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Separation of Anticomplementary Material and Plasminogen from a Cytotoxic Factor Active Against Ehrlich Ascites Cells in Cohn Fractions I-III by Fluorocarbon and n-Butanol.* (32507)

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It has been previously shown that normal human serum possesses a complement-dependent cytotoxic factor (CyF) active against Ehrlich ascites tumor cells(1-7). The CyF, probably a "natural antibody"(5) is found in the β-globulin fraction of the serum (Cohn's Fraction III) and is absorbed by human tumor cells but not by normal cells(4,6). Although HeLa cells absorb CyF, they do not subsequently undergo cytolysis on addition of complement. However, such cells are protected against the cytolytic effect of rabbit anti-HeLa antibodies(4).

Cohn's Fraction III, containing CyF, is also known to contain plasminogen and isohemagglutinins; it is strongly anticomplementary because of its high content of phospholipids and β-lipoproteins. In order to show CyF activity, it is therefore necessary to incubate the tumor cells with Fraction III, wash repeatedly to remove anticomplementary factors and finally to add complement to demonstrate cytotoxicity.

The present report describes a simple method for isolation of CyF with high recovery from human Cohn Fraction I-III, free of the accompanying anticomplementary material. This method involves extraction with fluorocarbon or n-butanol followed by gel-filtration through Sephadex G-25 and ion-exchange chromatography on DEAE-Sephadex. Also, by this method plasminogen present in Cohn's Fraction III is obtained in good yield.

Materials and methods. Isolation of CyF. Cohn's Fraction I-III was kindly supplied by the Marcus Memorial Blood Fractionation Institute of Tel-Aviv-Jaffa. One gram of Fraction I-III was dissolved in 10 ml of isotonic complement diluent (barbital-saline buffer containing Ca^{++} and Mg^{++}, pH 7.4(8)) by grinding in a mortar at 1°C. The highly opalescent solution was then extracted at 1°C with n-butanol 20% V/V or with fluorocarbon 1:1 V/V (Arcton-113, Imperial Chemical Industries) by stirring on a magnetic mixer in the cold for 30 minutes. Following extraction, the mixtures were centrifuged at 10,000 × *g* for 10 minutes in a refrigerated centrifuge.

In the butanol extraction 3 layers appeared in the tubes. An upper layer containing excess butanol and a middle layer containing a yellow insoluble "cake" were discarded, and a lower opalescent aqueous solution was aspirated and kept in the cold for further treatment.

In the fluorocarbon extraction the upper opalescent layer containing protein was removed and kept in the cold. The middle "cake" and a lower layer of excess fluorocarbon were discarded.

Both the butanol and fluorocarbon extracts were filtered through Whatman 41 filter paper. After 30 minutes at 1°C, a heavy precipitate appeared in both extracts. This was removed by centrifugation. The butanol and fluorocarbon extracts were passed through Sephadex G-25 column 15 × 2.5 cm (Pharmacia, Uppsala, Sweden) and then were chromatographed on DEAE-Sephadex (see below).

Determination of CyF activity. Various fractions were incubated with Ehrlich ascites cells (5 × 10^6/ml) suspended in barbital buffer in the presence of 5 CH_{50} units of complement(8), the 1 ml of reaction mixture containing cells at a final concentration of 5 × 10^6/ml and protein at 5-150 μg/ml. Rabbit serum was used as a complement source since it has been shown that the CyF did not

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TABLE I. Extraction of CyF from Fraction I-III by Fluorocarbon.

	Original Fraction I-III (mg)	Fluorocarbon extract (mg)	Gel-filtration on Sephadex G-25		Chromatography on DEAE-Sephadex*			
			Applied to column	Recovered (mg)	Applied to column	Fractions A (mg)	B	C
Protein in fraction	200	160	80	24	3.5	1.3	1.2	2.0
Cytotoxic activity, LD_{50}/mg	16	—†	—†	50	50	0	0	40

* Conditions given in Fig. 1.
† Not done since fluorocarbon was present.

utilize guinea-pig complement effectively(4). After 30 minutes at 37°C the percentage of cells stained with trypan blue (non-viable cells) was determined and the results were expressed as LD_{50} per mg protein(4). Protein was determined according to Lowry *et al*(9). To determine the CyF activity of the original Fraction I-III, the tumor cells were first incubated with this material for 30 minutes at 37°C, washed a few times with buffer, and resuspended in buffer containing complement. The tubes were further incubated for 30 minutes at 37°C and the LD_{50} was determined as above.

