CHROMSYMP. 2939

Purification of human tumour necrosis factor by membrane chromatography

Janja Luksa, Viktor Menart, Stjepan Milicic and Breda Kus

Lek, Pharmaceutical and Chemical Company, Research and Development, Celovska 135, 61000 Ljubljana (Slovenia)

Vladimira Gaberc-Porekar

Institute of Chemistry, Hajdrihova 19, 61000 Ljubljana (Slovenia)

Djuro Josic*

Octapharma Pharmazeutika GmbH, Oberlaaer Str. 235, A-1100 Vienna (Austria)

ABSTRACT

The recombinant human tumour necrosis factor α from an extract of *Escherichia coli* was enriched to homogeneity according to specific activity and sodium dodecyl sulphate–polyacrylamide gel electrophoresis by purification using anion-exchange HPLC and hydrophobic interaction HPLC. Parallel experiments with the same separation methods, but carried out with membrane chromatography on compact discs, gave similar results in terms of yield and purity of the product. The active form of the protein is a trimer. The second isolation step, hydrophobic interaction chromatography, causes dissociation of the trimer into monomers and a partial loss of the biological activity of the protein. The phenomenon occurs on both the column and the disc. This in turn indicates strongly that the dissociation of the protein is a consequence of interaction between the sample and the hydrophobic ligand, and is not caused by non-specific interaction with the matrix.

INTRODUCTION

Tumour necrosis factor α (TNF-α) is secreted by macrophages and belongs to the cytokines, a group of molecules which includes also interferons and interleukins. These polypeptides with molecular masses of up to 25 000 are involved in the complex regulation of cellular growth and differentiation. They also play a key role in inflammation, immunity and haemopoiesis [1]. TNF-α has antitumour properties and it displays antiviral activity and *in vitro* cytotoxicity to certain transformed cell lines [2,3]. However, TNF is also implicated in the pathogenisis of cachexia (wasting), rheumatoid arthritus and inflammatory tissue destruction [4,5]. Because of these properties, the molecule is of considerable interest as a potential therapeutic agent and for purposes of diagnosis.

Human TNF-α is an unglycosylated polypeptide, containing 157 amino acids, with a molecular mass of 17 350. The active form of the molecule is a trimer [6]. Using recombinant DNA technology, the TNF gene was cloned. The recombinant protein was expressed in *E. coli* [7]. The biologically active protein can be purified by anion-exchange HPLC (HPAEC), followed by hydrophobic interaction HPLC (HPHIC) [8]. However, the recovery of biologically active TNF-α after HPHIC is low owing to partial dissociation of the trimer into monomers [9].

---

* Corresponding author.

High-performance membrane chromatography (MC) [10–14] was introduced chiefly for biopolymer separations. Several techniques of membrane construction exist. One strategy consists in bundling several thin membranes made of synthetic hollow fibres of cellulose, another involves compact, porous, disc-shaped membranes, made of silica gel or polymer supports, and a third option is a combination of these two strategies. Excellent results in biopolymer separations have been achieved especially with separation devices that consist of several bundled membranes and with compact discs made of poly(glycidyl methacrylate) [10–13].

Membranes have several important advantages over HPLC columns. The chromatographic separation is carried out on a wide, thin disc, so that there is only a low pressure drop even at high flow-rates [10,11]. The fact that separation can be carried out very quickly is due to the fast reaction kinetics in such systems [15,16]. Membrane chromatography has been successfully used for separations of enzymes [16,17], serum and membrane proteins [11,18] and even for the isolation of such large molecules as plasmid DNA [19]. This method allows a separation within a shorter period of time than a chromatographic column with the same capacity. Because of the structure of the support, its surface is reduced. Therefore, the possibility of non-specific interactions of the sample with the surface of the support is much lower [10,11]. A comparison between MC and HPLC of TNF-α was undertaken in order to find out whether the dissociation of TNF and consequently the loss of its biological activity take place during interaction with the hydrophobic ligand, or whether it is a consequence of non-specific interaction with the support material.

