PRODUCTION OF LUMINESCENT SERA WITH THE AID OF DICHLOROTRIAZINYLAMINOFLUORESCEIN

V. A. Chibisova, K. L. Shakhanina,
Yu. E. Sklyar, and G. I. Mikhailov

UDC 615.373:576.8.073.4].012

A method of obtaining luminescent sera by means of dichlorotriazinylaminofluorescein (DCTAF) has been developed. Luminescent anthrax, tularemia, and brucellosis sera and also a luminescent serum against Coxsackie B3 virus have been prepared. A comparative study of luminescent sera labeled with fluorescein isothiocyanate (FITC) and DCTAF has shown that both preparations can be used equally successfully and localize antigens and antibodies in the tissues.

* * *

The method of luminescent antibodies has been widely applied in medical practice because of its high sensitivity, its reliability, and its rapidity of performance [3].

Fluorescein isothiocyanate (FITC) is most commonly used at the present time as the fluorochrome for labeling immune proteins. However, although this dye has advantages over others, its synthesis is complicated and is dependent on the use of thiophosgene, which is in short supply and dangerous to use. In an attempt to broaden the range of fluorescent protein tags experiments were carried out to develop a method of preparing luminescent sera by the use of dichlorotriazinylaminofluorescein (DCTAF). DCTAF was prepared by the use of chromatographically pure aminofluorescein, cyanuric chloride, and anhydrous acetone. The synthesis of DCTAF was carried out as follows.

Into a flask fitted with a stirrer, drop funnel, thermometer, and reflux condenser, 2.91 g aminofluorescein in 100 ml anhydrous acetone was poured. During energetic mixing, a solution of 1.77 g cyanuric chloride in 10 ml anhydrous acetone, cooled to 0°, was added drop by drop. The reaction mixture was stirred at 0° for a further 2 h. The pale yellow precipitate thrown down was separated by filtration and washed with 10 ml anhydrous acetone, after which it was dried in an exsiccator. Yield 3.77 g (84.3%). Melting point above 300°.

The fluorochrome thus obtained is a finely crystalline powder, pale yellow in color, and soluble in alcohol, aqueous solutions of sodium carbonate and bicarbonate, and sodium and calcium phosphate.

To prepare luminescent antibodies, several antisera (against anthrax, tularemia, and brucellosis) and antiserum against Coxsackie B3 virus were used.

The globulin fractions of the sera were separated by means of satured ammonium sulfate solution at 40% saturation. The globulins were purified from excess of ammonium sulfate by chromatography on a Sephadex G-25 (coarse) column in 0.1 M phosphate buffer, pH 8.6.

The protein concentration in the globulins was determined on a type SF-4A spectrophotometer at wavelength 280 mμ. The protein concentration was adjusted to 20-25 mg/ml by means of 0.1 M phosphate buffer, pH 8.6.

DCTAF was added to the solution of globulins in phosphate buffer, pH 8.6, at the rate of 20 mg/1000 mg protein. The conjugation reaction was carried out at 10° for 18 h with constant stirring on a magnetic mixer.

N. F. Gamaleya Institute of Epidemiology and Microbiology. All-Union Research Institute of Chemical Reagents and Especially Pure Chemical Substances (IREA), 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. 68, No. 11, pp. 120-122, November, 1969. Original article submitted April 4, 1969.

TABLE 1. Characteristics of Luminescent Sera Labeled with FITC and DCTAF

Immune globulin	Fluorochrome	Mf/Mp	Protein concentration (in %)	Dye titer	
				in liquid form	after drying
Anthrax	FITC	2.2	1.1	1:128	1:128
	DCTAF	2.0	1.1	1:128	1:128
Tularemia	FITC	3.0	1.0	1:32	1:32
	DCTAF	2.8	0.9	1:32	1:32
Brucellosis	FITC	2.5	0.9	1:32	1:32
	DCTAF	2.1	0.7	1:32	1:32
Against Coxsackie B3 virus	FITC	3.2	0.83	1.5	–
	DCTAF	2.8	0.80	1.5	–

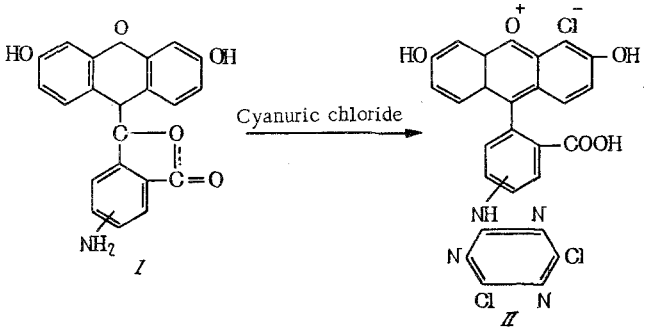

Fig. 1. I) Aminofluorescein, II) dichlorotriazinylamino-fluorescein. Molecular weight 531.76.

The free dye was removed by gel-filtration on Sephadex G-25 (coarse) in 0.01 M phosphate buffer, pH 7.5. Dye adsorbed on protein and causing nonspecific luminescence of the preparations was removed with the aid of DEAE-cellulose.

As control for the study and comparison of the DCTAF-globulins thus obtained, the same antisera were used, but were labeled with chromatographically pure FITC, with a content of not less than 90% of the primary substance.

Labeling with both fluorochromes was carried out under identical conditions and with identical doses of dye per gram protein. The molar ratio of fluorochrome: protein (Mf/Mp), the protein concentration [3], and also the dye titer of stained films under the luminescence microscope were determined in the resulting FITC- and DCTAF-globulins.

To determine the dye titer of the antibacterial luminescent antisera, preparations were made from a suspension of homologous microorganisms and dry diagnostic sera produced by the Tarasevich State Control Institute of Vaccines and Sera [1]. The films were fixed, stained with luminescent serum, and washed to remove excess of conjugate by the usual methods.

The starting power of the luminescent sera toward Coxsackie B3 virus was tested on tissue culture cells infected with Coxsackie B3 virus by the usual methods.

Stained films were examined in the ML-2 luminescence microscope with an immersion objective and with a combination of BS-8-2, FS-1-2, and SZS-7-2 exciting and suppressing filters.

To determine the stability of sera labeled with DCTAF, some of the luminescent sera (against anthrax, tularemia, and brucellosis) were poured into ampules and lyophilically dried, while the rest were stored in the liquid form at 4°.

Luminescent sera labeled with FITC and DCTAF under identical conditions were specific and gave an identical dye titer. The molar ratio of fluorochrome:protein and the protein concentration in these preparations also were identical (Table 1).

The dye titer for luminescent anthrax antisera labeled with the various dyes was 1:128, compared with 1:32 for brucellosis and tularemia antisera. Luminescent sera against Coxsackie B3 virus in both cases had a dye titer of 1:5. All the sera studied were specific.

Tests of the luminescent sera after long storage in liquid and lyophilized states showed that DCTAF-globulins were stable for 1 year in a liquid state and for 2 years in a lyophilized state (the period of study).

LITERATURE CITED

1. I. I. Lifanova, Control and Standardization of Luminescent Sera. Candidate Dissertation [in Russian], Moscow (1967).
2. L. B. Mekler, V. A. Chibisova, V. K. Naumova, et al., Vopr. Virusol., No. 5, 631 (1964).
3. Yu. F. Kubitsa (editor), Immunofluorescence [in Russian], Moscow (1968).