Oligomeric tumour necrosis factor α slowly converts into inactive forms at bioactive levels

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The stability of oligomeric human tumour necrosis factor α (TNF) at bioactive levels has been studied by two immunoenzymatic assays: one able to specifically detect oligomeric and not monomeric TNF (O-e.l.i.s.a.) and the other able to detect both forms (OM-e.l.i.s.a.). The selectivity of O-e.l.i.s.a. and OM-e.l.i.s.a. for oligomeric and monomeric TNF was demonstrated with isolated forms prepared by partial dissociation of recombinant TNF with 10% (v/v) dimethyl sulphoxide and gel-filtration h.p.l.c. Evidence for instability of oligomeric TNF were obtained in physiological buffers, as well as in serum and cell-culture supernatants, as a function of TNF concentration. In particular, only a half of the TNF antigen was recovered in the oligomeric form after 72 h incubation (37 °C) at 0.12 nM, whereas no apparent dissociation was detected at 4 nM. The structural changes observed at picomolar concentrations were rapidly reversed by raising the concentration of TNF to about 2 nM by ultrafiltration, suggesting that subunit dissociation and reassociation reactions occur in the picomolar and nanomolar range respectively. The cytolytic activity of L-M cells correlates with oligomeric-TNF levels after incubation at picomolar concentrations. Moreover, isolated oligomeric TNF was cytotoxic towards L-M cells, whereas monomeric TNF was virtually inactive. In conclusion, the results suggest that bioactive oligomeric TNF is unstable at picomolar levels and slowly converts into inactive monomers, supporting the hypothesis that quaternary-structure changes in TNF may contribute to the fine regulation of TNF cytotoxicity.

INTRODUCTION

Tumour necrosis factor α (TNF) is a pleiotropic mediator of the inflammatory and cellular immune response. This cytokine, originally identified by its antitumour properties and cytotoxicity to certain transformed cell lines [1], was found to be implicated in the pathogenesis of endotoxic shock, cachexia, rheumatoid arthritis and inflammatory tissue destruction (see [2–4] for reviews).

Two different soluble and cell-surface receptors have been discovered and are thought to mediate and regulate the pleiotropic effects of TNF [5–8].

Three-dimensional-structure studies have shown that TNF, in crystalline form, is a compact trimer composed of three identical 17.5 kDa subunits [9–10]. Analytical-ultracentrifugation [11,12], cross-linking [13], gel-electrophoretic [14] and X-ray-scattering [15] studies have shown that TNF is also oligomeric in solution. Nevertheless, the quaternary structure of TNF at bioactive levels in biological fluids needs more investigation, since TNF is bioactive at concentrations lower than the detection limits of most analytical systems exploited so far.

Oligomeric TNF is considered to be the biologically active form, whereas monomeric TNF is thought to be poorly, or not, active [16]. Monomers could be poorly cytotoxic because they are unable to efficiently bind receptors, as has been observed with Hela and LAK cells [16–17] or because polyvalency is required for triggering cellular cytotoxicity, e.g. by receptor microclustering [18]. Thus it has been hypothesized that changes in quaternary structure could play a role in the regulation of TNF activity [16]. This hypothesis is also supported by the observation that radiolabelled oligomeric TNF is unstable and

dissociates to monomeric TNF after prolonged incubation in buffer [17]. In the present study the stability of native (unlabelled) oligomeric TNF at picomolar concentrations has been examined by means of sandwich e.l.i.s.a.s able to detect specifically only oligomeric TNF (O-e.l.i.s.a.) or both oligomeric and monomeric forms (OM-e.l.i.s.a.). Moreover, the relationships between quaternary-structure changes and cytolytic activity have also been examined.