EXPERIMENTAL

Production of recombinant TNF-α

Synthetic gene human TNF-α with codons optimized for expression in E. coli in plasmid BBG4 was purchased from British Biotechnology (Oxford, UK). The gene was properly inserted into the expression vector pCYTEXPl, supplied by MEDAC (Hamburg, Germany).

Intracellular expressison of biologically active TNF-α was achieved by growing cells at 28°C to an absorbance of 0.3–0.35, followed by incubation for a further 4–5 h at 41°C. In E. coli TG1 the high copy number of expression plasmid was maintained by addition of 50–70 μg/ml of ampicillin in Luria-Bretani medium made according to the laboratory manual [23]. With this system, a TNF expression level of about 1% total cellular protein in laboratory flask culture was reproducibly achieved.

An extract of bacterial cells was the starting material for the purification of human TNF-α. For this the cells were harvested, treated with a lysozyme solution (1 mg/ml) and subsequently sonicated (W358 sonicator; Ultrasonic, New York, USA). The clear supernatant after centrifugation, containing 1.3 mg/ml of protein, was subjected to chromatographic purification. The biological activity of TNF-α at the beginning was $5 \cdot 10^5$ U/mg of protein. The activity was based on its cytotoxic effect on transformed mouse fibroplasts, cell line L 929, measured according to the method of Marnenout et al. [20].

Chemicals

All chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany). Water of HPLC grade was obtained either from Baker (Gross-Gerau, Germany) or from Merck. All chemicals required for electrophoresis were purchased from Pharmacia (Vienna, Austria).

Instrumental

A gradient liquid chromatographic system was used, consisting of two FPLC pumps, type P-500, an LCC-50 plus control unit, a UV monitor with a 280-nm filter, an REC 102 recorder and a FRAC-100 fraction collector (all from Pharmacia). Sample application loops with volumes between 0.5 and 10 ml were used. Equipment for electrophoresis was obtained from Hoefer Scientific (Vienna, Austria) or Pharmacia.

Chromatography on compact discs and on HPLC columns

For HPAEC, a Mono-Q column (50 × 5 mm I.D.) (Pharmacia) was used. A Quick Disk Q

compact disc 25 mm in diameter and 3 mm thick (Säulentechnik Knauer, Berlin, Germany) was used for anion-exchange (AE) MC. For HPHIC a phenyl-Superose column (50 × 5 mm I.D.) (Pharmacia) and a butyl-Sepharose fast flow column (60 × 8 mm I.D.) were used. Parallel HI-MC was carried out on a Quick Disk C4 unit 25 mm in diameter and 3 mm thick (Säulentechnik Knauer).

The buffers for HPAEC were (A) 10 mM Tris–HCl (pH 8.0) and (B) 10 mM Tris–HCl containing 1.0 M NaCl. For HPHIC, buffer A was 1.7 M ammonium sulphate in 0.1 M potassium phosphate (pH 7.0) and buffer B was 0.1 M potassium phosphate (pH 7.0). Both the column and the disc were regenerated with 0.1 M acetic acid. Other chromatographic conditions are given in the figure captions.

