Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 186-194

ISOLATION, PARTIAL CHARACTERISATION AND COMPLEMENT INHIBITING ACTIVITY OF A NEW GLYCOPROTEIN FROM *CUSCUTA EUROPEA*

Zhivko D. Zhelev, Spaska A. Stanilova, and Brian G. Carpenter *

Department of Molecular Biology and Immunology, Higher Medical Institute, Stara Zagora 6000, Armeiska 11 St, BULGARIA

* School of Pharmacy, University of Portsmouth PO1 2DZ, UK

Received May 24, 1994

Summary: A new complement inhibiting factor (CIF) was isolated from the seeds of *Cuscuta europea* parasitic plant. When activated via both classical and alternative pathway, the complement activity was completely depleted by CIF at a concentration of 0,25 mg per ml serum. Studies concerning the precipitation showed that CIF developed one or two precipitin bands against human sera. It was established that the precipitation is as a result of the specific association of CIF to the C3 component of complement. A partial characteristic of the CIF was carried out. It is a glycoprotein with molecular weight between 27000 and 28000 Da. Its molecule consists of one polypeptide chain.

The complement system consists of about 30 glycoproteins which, after having been activated, form a membrane-attack complex (1). Its activation can be affected in two pathways - classical and alternative, differing in their initial phase, before the formation of C3 - convertase (2,3). This enzyme cleaves the intact C3 - molecule into two fragments - an anaphylatoxin C3a and a cytolytically active C3b. Complement component C3 plays a key role both in the classical and in alternative pathway of complement activation. Binding of C3b fragment to the target cell surface constitutes the central mechanism of antibody independent opsonization and initiates complement dependent lysis or phagocytosis (3,4).

At present only a few substances are known to block complement activity by affecting intact C3 or C3b subfragment. These are conglutinine, which binds to carbohydrate residue of the C3 alpha chain (5,6) and cobra venom factor (CVF),(7). CVF does not "block" complement activation but, conversely, binding of CVF to factor B, results in a long lasting C3 convertase activity which activates and consumes entirely and rapidly the C3 activity of the serum (8,9). Some other communications about anticomplementary factors, isolated from blood parasites are also of interest. These factors are cell surface glycoproteins interfere with C3 convertase formation or action in a manner similar to the decay accelerating factor (10,11).

Based on our programme in search of new plant lectins, we established that the parasitic plant *Cuscuta europea* produces a factor which completely depletes the human serum complement

0006-291X/94 $5.00

activity. In this paper we are presenting its isolation, molecular properties and an evidence of the anticomplementary action of this factor.

Materials and Methods

Cuscuta europea seeds were collected in August 1991 and in August 1992 in Bulgaria. Sephadex G - 200, BrCN - activated Sepharose 4B, Protein A Sepharose 4B and molecular weight standard markers were purchased from Pharmacia - LKB (Uppsala, Sweden). All other reagents were analytical grade. Male rabbits, weighting 3.5 - 4.0 kg, were used for immunisation.

Preparation of a crude extract from *Cuscuta europea* seeds (CECE). The seeds (5g) were ground and suspended in 50 ml of phosphate-buffered saline, pH 7.2 (PBS). The extraction was carried out with continuous magnetic stirring at room temperature (20-22^{o}C) for 2 hours. After centrifugation (20 min at 5000 g) the supernatant was collected and used for further experiments.

Ammonium sulphate precipitation. (20-50%) Ammonium sulphate in various quantities was added to separate aliquots of CECE in order to determine conditions for optimum protein production. After addition, solutions were incubated at room temperature for 2 hours and then centrifuged 20 min at 5000 g. The pellets produced were dissolved in PBS and dialysed against the same buffer.

Raising specific antibodies against CIF. An antigen was prepared by mixing together 5 ml of normal human serum and 10 ml of CECE followed by the addition of calcium chloride to a final concentration of 1 mM (preliminary results showed that the calcium ions were necessary for precipitation to occur). The mixture was incubated at room temperature for 1 hr with stirring and then centrifuged for 20 min at 5000 g. The pellet produced was re-suspended in 2 ml of PBS buffer, followed by further centrifugation 20 min at 5000 g; This washing procedure was repeated a total of five times. The final suspension, 2 ml was mixed with an equal volume of Freund's complete adjuvant and the emulsion injected intradermally, at multiple sites, into the backs of rabbits (0.2 ml per rabbit). The same procedure was repeated four times with one week interval between each application. On the seventh day after the last injection the rabbits were bled and their sera tested by immunodiffusion against CECE and human sera. The immune serum showing the highest titter was collected and the IgG fraction isolated from it. A second antigen was prepared by mixing 15 mg of the isolated IgG with 10 ml of CECE and incubating for 1 hr at 37^{o}C. After centrifugation the immune pellet was collected, washed three times with PBS and resuspended in the same buffer at a final concentration of 3 mg/ml. This suspension was used for immunisation of other rabbits under the same conditions as described above. The immune sera obtained from the second series of immunisations were tested against CECE and human sera and the monospecific anti-CIF IgG was isolated.