MATERIALS AND METHODS

Materials

Human recombinant TNF expressed in yeast was from Esquire Chemie AG (Zurich, Switzerland); rabbit anti-TNF antiserum (IP300) was from Genzyme Corp. (Boston, MA, U.S.A.). Neutralizing monoclonal antibody 78 (Mab78) was produced by Farmitalia Carlo Erba (Milan, Italy). Poly(vinyl chloride) (PVC) microtitre plates (Falcon MicroTestIII flexible assay plates) were obtained from Becton Dickinson and Co. (Oxnard, CA, U.S.A.).

D-Biotinyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester, goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate, streptavidin-HRP conjugates, BSA (Cohn Fraction V), normal goat serum, Tween 20 and dimethyl sulphoxide (DMSO) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A kit containing the chromogenic substrate 2,2'-azinobis-(3-ethylbenzthiazolinesulphonic acid) (ABTS) was from Kirkegaard and Perry Laboratories (Gaithersburg, MD, U.S.A.). Cell-culture reagents, media and fetal bovine serum were from Flow Laboratories (McLean, VA, U.S.A.). All other reagents were analytical-grade products from Carlo Erba (Milan, Italy).

Abbreviations used: TNF, human tumour necrosis factor- α ; O-TNF, TNF antigen detected by O-e.l.i.s.a. (which detects only oligomeric TNF); OM-TNF, TNF antigen detected by OM-e.l.i.s.a. (which is able to detect both oligomeric and monomeric TNF); PBS, phosphate-buffered saline (0.05 M-sodium phosphate/0.15 M-NaCl, pH 7.3); PBS-T, PBS containing 0.05% Tween 20; PBS-BT, PBS-T containing 1% BSA; HRP, horseradish peroxidase; DMSO, dimethyl sulphoxide; ABTS, 2,2'-azinobis-(3-ethylbenzthiazolinesulphonic acid); Mab, monoclonal antibody; PVC, poly(vinyl chloride); MTT, 3-(4,6-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Preparation of biotinylated Mab78

Mab78 was biotinylated as follows: 600 μ l of 1 mg/ml protein solution, in 0.1 M-sodium borate buffer, pH 8.8, was mixed with 6 μ l of D-biotinyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester (10 mg/ml in DMSO) and incubated for 4 h at room temperature. Then 60 μ l of 1 M-lysine solution was added to the reaction mixture, incubated for 1 h at room temperature and extensively dialysed overnight at 4 °C against phosphate-buffered saline (PBS). The product was diluted to 3 ml with PBS and stored as stock solutions at -20 °C.

O-e.i.s.a.

O-e.i.s.a. was carried out as follows: PVC microtitre plates were incubated with Mab78 (2 μ g/ml; 50 μ l/well) in PBS overnight at 4 °C. After the coating step the plates were washed with PBS and incubated for 2 h with 200 μ l of PBS containing 3% BSA. The microtitre plates were then washed with PBS and filled with TNF samples or standards in triplicate (50 μ l/well) that had previously been diluted with PBS containing 1% BSA. Standard solutions were freshly prepared from a 1 mg/ml TNF stock solution kept frozen at -20 °C. After 1.5 h incubation at 37 °C, the plates were washed eight times with PBS-T (PBS containing 0.05% Tween 20) and filled with biotinylated Mab78 [50 μ l/well; 1:500 dilution in PBS-T containing 1% BSA (PBS-BT) stock solution]. The plates were incubated again for 1 h at 37 °C and washed eight times with PBS-T. Then, 50 μ l of streptavidin-HRP (1:1000 in PBS-BT) was added to each well. The plates were left for 1 h at 37 °C in a humid covered box, washed with PBS-T (eight times) and filled with 100 μ l of freshly prepared ABTS chromogenic substrate solution (0.78 mM). The colour was allowed to develop for 30 min and the A_{405} of each well was measured. TNF antigen concentrations, as measured by O-e.i.s.a. (O-TNF), was calculated by interpolation of absorbance values on the calibration curve.

OM-e.i.s.a.