Electrophoresis

The dialysed and freeze-dried samples were dissolved in 62.5 mM Tris–HCl buffer (pH 6.8) containing 3% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) mercaptoethanol, 10% (v/v) glycerol and 0.0015 (w/v) bromophenol blue. In the other experiments, 20–50 μl of sample were taken from the collected fractions after HPLC or MC separation. These samples were subsequently mixed with a buffer that contained five times higher concentrations of the substances mentioned above. The amount of buffer used in the experiments was measured in such a way as to restore the original concentration after dilution by the sample. SDS–polyacrylamide gel electrophoresis (PAGE) was carried out by the Laemmli method [21] on a 15% separating gel with a 3% stacking gel. The amount of protein applied
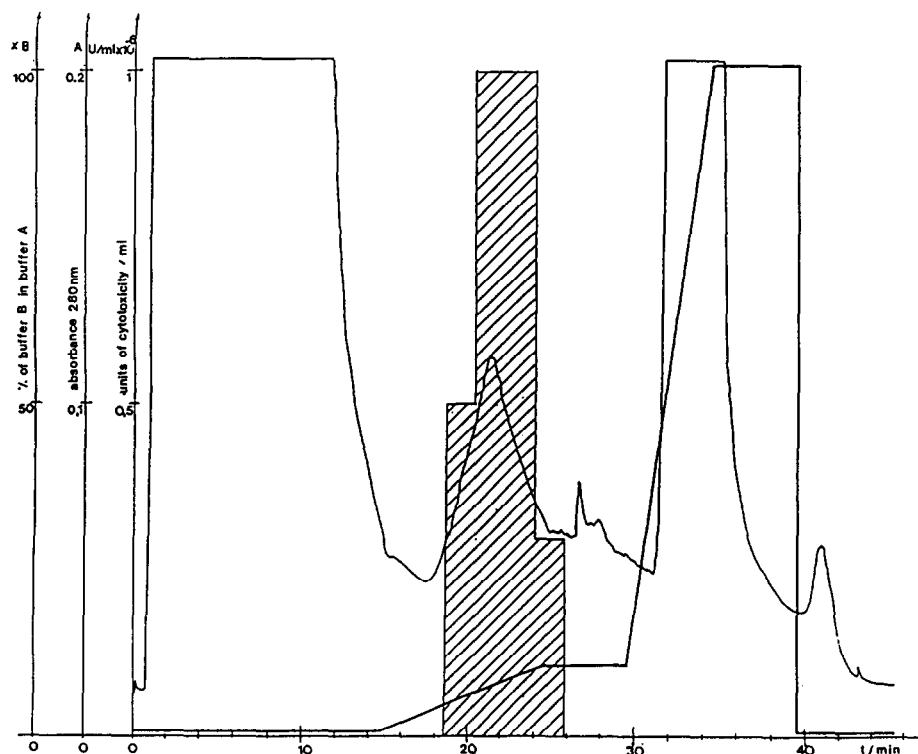

Fig. 1. First purification step: HPAEC of TNF-α. A 10-ml volume of extract from bacterial cells, containing 13 mg of protein and $6.5 \cdot 10^6$ units of TNF-α, were applied to a Mono-Q column and eluted with a sodium chloride gradient (see Experimental). Chromatographic conditions: flow-rate 1 ml/min; pressure, 15–20 bar; room temperature. The gradient is shown in the chromatogram. The fractions containing TNF activity are hatched. The presence of TNF-α in these fractions was also confirmed by subsequent dot blot analysis.

was between 10 and 75 μg per line, depending on the purity of the sample.

Dot blot analysis

The dot blot method was adopted from protocols for Western blotting [22,23]. A nitrocellulose membrane (Pharmacia) was spotted with samples (5 μl of each) from fractions which were collected after separation and dried. The remaining free groups of the membrane were blocked with excess of protein (3% bovine serum albumin in Tris-buffered saline, pH 7.4). Primary polyclonal rabbit anti-human recombinant TNF-α antibodies (Sigma, Deisenhofe, Germany), were bound to TNF-α from collected fractions. In a second step, goat anti-rabbit antibodies conjugated with horseradish peroxidase (Bio-Rad, Vienna, Austria) were bound and detected as blue spots by adding 4-cholor-1-naphthol solution.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the two HPLC steps for the purification of recombinant human TNF-α, *i.e.*, HPAEC and HPHIC. The parallel experiments carried out with AE-MC and hydrophobic interaction (HI) MC are shown in Figs. 3 and 4, respectively. The purification steps agree with those presented by Lin and Yamamoto [8] in their purification scheme for TNF. The enrichment in the final product was from $5 \cdot 10^5$ units/mg of protein in the cell culture lysate to about $5 \cdot 10^7$ units/mg of protein after HPHIC. The initial concentration of TNF-α in the cell lysate was 1%, which means that the product was purified practically to homogeneity. This also agrees with the results that were obtained by Lin and Yamamoto [8].