Affinity purification of CIF. The anti-CIF IgG isolated from the immunisation experiments described above was coupled to BrCN activated Sepharose 4B (Pharmacia - LKB) and packed into a column, of 0,9 x 10 cm bed volume. The column was equilibrated with PBS. The CECE fraction achieved by 25% ammonium sulphate precipitation was applied to the column and eluted with the same buffer . After elution of inbound components a second eluent (glycine - HCL buffer pH 2.5) was used to desorb the specifically bound CIF from the column.

Assay for inhibition of complement activity. To samples of fresh human sera were added various quantity of CIF (0; 0.05; 0.10; 0.15; 0.25 mg per 1 ml serum). The sera were incubated for 60 min. at room temperature on a magnetic stirrer. After centrifugation for 15 min. at 5000g the supernatants were tested for complement activity via both the classical and alternative activation pathway. The complement activity has been recorded by CH50 haemolytic units using method of Mayer (12).

Isolation of IgG. IgG was isolated from immune sera by passing down through a protein A - Sepharose 4B column, as a described by Pharmacia - LKB (Uppsala, Sweden).

Preparation of affinity sorbents. Two different affinity sorbents were prepared by coupling to BrCN - activated Sepharose 4B the CIF and rabbit anti-CIF IgG respectively. The coupling procedures were carried out according to the recommendations of Pharmacia - LKB.

Depletion of C3 complement of human serum by means of immobilised CIF. 4 ml of human serum was applied to a column containing CIF-affinity sorbent (3 ml). The elution was carried out according to the recommendations of Pharmacia - LKB.

Inducing of C3 cleavage in human serum. The experiments were performed according to Molenaar et al. (13).

Immunodiffusion. The immunodiffusion experiments were carried out according to Ouchterlony (14). Agar , 1 % in saline, containing 1 mmol $CaCl_2$ was used. The gels were stained by Coomassie Brilliant blue R-250.

Gel electrophoreses. The electrophoresis in polyacrylamide gel (PAGE) was performed in a system, containing concentrating (3%) and distributing (7.5%) gels (15). The electrophoresis in polyacrylamide gel with sodium dodecyl sulphate (SDS - PAGE) was carried out according to Laemmli (16) The gels were stained by Coomassie Brilliant blue R-250 or Schiff's reagent.

Ultraviolet (UV) spectroscopy. UV absorption spectra were recorded using a Perkin Elmer Lambda 2 Spectrophotometer in 0.1 M phosphate buffer pH 7.

Results

Isolation and purification of CIF. Ammonium sulphate precipitation was employed as a first step in the isolation of CIF. We established that 25 % saturation was the most appropriate. Under these conditions the CIF was completely precipitated from the seed extract and was contaminated with only a further 5-6 proteins, as revealed by PAGE. Affinity chromatography was then used to obtain CIF in a pure state.

The elution profile obtained from Sepharose 4B column is presented in Fig. 1. This profile showed two peaks, the first was eluted with the PBS starting buffer and the second after application of the

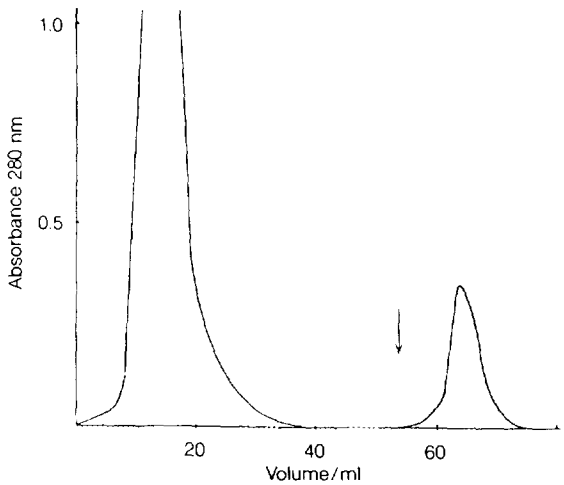


Figure 1. Affinity chromatography of the fraction from 25% ammonium sulphate precipitation. Immunosorbent - anti-CIF rabbit IgG, immobilised on BrCN -activated Sepharose 4B. The sample (20 mg protein) was applied on a column 0,9 x 10 cm. equilibrated with PBS. Elution was performed with the same buffer, followed by 0.1 M glycine -HCl buffer, pH 2.5 (arrow). Fractions (3 ml) were collected. Each of collecting tube contains 0.5 ml 0.75M TRIS - HCl buffer, pH 8.6, for neutralisation of acid buffer.

