ADVANTAGES OF DICHLOROTRIAZINYLAMINOFLUORESCEIN CONJUGATES ISOLATED BY ONE-STAGE GEL FILTRATION IN IMMUNOFLUORESCENCE

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UDC 612.017.1-087.4:543.426

A simple method of obtaining dichlorotriazinylaminofluorescein (DCTAF) conjugates against human immunoglobulins and of isolating their optimally labeled fractions is described. The one-stage method of liberation of the conjugates from unbound fluorochrome and of simultaneous separation of optimally labeled fractions is carried out on Sephadex G-25 at pH 5.6-6.6 in an eluting solution of low ionic strength. Proteins loaded with dye are adsorbed on the gel in accordance with their isoelectric points.

KEY WORDS: *dichlorotriazinylaminofluorescein; fluorescein isothiocyanate; conjugate; immunofluorescence.*

The introduction of an immunological method into routine clinical practice makes it necessary to seek reliable and reproducible methods of obtaining highly specific conjugates. The quality of the latter is largely dependent on the purity and activity of the dye. Commercial preparations of fluorescein isothiocyanate (FITC), such as are widely used in immunofluorescence techniques, are very heterogeneous. For instance, among 18 specimens of FITC marketed by different firms, the content of the basic dye varied from 65 to 100% [9]. Many of them are unsuitable, for it is essential in practice to use chromatographically pure FITC with a content of the basic substance of not less than 90% [4]. In this respect dichlorotriazinylaminofluorescein (DCTAF) has advantages for it has a high content of the basic substance (90-92%) and a high degree of purity [3]. However, in the literature there are only isolated reports of the use of this dye for the preparation of luminescent sera [3, 4]. There are no data on the possibility of isolating optimally labeled fractions of DCTAF-conjugates by means of gel filtration at a low ionic strength.

The object of this investigation was to obtain a highly specific serum labeled with DCTAF and then to isolate optimally labeled fractions on Sephadex G-25 in accordance with their isoelectric points.

EXPERIMENTAL METHOD

Rabbit antisera, both polyvalent toward human immunoglobulins and monospecific toward individual classes of immunoglobulins (G, M, and A) were used. The antisera were obtained by the following scheme: Rabbits were given an intraperitoneal injection of 0.1-0.2% solution of protein containing 1-2 billion killed whooping cough bacteria. A second intraperitoneal injection of 0.2-0.3% protein solution containing 2-3 billion whooping cough bacteria was given 2-3 months later. Blood was taken on the 9th-16th day from the auricular vein.

The immune sera were exhausted by means of immunosorbents from umbilical blood and $F(ab)_2$ fragments of human immunoglobulins [5]. The specificity and monospecificity, and also the activity of the sera, were determined by the precipitation test in agar against individual classes of human immunoglobulins and blood serum. The titer of the sera was 8-32

Laboratory of Clinical Immunology, Department of Immunology, Scientific-Research Center, N. I. Pirogov Second Moscow Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR Yu. M. Lopukhin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 83, No. 4, pp. 505-506, April, 1977. Original article submitted November 30, 1976.

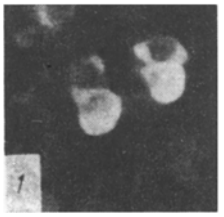

Fig. 1

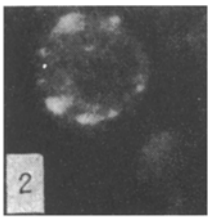

Fig. 2

Fig. 1. Specific fluorescence of immunoglobulin-synthesizing cells of human lymph node. Section treated with antiserum against human immunoglobulin G labeled with DCTAF. 200×.

Fig. 2. Specific punctate fluorescence of lymphocyte surface with immunoglobulin determinants of human tonsil. Suspension of living lymphocytes treated with antiserum against human immunoglobulin M labeled with DCTAF. 450×.

precipitation units during agar diffusion against 1 mg/ml of the corresponding immunoglobulin.