Using membrane chromatography with compact discs as separation units, a considerable decrease in separation time was achieved, from
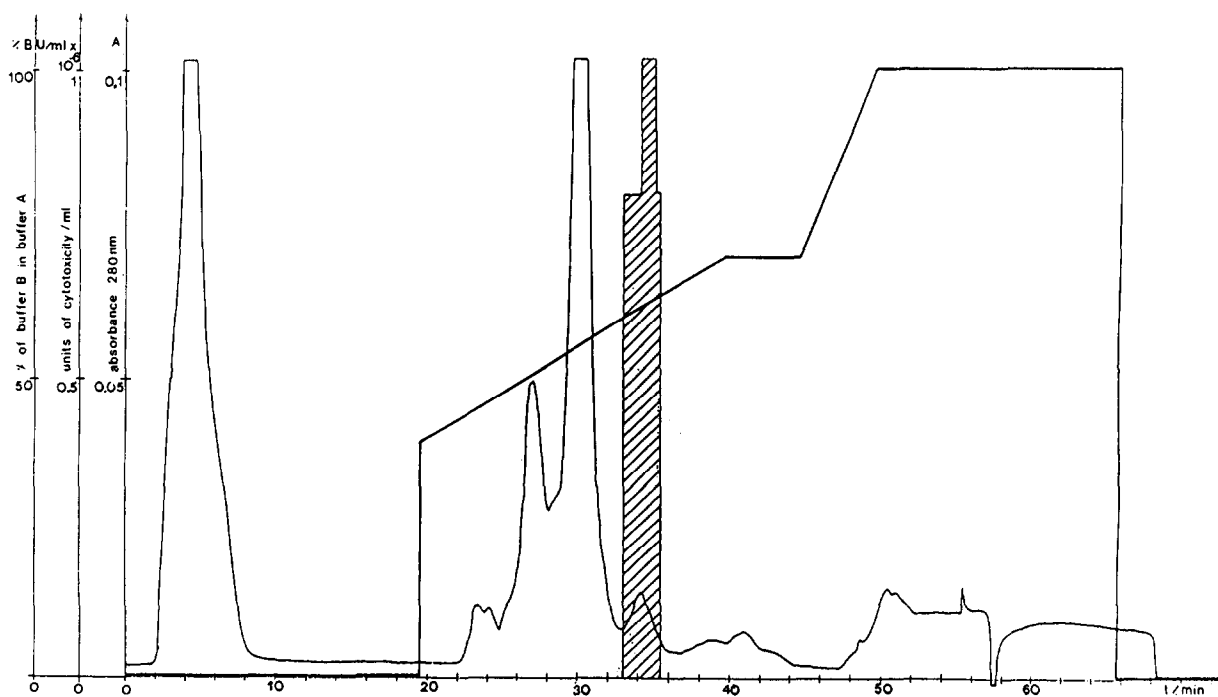