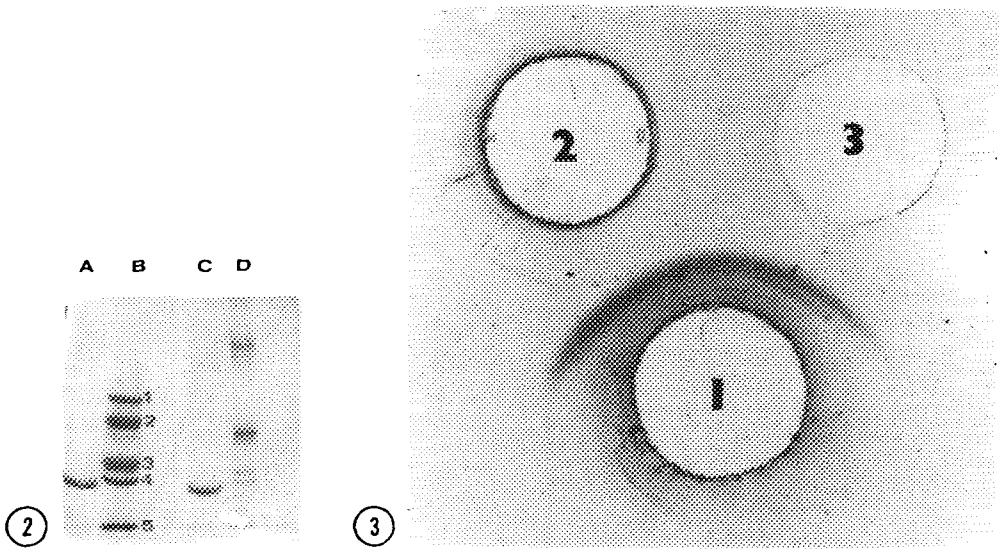


Figure 2. SDS - PAGE electrophoresis. The gel is stained with Coomassie blue. Line A end line C - CIF (2 mg). Line B - Molecular weight calibration kit (low molecular weight). Markers proteins: 1 - phosphorilase B (94,000); 2 - bovine serum albumin (67,000); 3 - ovoalbumin (43,000); 4 - carbonic anhydrase (30,000); 5 - trypsin inhibitor. Line D - Molecular weight calibration kit (high molecular weight).

Figure 3. Double immunodiffusion in 1% agar gel in saline, containing 1 mmol $CaCl_2$. Sample volume - 0,2 ml. 1. CIF, 2 mg/ml concentration. 2. Fresh normal human serum. 3. Fresh human serum containing C3b immune complex.

acid eluent. The electrophoresis (Fig. 2) shows that the second peak contains just one protein band. This protein was immunologically tested against human sera, and a precipitation line was observed. (Fig. 3). This protein was used for further experiments as CIF.

Molecular properties. The molecular weight of native CIF was estimated by gel filtration chromatography, using molecular weight standard markers and Sephadex G-200, a value of 27000 Da was obtained (Fig. 4). The molecular weight (under denaturating conditions) was determined by SDS-PAGE and a value of 28 000 Da (Fig. 2) was achieved. These results suggest that the native CIF is composed of one polypeptide chain only.

The polyacrylamide electrophoresis band corresponding to CIF stained very strongly with Schiff's reagent as well as with Coomassie blue indicating that the protein is highly glycosylated.

UV-absorption spectrum is presented in Fig.5. Absorption maximum was determined at 268 nm. and a second subpeak at 315 nm. was observed.

Depletion of complement activity by CIF. Changes of the complement activity via classical pathway in human sera after incubation with CIF are shown in Fig. 6. The addition of CIF to the sera decreases complement activity , producing complete inactivation at a concentration of 0.25 mg per ml serum. The same manner of complement inactivation was observed by the alternative complement activation pathway (results are not shown).