Determination of plasminogen. One mg/ml of the fraction to be tested was incubated for 10 minutes at 37°C with 100 units/ml of Streptokinase (Varidase, Lederle Lab.) to activate plasminogen. Bovine fibrin (prepared from 10 mg/ml of fibrinogen clotted with 5 units of thrombin) was added, and after 30 minutes at 37°C, the extent of lysis was determined. One unit of plasmin was taken as the highest dilution which caused lysis.

Results. Table I summarizes the results of the recovery of CyF activity from Fractions I and III following extraction with fluorocarbon. As can be seen, 75% of the protein was recovered following the extraction but only 35% was recovered from the gel-filtration. CyF activity was found in the fluorocarbon extract, with considerable loss but with a 3-fold increase in specific activity on gel-filtration (Sephadex G-25 in 0.02 M barbital buffer, pH 7.4). Similar results were obtained by extraction with n-butanol.

Further purification of CyF was achieved by ion-exchange chromatography on DEAE-Sephadex columns. In a typical experiment 1 ml of fluorocarbon extract containing 3.5 mg protein and CyF activity of 50 LD_{50} units/mg protein were chromatographed on 10 × 1.2 cm columns by stepwise elution, with 0.02 M barbital buffer, pH 7.4, and 0.1 M NaCl and 0.25 M NaCl in this buffer (Fig. 1). CyF activity was found in the fraction eluted at 0.25 M NaCl, with a specific activity of 40 LD_{50} units/mg protein (Table I). Immunoelectrophoretic analysis of this fraction showed two precipitin lines *vs* rabbit antiserum to total human serum protein, in comparison with five such lines in the fluorocarbon extract.

It was also found that the 0.25 M NaCl eluate contained considerable amounts of plasminogen. Since plasminogen may be converted autocatalytically to plasmin, and the latter is known to inactivate complement(10), it was desirable to eliminate plasminogen from CyF preparations. It was found that heating of CyF-containing material to 58°C for one hour resulted in the inactivation of plasminogen, leaving CyF activity unaltered.

In another series of experiments it was found that plasminogen with high specific activity and free of CyF was obtained by gel-filtration of the fluorocarbon extract on a column of Sephadex G-25 (15 × 1.5 cm)

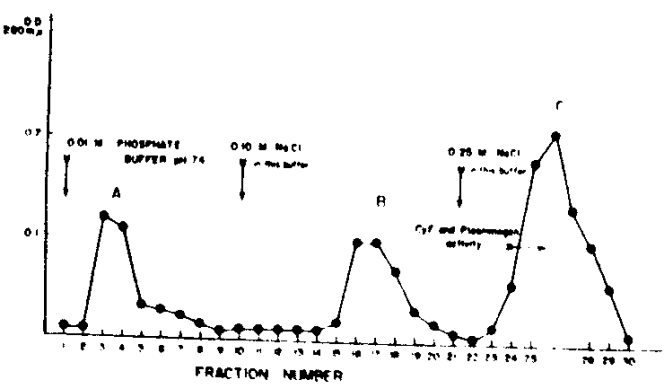

FIG. 1. Stepwise elution chromatography of Fraction I-III extracted with fluorocarbon on DEAE-Sephadex A-50.