Fig. 2. Second purification step: HPHIC of TNF-α. A 10-ml volume of AE-HPLC product containing biologically active TNF-α was applied to a phenyl-Superose column and eluted by lowering the ammonium sulphate concentration (see Experimental). Chromatographic conditions: flow-rate, 0.5 ml/min; pressure, 15–20 bar; room temperature. The gradient is shown in the chromatogram. The fractions containing TNF activity are hatched.
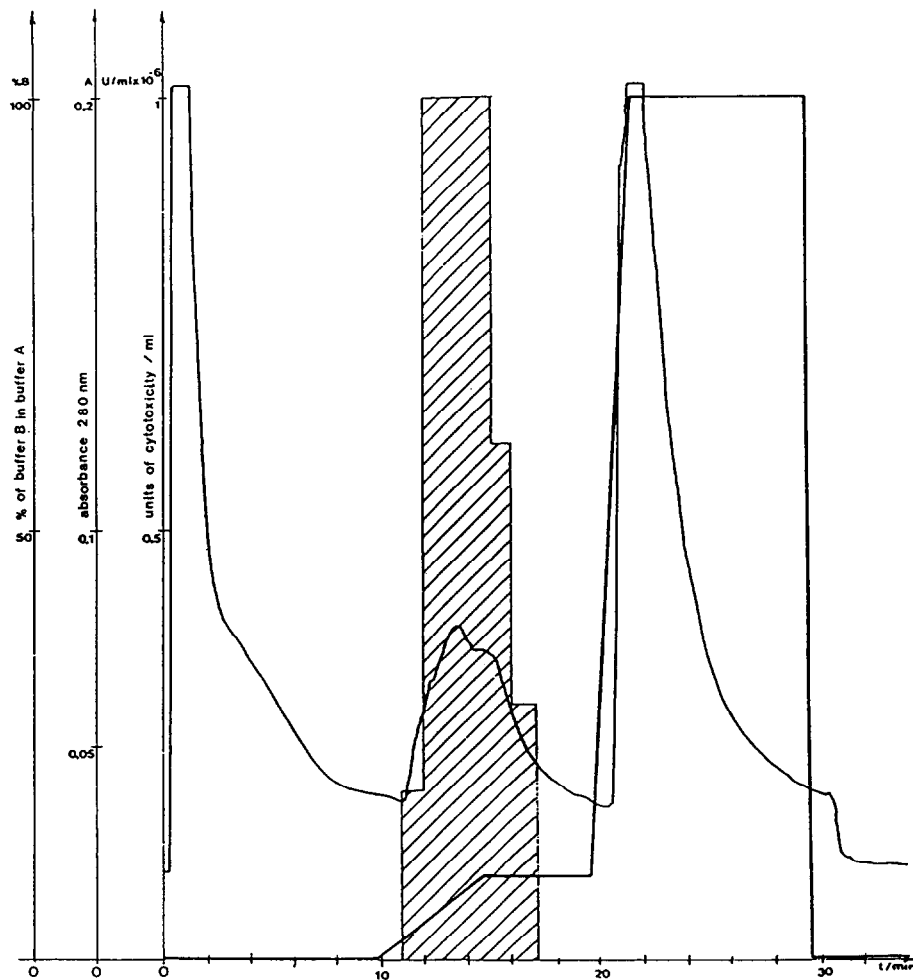
Fig. 3. AE-MC of TNF-α. The separation unit was a Quick Disk Q membrane. Chromatographic conditions: flow-rate, 3 ml/min; pressure, 5 bar; room temperature. Other conditions as in Fig. 1.

120 min in HPLC to about 60 min in MC. Fig. 5 shows the SDS-PAGE of samples with TNF-α activity from separations by HPLC and MC.