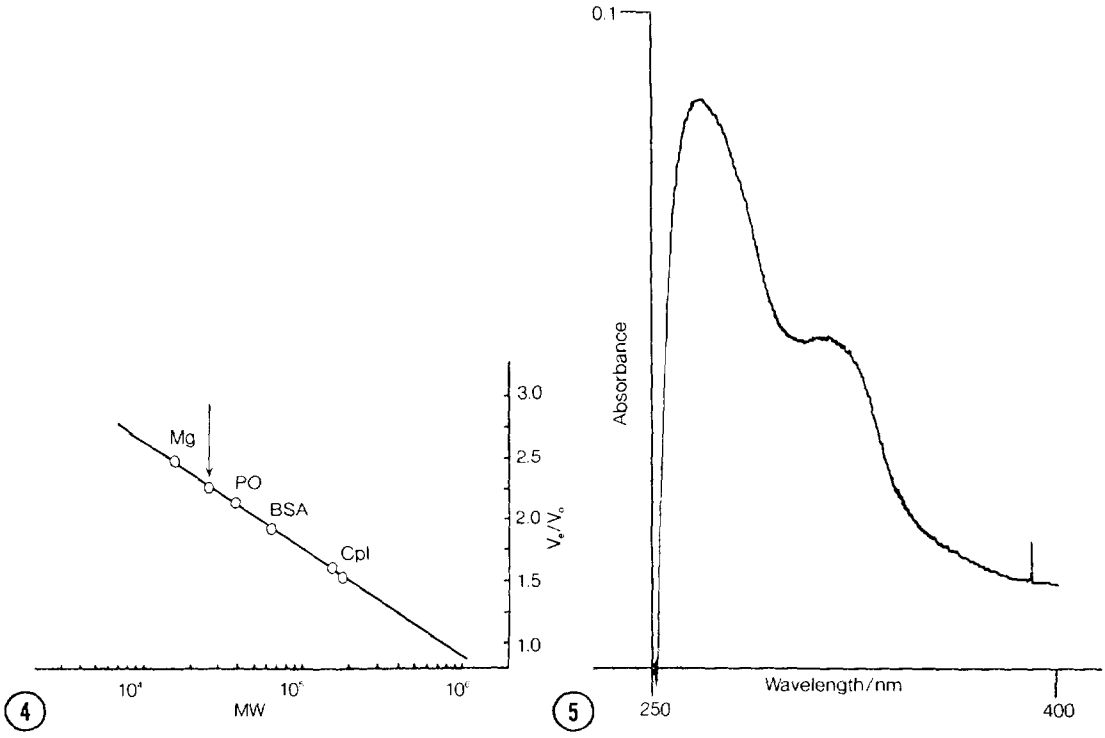


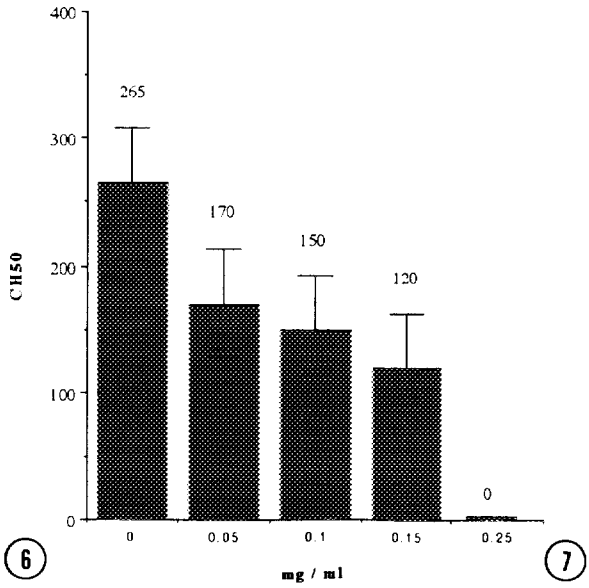
Figure 4. Molecular weight determination of CIF by gel filtration. The purified CIF (fraction No 2 from affinity chromatography) was applied to a gel G -250 column (75 cm x 1,6 cm inner diameter) pre equilibrated in PBS, pH 7.2. The flow rate was 30 ml/hour and absorbance was monitored at 280 nm. Molecular weight standards, which were chromatographed in the same conditions included: ceruloplasmine (Cpl) - 140.000, bovine serum albumin (BSA) - 67,000, peroxidase (PO) - 40,000, mioglobin (Mg) - 17, 200. The elution position of the CIF is indicated by an arrow and a molecular weight of 27,000 was estimated.

