studies, we have advantageously combined the last two operations using the column for filtration and liver preparation with dia-

¹We may work here with a 0.5 M solution with coupling with Lissamine Rhodamine B-200 sulfochloride or dimethylamino naphthyl derivative.

tomaceae for Sephadex G-25 filtration. The column is rinsed with this 0.15 M solution of sodium chloride phosphate-buffered, pH 7.2, 0.01 M.

The fluorescent globulin solution and merthiolate 1:10,000 is preserved in closed receptacles (test tube with a rubber stopper) in the refrigerator at 4°C.

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