The HPAEC separation was performed on a Mono-Q column and the MC separation with a Quick Disk Q unit. The quaternary amine ligand density on a poly(glycidyl methacrylate) membrane (Quick Disk Q) is about 2.2 μM/g, which is two orders of magnitude lower than ligand density on a Mono-Q support (about 600–700 μM/g). However, the capacity of this anion-exchange membrane is about 30–40 g of BSA per gram of support, which is comparable to the capacity of Mono-Q material [11]. A similar relationship for ligand density exists between the butyl-Sepharose fast flow support (about 50 μM/ ml) and a Quick Disk butyl membrane (0.85 μM/g). The separation and recovery of TNF-α on a butyl-Sepharose fast flow column (not shown here) was comparable to those on the phenyl-Superose column (Fig. 2). Because of the different geometry and mass transfer on a chromatographic column and on a membrane unit, a direct comparison of these two devices is difficult. In this paper, a comparison according to TNF-α purity and recovery of biologically active factor was made.
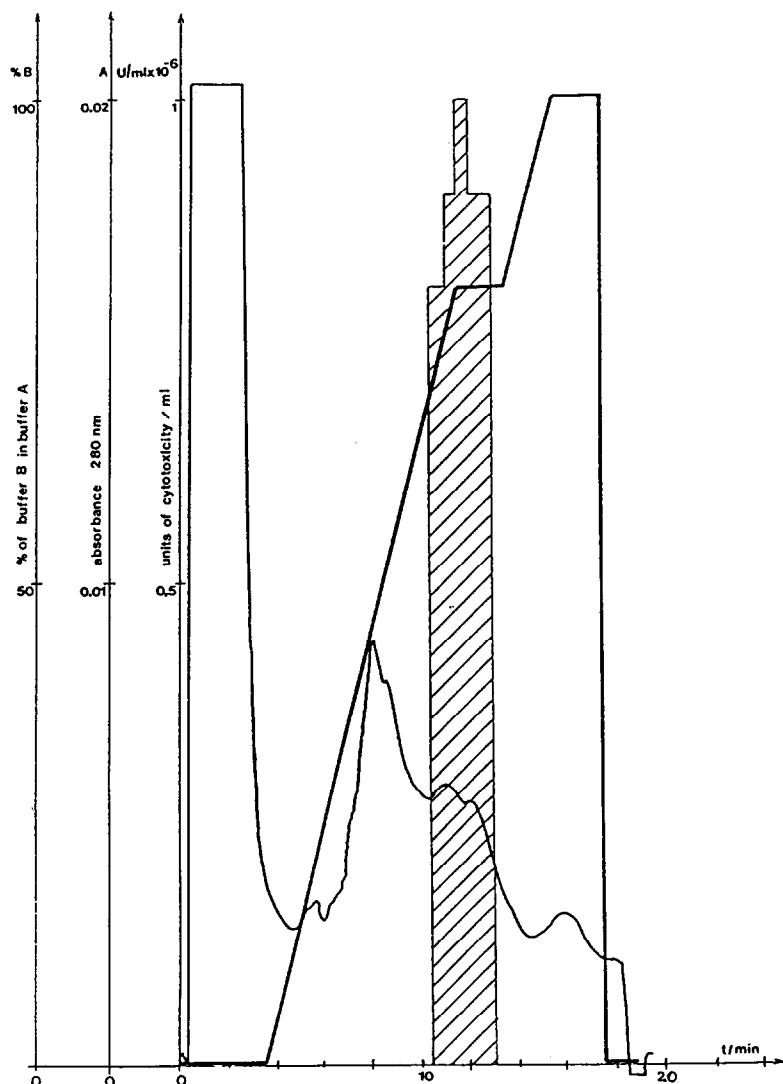
Although the chromatograms from HPLC

Fig. 4. HI-MC of TNF-α. The separation unit was a Quick Disk C4 (butyl) membrane. Chromatographic conditions: flow-rate, 3 ml/min; pressure, 5 bar; room temperature. Other conditions as in Fig. 2.

show superior peak resolution, the purity was comparable according to the electrophoretic presentation (cf., Fig. 5): after HPAEC (lane 4), after AE-MC (lane 6), after HPHIC (lanes 2 and 3) and after HI-MC (lane 7). The specific activity of the final product was identical after purification by either HPLC or MC, i.e., $5 \cdot 10^7$ units/ mg protein.