OM-e.i.s.a. was carried out as described for O-e.i.s.a., on the same plates and with identical buffers and reagents, except that a rabbit anti-TNF serum (IP300), diluted 1:500 with PBS-BT, was used in place of biotinylated Mab78, and that HRP-labelled goat anti-(rabbit IgG) immunoglobulin solution, diluted 1:1000 with PBS-BT containing 1% normal goat serum, was used in place of streptavidin-HRP solution. TNF antigen concentrations, as measured by OM-e.i.s.a. (OM-TNF), were calculated by interpolation of absorbance values on the calibration curve.

Gel-filtration h.p.l.c.

Gel-filtration h.p.l.c. of TNF was performed on a TSK-3000SW column (LKB-Pharmacia) by using an HPLC DfB (LKB-Pharmacia) apparatus. The column was pre-equilibrated and eluted with PBS containing BSA (50 μ g/ml) at a flow rate of 0.5 ml/min. When specified, 10% DMSO was added to the eluent.

Cytolytic assay

TNF cytotoxic activity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytolytic assay on mouse L-M fibroblasts (A.T.C.C. CL12) as described in [19].

RESULTS

OM-e.i.s.a. and O-e.i.s.a. of recombinant TNF

To evaluate the molecular forms of TNF in solution, two different sandwich e.i.s.a.s have been developed. They are based on: (a) a 'sandwich' reaction between Mab78 immobilized on

microtitre plates, TNF and rabbit anti-TNF polyclonal antibodies, followed by goat anti-rabbit IgG-HRP conjugate (OM-e.i.s.a.); (b) a sandwich reaction between Mab78, immobilized on microtitre plates, TNF and biotinylated Mab78, followed by streptavidin-HRP conjugate (O-e.i.s.a.).

Calibration curves

The ability of OM- and O-e.i.s.a. to detect TNF irrespective of its molecular form was first examined. Recombinant TNF solutions at various concentrations were freshly prepared from a 1 mg/ml stock solution, kept frozen at -20 °C, and tested. As shown in Fig. 1(a), a dose-response curve was obtained by OM-e.i.s.a. with TNF in the range between 0.1 and 25 ng/ml. This indicates that Mab78 and rabbit polyclonal antiserum were efficiently able to form 'molecular sandwiches' with TNF. The binding is specific, since no sandwich reaction occurred in the absence of capturing Mab78.

Recombinant TNF was also able to form detectable molecular sandwiches in the O-e.i.s.a. analytical system, as shown by the dose-response curve obtained in the range between 1 and 50 ng/ml (Fig. 1b). No detectable signals were obtained when Mab78 or biotinylated Mab78 were omitted, indicating that the binding is specific. When repeated, similar binding curves and sensitivity were obtained in the same range of concentration, even with different batches of reagents.

Since the antibody used in the 'capture' and 'detection' step is the same (Mab78), this result indicates that Mab78 is able to recognize at least two accessible sites on recombinant TNF that are probably related to its oligomeric structure.

Detection of oligomeric TNF and monomeric TNF by OM- and O-e.i.s.a.

The specificity of OM- and O-e.i.s.a. for monomeric and oligomeric TNF was then examined with isolated forms. Hydrophobic interactions are known to play a role in the TNF subunit interaction [9,16,20], and thus treatment with DMSO, followed by gel-filtration chromatography, were successful in dissociating and separating TNF subunits. To this end a series of chromatographic runs were carried out with TNF in the presence or absence of DMSO on a TSK-3000SW column. Fractions were analysed by OM-e.i.s.a..

As Fig. 2 (chromatogram a) shows, only one immunoreactive peak, corresponding to 35-40 kDa, was observed when native TNF (400 ng/ml) was run in the absence of DMSO. This chromatographic behaviour of TNF is in agreement with the results of other gel-filtration-chromatographic studies, where

Fig. 1. (a) OM-e.i.s.a. and (b) O-e.i.s.a. calibration curves with recombinant TNF

Binding curves are expressed as percentage of maximum binding (B/B_{max} , %) (obtained at saturation with 400 μ g of TNF/ml) versus TNF concentration.