The γ-globulin fraction was isolated by the sulfate–rivanol method [12]; DCTAF was added to the γ-globulin in the ratio of 1:20-1:50 and DCTAF (RIAPT Factory, Kiev) was used as a rule in a dose of 15-20 mg per gram protein. The dye, previously dissolved in 0.2 M Na_2HPO_4, was added in a concentration of 1-2 mg/ml to the protein solution drop by drop with constant stirring. The pH of the resulting mixture was adjusted to 9.0-9.5. The reaction was carried out at 22-37°C for 20-50 min. The initial protein concentration in 0.05-0.1 M Na_2HPO_4 was 15-25 mg/ml. The protein concentration was determined spectrophotometrically.

Conjugates containing 40-400 mg protein were applied to columns containing Sephadex G-25 fine (2 × 20 cm, 3 × 50 cm), equilibrated with 0.0175 M Na_2HPO_4, pH 5.7, or 0.01 M CH_3COONa, pH 5.6. Two fractions were obtained by elution with these solutions of low ionic strength. Fraction 1 contained conjugate with the optimal molar fluorochrome:protein (M_f:M_p) ratio, namely 1.5-5. A conjugate loaded with dye (M_f:M_p = 6) was eluted in fraction 2. The molar ratio of dye and protein and the protein concentration were determined spectrophotometrically [2]. The pH of the conjugate of fraction 1 was adjusted to 7.2-7.4. The preparations were preserved with merthiolate up to 1:10,000 or sodium azide to 0.1%.

The DCTAF conjugates thus obtained were subjected to immunodiffusion and immunofluorescence analysis in order to determine their specificity and activity. The titer of the conjugates with a protein concentration of 10-20 mg/ml, determined by the agar precipitation test against individual classes of human immunoglobulins and serum, was 4-8 units.

Immunofluorescence analysis was carried out on paraffin sections of human lymph nodes and tonsils by examination for cells producing γ-globulin [11] (Fig. 1). Lymphocytes from human peripheral blood, tonsils, lymph nodes, and spleen were used to investigate surface immunoglobulin determinants [1, 10] (Fig. 2). High activity of DCTAF conjugates was revealed by fluorescence of the antigens of lymphoid tissue and cells when treated in a dilution 1:8-1:256.

EXPERIMENTAL RESULTS

The specificity of the conjugates thus prepared was confirmed by various combinations of controls: 1) incubation of the preparations with labeled nonimmune serum, 2) with labeled antiserum not containing antibodies against the test antigens, 3) neutralization of serum with the test antigen, 4) treatment of sections not containing the test antigens, 5) complete extinction of immunofluorescence after incubation of preparations with unlabeled immune serum in three successive dilutions with labeled antiserum (1:32-1:128).

The advantages of the DCTAF conjugates over FITC conjugates showed most clearly in the last, most convincing, control test. The former always gave more complete extinction of specific fluorescence even when diluted twice as much as the latter. The results showing higher specificity of DCTAF conjugates are in agreement with data in the literature [4].

Amino acids and peptides are known to be adsorbed on Sephadex equilibrated with buffer of low ionic strength at a pH equal to their isoelectric points [7]. Adsorption of FITC-F(ab)$_2$ fragments on Sephadex with buffer of low ionic strength at pH values of below 5.0 is described in the literature [8]. There are also similar data for γ-globulin fractions, highly labeled with FITC at pH 6.5-5.5 [6]. There is no such information for DCTAF conjugates. There is only a statement that to purify DCTAF conjugates from free unbound dye, Sephadex equilibrated with 0.1 M phosphate buffer, pH 9.5, should be used, for on elution with 0.01 M phosphate buffer, pH 7.5, denaturation of the conjugate and precipitation take place [6].

The results of the present investigation contradict these data. Under the conditions described above, purification of DCTAF conjugates from unbound dye and, at the same time, from fractions loaded with dye, was carried out in one stage on Sephadex equilibrated with buffer of low ionic strength at pH values below 7.0. Precipitation by individual fractions of conjugates in accordance with their isoelectric points, by contrast with denaturation of proteins, is a reversible phenomenon. With an increase in the pH of the solution, these precipitates of conjugates dissolve and they exhibit high specificity and activity in immunofluorescence and immunodiffusion analyses.

The method of obtaining DCTAF conjugates and of single-stage isolation of optimally labeled fractions, as described above, is therefore a convenient, simple, and reliable method, giving reproducible results in clinical practice.

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