The recovery of biologically active TNF was about 20% and therefore fairly low with both purification methods, HPLC and MC. A large loss in yield occurs in the second purification step, HPHIC, using both the column and the compact disc (membrane) methods. In order to establish what actually becomes of the TNF-α polypeptide, dot blot analyses of the biologically active fraction and of the other fractions from HPLC and MC were carried out. The results of the dot blot analysis are shown in Fig. 6. Apart from the peak where biological activity was found (hatched in Figs. 2 and 4), intense blue dots appear in fractions Ac1–Ac5 also. These are
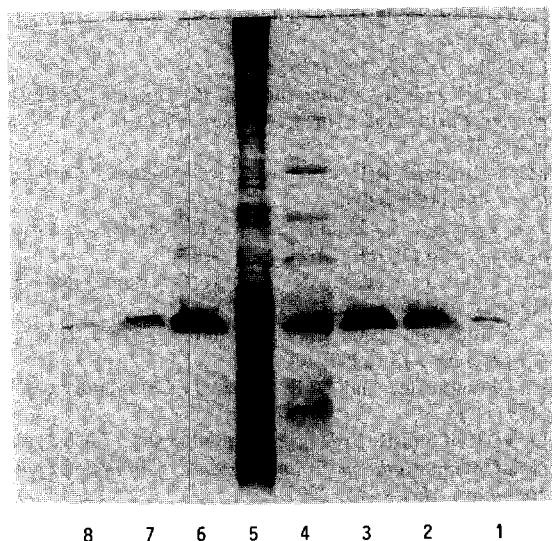
Fig. 5. SDS-PAGE of the fractions from anion-exchange HPLC and MC and from hydrophobic interaction HPHIC and MC. Lanes: 1 = myoglobin (marker protein for molecular mass 17 000); 2 = TNF-α purified by HPHIC column (C₄ functional group); 3 = TNF-α purified by HPHIC column (phenyl functional group); 4 = mixture of proteins obtained in HPAEC purification step; 5 = cell proteins of recombinant E. coli in extract; 6 = mixture of proteins obtained in AE-MC purification step; 7 = TNF-α purified by HI-MC compact disc (C₄ functional group); 8 = myoglobin.

the fractions that were obtained by rinsing the column with acidic acid. The protein in these fractions has lost its biological activity.

Kunitani et al. [9] investigated the behaviour of TNF-α in HPHIC. They found that the biologically active trimer dissociates on the column into biologically inactive monomers. They also found that the dissociation is reversible, i.e., the monomers can be re-associated into the native, biologically active trimer. Re-association could not be achieved in our experiments, because the TNF-α monomers could not be eluted from either the column or the disc (membrane) unless strongly denaturing conditions (acetic acid) were applied. This causes a further decrease in biological activity in this fraction of recovered protein. However, Kunitani et al. [9] reported that an increase in sample volume favours the dissociation and therefore denaturation on the HPHIC column. The dissociation of TNF on a hydrophobic interaction column seems to be dependent on the form of the ligand. When butyl-Sepharose fast flow material was used, a similar separation performance and recovery to those on a phenyl-Superose column were
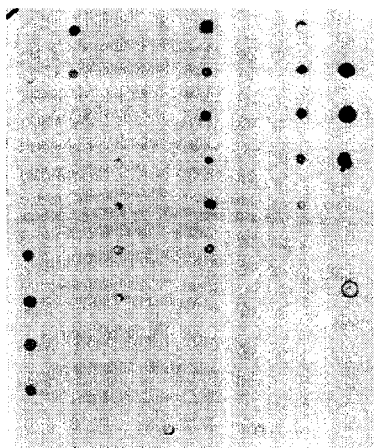

Fig. 6. Dot blot analyses of the fractions from hydrophobic interaction chromatography. 1d–14d = Fractions from Quick Disk Q separation. Fractions 6d–9d show also TNF-α activity in test with transformed mouse fibroblasts (see Experimental). These fractions correspond to the hatched region in Fig. 3 and lane 6 in SDS-PAGE in Fig. 5. 41–59 = Fractions from Quick Disk C4 separation. Fractions 51–54 show also TNF-α activity in biological test (see above). These fractions correspond to the hatched region in Fig. 4 and lane 7 in SDS-PAGE in Fig. 5. 1–37 = Fractions from HPHIC column. Fractions 8–13 show also TNF-α activity in biological test and correspond to the TNF-α peak in the chromatogram (hatched region in Fig. 2). Some TNF-α could be subsequently washed out with 0.1 M potasium phosphate buffer (spots 28–31). However, these fractions did not have biological activity. Fractions 8–13 correspond also to lane 2 in SDS-PAGE in Fig. 5. Ac1–Ac5 = Fraction collected during washing of hydrophobic compact disc (Quick Disk C4) with acetic acid. XXX = No sample.