Fig. 2. Gel-filtration chromatography of TNF treated or not with DMSO

TNF (400 ng/ml) was preincubated overnight at 4 °C in the absence of DMSO (chromatograms a and c), in the presence of 5 % DMSO (d) and 10 % DMSO (b and e), prior to gel filtration through a TSK-3000SW column. The column was pre-equilibrated and eluted with buffer not containing (a and b) and containing (c, d and e) 10 % DMSO. Fractions (0.5 ml) were tested by OM-e.l.i.s.a. Arrows indicate the elution volumes of molecular-mass (*M*) standards: BSA (69 kDa), thermolysin (34 kDa) and bovine neurophysin (10 kDa). Abbreviation: AUFS, absorption units full scale.

oligomeric TNF has been reported to exhibit an apparent molecular mass of 34–40 kDa [17,20–21], and indicate that TNF was homogeneous and fully oligomeric at 400 ng/ml.

When TNF was pretreated overnight at 4 °C with 5 % DMSO and gel-filtered through a column pre-equilibrated with 10 % DMSO, a second immunoreactive peak with an elution volume expected for monomeric TNF (17 kDa) was revealed by OM-e.l.i.s.a. (Fig. 2, chromatogram d). The second peak increased when TNF was treated with 10 % DMSO, whereas the first peak decreased (Fig. 2, chromatogram e). Only the first peak was observed when native TNF was subjected to chromatography through a column pre-equilibrated with 10 % DMSO, indicating that preincubation with DMSO was necessary for dissociation (Fig. 2, chromatogram c).

Chromatography of DMSO-treated TNF on the column pre-equilibrated without DMSO resulted in abnormal retardation of the second peak, the elution volume being higher than the column total volume (Fig. 2, chromatogram b). Matrix absorption phenomena were likely occurring with monomeric TNF in this case, possibly because of the protein's high hydrophobicity [20].

In conclusion, the results of gel-filtration experiments indicate that the oligomeric structure of TNF is destabilized and dissociated by DMSO and that both monomeric and oligomeric TNF can be detected by OM-e.l.i.s.a.

The total immunoreactivity of TNF, as measured by OM-e.l.i.s.a., was fairly conserved after dissociation, suggesting that oligomeric and monomeric TNF are detected with similar efficiency.

The specificity of O-e.l.i.s.a. for oligomeric TNF was then examined. As Table 1 shows, when h.p.l.c. fractions corresponding to oligomeric and monomeric TNF were tested by O-e.l.i.s.a., monomers were undetectable, whereas comparable amounts (by OM-e.l.i.s.a.) of oligomers were efficiently detected.

Table 1. O-e.l.i.s.a. and OM-e.l.i.s.a. of chromatographic fractions corresponding to the first (oligomeric TNF) or the second (monomeric TNF) peak of chromatogram e (Fig. 2)

Fraction no.	TNF antigen (ng/ml)*	
	OM-e.l.i.s.a.	O-e.l.i.s.a.
35 (first peak)	20	16.2
42 (second peak)	16	ND†

* After chromatography; fractions were rapidly diluted 2-fold with assay buffer and tested.
† ND, not detectable.

Fig. 3. Effect of DMSO on TNF as monitored by O-e.l.i.s.a. and OM-e.l.i.s.a.

TNF solutions at various concentrations were tested by O- and OM-e.l.i.s.a. after overnight incubation (4 °C) in the presence of various amounts of DMSO. Samples were diluted 1:4 with analytical buffer before testing. The results are expressed as [O-TNF]/[OM-TNF] ratio (%). [TNF] (ng/ml): □, 400; ▨, 50; ▩, 25; ■, 12.5; ▀, 6.25.

This confirms that monomeric TNF, although able to bind Mab78, is unable to form molecular sandwiches in the O-e.l.i.s.a. system, probably because of its univalency. Thus the assay is specific for oligomeric TNF. The [O-TNF]/[OM-TNF] ratio is therefore a function of the proportion of the oligomeric form over the total TNF antigen.