Figure 5. Ultraviolet absorption spectrum of CIF in 0.1 M phosphate buffer, pH 7. Absorption maximum was determined at 268 nm. with a second subpeak at 315 nm.

Precipitating activity of CIF. Immunodiffusion tests showed one, and occasionally two, precipitin lines developed between CIF and human sera. Two lines were only observed when the tested human sera contained immune complexes (Fig. 3). Results of the testing of human serum depleted by CIF against anti - C3 monospecific immune serum, are presented in Fig. 7. A very strong precipitin line was formed between the native serum and the immune serum but no reaction was observed between the immune serum and the CIF depleted serum.

The precipitating activity of the CIF against the C3 and C3 - subfractions is presented in Fig. 8. Only fresh human serum samples, containing C3 and C3b, produced a precipitation line with CIF. There was no reaction in these samples previously incubated to produce C3 cleavage .

Precipitating activity of immune rabbit serum. As is seen on figure 9 the rabbit immune serum developed one precipitin line against CIF and another against C3 from normal human serum.



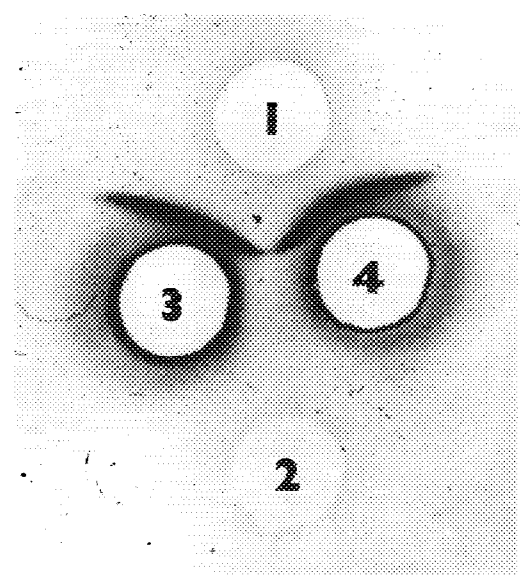


Figure 6. Depletion of complement activity in human sera by CIF. The complement activity was recorded as CH50 haemolytic units after addition of 0.05; 0.10; 0.15; 0.25 mg to each sample (1 ml) of fresh human serum. After 60 minutes incubation the precipitate has been removed and the supernatant has been tested for complement activity.

Figure 7. Double immunodiffusion in agar gel. The experimental conditions were the same as figure 3. 1. Normal human serum. 2. The same serum, depleted by CIF. 3 and 4 - Goat anti-human C3 serum.

Discussion

Two stages - rough fractionation by precipitation with ammonium sulphate and an affinity purification by immobilised on Sepharose rabbit anti-CIF IgG, have been carried out for isolation of CIF in a purified form. Monospecific polyclonal antibodies against CIF have been obtained by an "immunological purifying" - two consecutive immunisation steps. At the first step, a precipitate formed after reaction of CECE with normal human serum was used for immunisation. The resulting immune rabbit serum recognised both the CIF (from the extract) and the C3 (from the human serum) A second immunisation to be prepared monospecific antibodies towards the CIF only, was carried out. In this case another precipitate, between CIF and rabbit IgG was used. It was formed after mixture of CECE and rabbit immune IgG (isolated from the sera of the first group rabbits) and was injected as an antigen. The immune sera obtained after the second immunisation developed one precipitin line with the CECE only and showed no reaction with normal human sera (the results are not presented). IgG fraction of this serum was utilised for preparation of an immune sorbent which further was applied for CIF affinity purification. Thus, CIF was isolated in an electrophoretic purified form. By PAGE and SDS-PAGE analyses the availability of a single intensive stained band was demonstrated with both Coomassie blue and Schiff's reagent. These results suggested a high sugar contents in CIF molecule.