achieved (see above). The large application volumes that were used here may explain the large amount of dissociated TNF-α. Apparently the interaction of the TNF-α takes place with the hydrophobic ligand, i.e., the phenyl or butyl groups in HPHIC and the butyl groups in HI-MC. The surface of the support does not seem to be involved. The experiment with a disc, with contains hydroxy groups instead of quaternary amine groups, supports this assumption. In this experiment, the sample was applied under identical conditions on such a membrane unit, i.e., means in 1.7 M ammonium sulphate. Neither retention nor loss of biological activity of TNF-α was observed (not shown here). Further, the identical behaviour of TNF-α in both HPLC and MC is in agreement with this conclusion. Despite the much smaller surface area of the support, due to the structural characteristics of the disc [10,11], the amount of dissociated TNF-α did not decrease when the separation was carried out by MC. The portion of dissociated TNF-α also could not be decreased by increasing the flow-rate, i.e., by shortening the period of contact between the sample and the disc.

REFERENCES

1 L.J. Old, Nature, 326 (1987) 330.
2 E.A. Carswell, L.J. Old, R.L. Kassel, S. Green, N. Fiore and B. Williamson, Proc. Natl. Acad. Sci. U.S.A., 72 (1975) 3666.
3 G.H.W. Wong and D.W. Goeddel, Nature, 323 (1986) 819.
4 B. Beutler and A.A. Cerami, Rev. Biochem., 57 (1988) 505.
5 F.A. Wolheim, D. Heinegard, M. Palladino, T. Saxne and N. Tatal, Arthritis Rheum., 30 (1987) 129.

6 P. Wingfield, R.H. Pain and S. Craig, FEBS Lett., 211 (1987) 179.
7 L.J. Old, Science, 230 (1985) 630.
8 L.S. Lin and R. Yamamoto, in R. Burgess (Editor), Protein Purification: Micro to Macro, Alan R. Liss, New York, 1987.
9 M.G. Kunitani, R.L. Cunico and S.J. Staats, J. Chromatogr., 443 (1988) 205.
10 J.A. Gerstner, R. Hamilton and S.M. Cramer, J. Chromatogr., 596 (1992) 173.
11 Dj. Josic, J. Reusch, K. Löster, O. Baum and W. Reutter, J. Chromatogr., 510 (1992) 59.
12 T. Tennikova, B.G. Belenkii and F. Svec, J. Liq. Chromatogr., 13 (1990) 63.
13 B. Chompluvier and M.R. Kula, J. Chromatogr., 539 (1991) 315.
14 O.-W. Reif and R. Freitag, presented at the 17th International Symposium on Column Liquid Chromatography, Hamburg, May 9–14, 1993.
15 M. Unarska, P.A. Davies, M.P. Esnouf and B.J. Bellhouse, J. Chromatogr., 519 (1990) 53.
16 H. Abou-Rebyeh, F. Körber, K. Schubert-Rehberg, J. Reusch and Dj. Josic, J. Chromatogr., 566 (1991) 341.
17 V. Prpic, R.J. Uhing and T.W. Gettys, Anal. Biochem., 208 (1993) 155.
18 Dj. Josic, F. Bal and H. Schwinn, J. Chromatogr., 632 (1993) 1.
19 N. van Huguh, J.C. Motte, J.F. Pilette, M. Decleire and C. Colson Anal. Biochem., 210 (1993) 1.
20 A. Marnenout, L. Fransen, J. Tavernier, J. Van der Heyden, R. Tizard, E. Kawashima, A. Shaw, M.J. Johnson, D. Seman, R. Mueller, M.-R. Ruysschaert, A. Van Vliet and W. Fiers, Eur. J. Biochem., 152 (1985) 515.
21 U.K. Laemmli, Nature, 227 (1970) 680.
22 E. Harlow and D. Lane, in Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988, p. 471.
23 J. Sambrook, E.F. Fritsch and T. Maniatis, in C. Nolan (Editor), Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 2nd ed., 1989, p. 18.