Monitoring of TNF subunit dissociation and reassociation reactions by O- and OM-e.l.i.s.a.

Dissociation of oligomeric TNF is expected to reduce the [O-TNF]/[OM-TNF] ratio in TNF solutions. Thus it should be possible to monitor TNF subunit dissociation and reassociation reactions by measuring the [O-TNF]/[OM-TNF] ratio.

To verify this assumption, TNF solutions at various concentrations were incubated in the presence or absence of DMSO (5 and 10 %) and tested. As Fig. 3 shows, the [O-TNF]/[OM-TNF] ratio decreased, as expected, under dissociating conditions as a function of the TNF and DMSO concentration. The effect of DMSO on antibody binding, as investigated in preliminary experiments, was found to be negligible when samples were diluted at least 4-fold before assay. Interestingly, dissociation was higher at lower TNF concentrations, suggesting that DMSO causes a mild reduction of subunit affinity without heavy-denaturation phenomena.

It is noteworthy that, when DMSO was removed from samples containing 400 ng of TNF/ml, by dialysis, and re-association allowed to occur, the [O-TNF]/[OM-TNF] ratio increased to the original value. This suggests that the method can be reliably

Fig. 4. Kinetics of TNF structural changes in buffer and serum

TNF solutions (6 ng/ml) were prepared by diluting a 1 mg/ml TNF stock solution with PBS/1% (w/v) BSA or normal human serum. TNF solutions were tested by O- and OM-e.l.i.s.a. after incubation for various times at 4 °C (○) and 37 °C (●). Each point represents the mean result for three assay replicates.

Fig. 5. Binding kinetics of TNF to Mab78-coated microtitre plates

TNF at various concentrations was allowed to bind to Mab78 coated microtitre plates for various times. Binding was revealed by OM-e.l.i.s.a. (a) and O-e.l.i.s.a. procedures (b). [TNF] (ng/ml): ●, 20; ○, 10; ■, 5; □, 2.5; ▲, 1.25.

exploited to monitor TNF subunit dissociation/re-association reactions.

Stability of oligomeric TNF in physiological buffer and human serum

The stability of TNF quaternary structure at picomolar levels was examined by O- and OM-e.l.i.s.a. in the absence of dissociating agents.

The effect of time and temperature was first evaluated. A stock solution (1 mg/ml) of TNF, previously shown to contain only oligomeric TNF by gel-filtration chromatography, was diluted to 6 ng/ml with PBS containing 0.1% (w/v) BSA, 10 kallikrein-inhibitory units of aprotinin, 10 μ M-phenylmethanesulphonyl fluoride, 10 μ M-EDTA, 0.02% NaN_3 and tested by O- and OM-e.l.i.s.a. after incubation. As Fig. 4 shows, the [O-TNF]/[OM-TNF] ratio slowly decreased to about a half of initial value after 72 h at 37 °C, suggesting that oligomeric TNF was unstable under those conditions. The decrease was higher at 37 °C than at

Fig. 6. Structural changes in TNF after incubation at various concentrations as measured by O- and OM-e.l.i.s.a.

A TNF stock solution (1 mg/ml) was diluted to various concentrations with PBS containing 0.1% BSA, aprotinin (10 kallikrein-inhibitory units/ml), 10 μ M-EDTA, 10 μ M-PMSF, 0.02% NaN_3 and incubated for 0 (○) and 72 h (●) at 37 °C. All TNF samples were tested by O- and OM-e.l.i.s.a.. Each point represents the mean for three replicates.

4 °C, probably owing to the faster kinetics of structural change at higher temperatures. Similar effects were observed also with TNF diluted in human serum (results not shown).

It is noteworthy that, when the final product was 30-fold concentrated by ultrafiltration and further incubated for 2 h at room temperature, the [O-TNF]/[OM-TNF] ratio increased to the original value. This strongly suggests that the observed structural changes are not related to proteolytic reactions but, more likely, to TNF subunit dissociation and reassociation reactions.