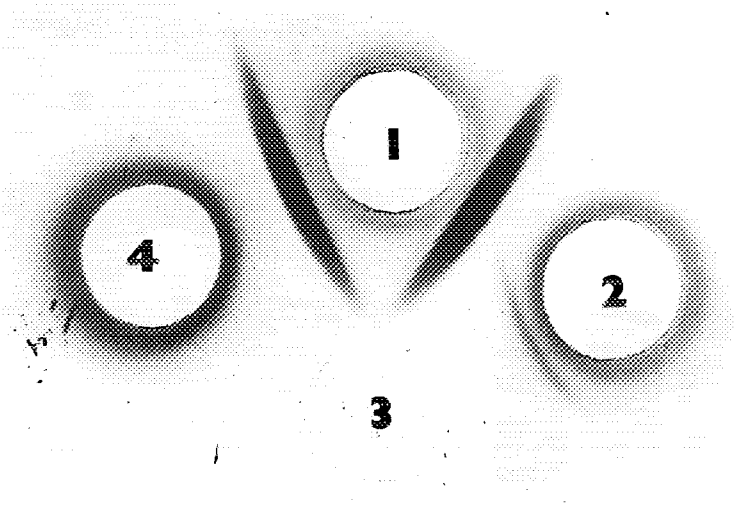


Figure 8. Double immunodiffusion in agar gel. The experimental conditions were the same as figure 3. 1. Goat anti-human C3 serum. 2. Normal human serum. 3. CIF. 4 Normal human serum, incubated to induce C3b cleavage.

By means of gel filtration chromatography, the native CIF molecular weight was estimated about 27000 Da. Similar molecular weight-28000 Da of the possible subunits was determined by SDS-PAGE, also. Based on these results it might be concluded that the native CIF is composed of one polypeptide chain only.

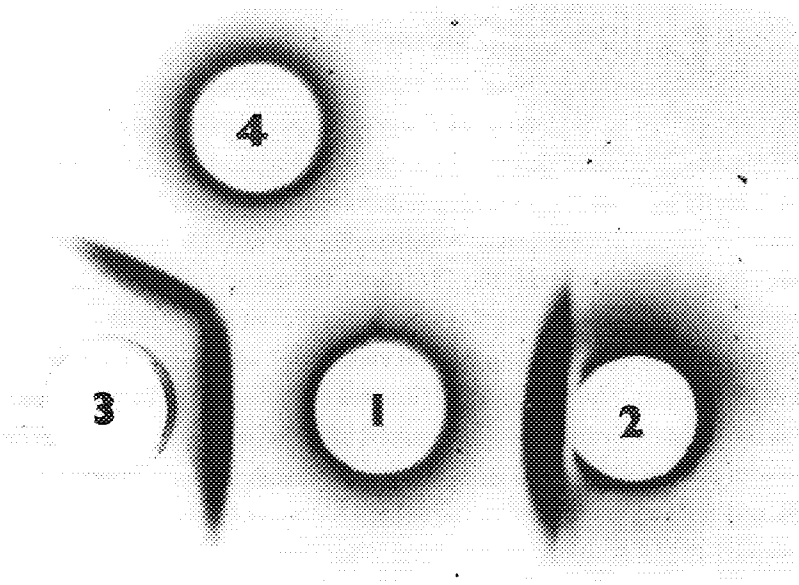


Figure 9. Double immunodiffusion in agar gel. The experimental conditions were the same as figure 3. 1. Immune rabbit serum after immunisation with a precipitate, formed between CECE and normal human serum. 2. CIF. 3. Normal human serum. 4. Goat anti-human C3 serum.

As is seen in Fig. 6, CIF fully depletes the complementary activity of the serum at concentration of 0.25 mg per ml serum. Apparently, this depletion has been accomplished directly by binding with the C3 component, consequently a new precipitate band is formed during agar gel diffusion or an precipitate is deposited after mixing of CIF with serum. It should be mentioned that such precipitate has not been formed against a normal serum exhausted with anti C3-antibodies or against the same serum, treated for C3 cleavage. The direct binding of CIF to C3-component of the complement was also verified by ELISA, carried out in the laboratory of Prof. Self, Department of Clinical Biochemistry, University of Newcastle upon Tyne, UK. The results will be published separately.

The properties of CIF suggest, that it can be used for a simple gel-diffusion analysis or quantitative immunoenzyme techniques for determination of C3 and C3b-containing immune complexes in human serum, without nowadays applied complicated procedures (17,18).

Finally, it is worth noting one of the "side" results from our experiments. As it is shown, after the immunisation with the complex CIF-C3-component of the complement, an immune serum towards the C3 fraction of the human complement with high titre was produced, which does not react with any other human serum proteins. This method for production of monospecific immune serum towards the C3-fraction of the complement is easier and more effective than the routinely used procedures, as it is not necessary the human serum to be fractionated in order to obtain a pure complement fraction for the immunisation (19, 20).

Acknowledgment

This research was supported by Commission of the European Communities to cooperation in science and technology with Central and Eastern European countries.

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