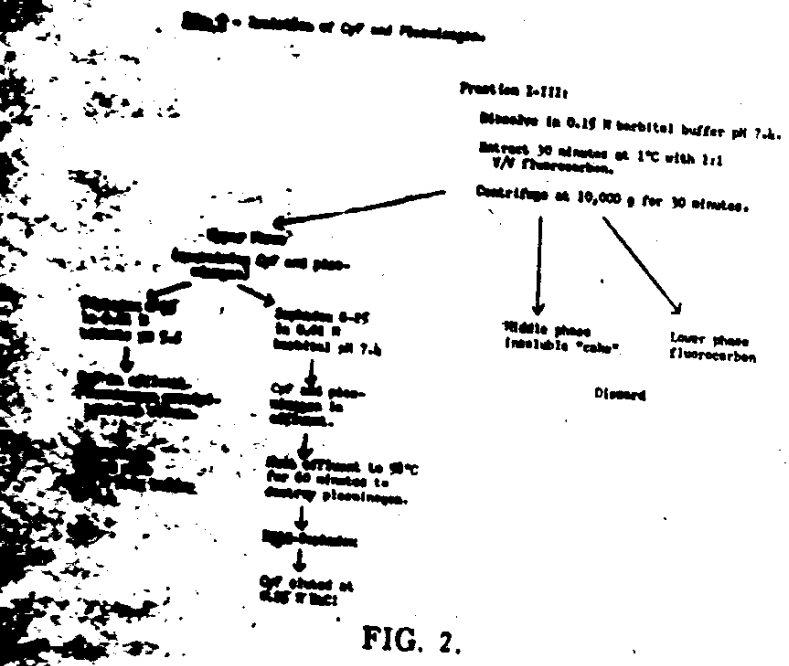

FIG. 2.

equilibrated with 0.01 M acetate buffer, pH 5.4. Under such conditions most of the protein, including plasminogen, was precipitated on the column, allowing CyF to pass through unaltered. Plasminogen was then eluted from the same column by washing with 0.2 M Tris NaCl buffer, pH 7.4. In a typical experiment (Table II) the specific activity of plasminogen was increased more than 10-fold compared with the original Fraction I-III. Fig. 2 gives the flow-chart for the isolation of CyF and plasminogen.

Discussion. It was previously shown(4) that normal human serum has a cytotoxic activity (CyF) for Ehrlich ascites tumor cells, with approximately 0.3 LD_{50} units/mg protein. Cohn Fraction I-III contains 16 LD_{50} units/mg protein, but its use in this cytotoxic reaction and its applications have been hampered by the anticomplementary factors which were also concentrated in this fraction. Fluorocarbon, which has been successfully used to extract antigens from tissues(11) and to purify viruses(12), was effective in separating the CyF from the anticomplementary material. Further, the use of low ionic-strength buffer in gel-filtration on Sephadex G-25 allowed the separation of CyF from other proteins insoluble under such conditions. It appeared also that gel-filtration increased the total plasminogen, probably by elimination of inhibitory materials present in the fluorocarbon extracts.

The separation of CyF from lipoproteins in the serum fractions suggests other possible uses of fluorocarbon extraction. This method might be useful in the treatment of hyperlipemic sera, where complement fixation tests are difficult or impossible to perform. It may also be of use for the elimination of non-specific antistreptolysin activity of sera rich in β-lipoproteins. Preliminary work performed in our laboratory has shown that treatment of a variety of antisera (iso-hemagglutinins, rabbit anti-human red blood cells, rabbit anti-Ehrlich ascites tumor cells) with fluorocarbon does not result in loss of either antibody, or of complement activity (to be published). Finally, the 2-step elution method of the fluorocarbon extract from Sephadex G-25 may be usefully employed in the preparation of large amounts of plasminogen.

Summary. 1. A method for the separation of a cytotoxic factor active against Ehrlich ascites tumor cells and of plasminogen from Cohn Fraction I-III is described. The method involves extraction by fluorocarbon, gel-filtration on Sephadex G-25, and ion-exchange chromatography on DEAE-Sephadex. 2. The fluorocarbon extract contains both CyF and plasminogen. Heating the extract to 58°C for

TABLE II. Isolation of Plasminogen from Fluorocarbon Extract of Fraction I-III* by Gel Filtration on Sephadex G-25.

Fraction	Protein (mg)	Protein recovery (%)	Specific activity of plasminogen (units/mg)	Total activity recovered (units)

1 hour inactivates plasminogen but leaves the CyF unaltered. 3. Separation of CyF from plasminogen in the fluorocarbon extract could be done on Sephadex G-25, using a column equilibrated with a buffer of such low pH and ionic strength as to precipitate the plasminogen selectively, and then recovering this with an eluting buffer in which plasminogen is soluble.

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