Further evidence for instability of TNF quaternary structure at low concentrations was obtained by examining the binding kinetics of TNF in the OM- and O-e.l.i.s.a. analytical systems. Typical binding curves were obtained on OM-e.l.i.s.a., with saturation reached after 5 h of incubation (Fig. 5a). In contrast, bell-shaped curves were obtained by O-e.l.i.s.a. (Fig. 5b). This is in agreement with the above observation that O-TNF is unstable and slowly converts into a form still detectable by OM-e.l.i.s.a. but undetectable by O-e.l.i.s.a.

The stability of oligomeric TNF was further studied at various concentrations. TNF solutions were tested before and after 72 h incubation at 37 °C. As Fig. 6 shows, the decrease in the [O-TNF]/[OM-TNF] ratio after incubation was clearly a function of concentration, being higher at lower concentrations (Fig. 6). It was noteworthy that no significant decrease was observed at 200 ng/ml, suggesting that oligomeric TNF is fairly stable at nanomolar levels and unstable at subnanomolar levels.

Correlation between oligomeric TNF instability and loss of bioactivity

The effect of oligomeric-TNF instability on its cytolytic activity was examined. A solution containing 6 ng of TNF/ml was incubated in cell-culture medium and tested at various times by O-e.l.i.s.a., OM-e.l.i.s.a. and a standard cytolytic assay on L-M cells.

As Fig. 7 shows, the decrease in the [O-TNF]/[OM-TNF] ratio observed upon incubation correlated with a decrease of

Fig. 7. Correlation between oligomeric TNF instability and loss of bioactivity

A TNF solution (6 ng/ml) was preincubated for various times and tested by O-e.l.i.s.a., OM-e.l.i.s.a. and standard cytolytic assay on mouse L-M cells. The results are expressed as [O-TNF]/[OM-TNF] ratio (●) and [bioactive TNF]/[OM-TNF] ratio (○).

Fig. 8. Effect of DMSO on TNF cytotoxicity

The effect of various amounts of DMSO on L-M-cell viability (A_{595}) and TNF cytotoxicity was measured by the MTT assay after 24 h of incubation in the absence (▲, △) or presence of TNF (250 pg/ml) (●, ○), and with (●, ▲) and without (○, △) actinomycin D in the cell-culture medium.

bioactivity, suggesting that oligomeric TNF slowly converts into inactive forms at subnanomolar concentrations.

Cytotoxic properties of oligomeric and monomeric TNF

To verify that dissociation of TNF is accompanied by loss of cytolytic activity on L-M cells, the effect of dissociating amounts of DMSO on TNF cytotoxicity was first studied.

TNF cytotoxicity was found to decrease in the presence of DMSO (Fig. 8). This could be due to TNF dissociation as well as to effects of DMSO on TNF-receptor binding and/or on L-M-cell responsiveness. The following experiments were done to clarify this point.

(a) A TNF solution (10 ng/ml) was pretreated overnight at 4 °C with or without 10% DMSO and tested for cytolytic

Fig. 9. Cytotoxic activity of isolated TNF oligomers and monomers

TNF (400 ng/ml) was treated overnight at 4 °C with 10% (v/v) DMSO and subjected to gel-filtration chromatography on a TSK-3000SW column. Fractions were tested by e.l.i.s.a. (○) and cytolytic test on L-M cells (●) after 20-fold dilution.

activity after 20-fold dilution (final [DMSO] in the assay less than 0.5%). Also, in this case, the cytolytic activity of DMSO-treated TNF was decreased to about 20% of that of untreated controls. The effect of 0.5% DMSO on TNF cytotoxicity was determined in a preliminary experiment and found to be negligible. These results thus suggest that the loss of cytolytic activity after exposure to DMSO is more likely to be related to TNF structural changes than to effects on cell responsiveness. Interestingly, the residual cytolytic activity of TNF after incubation in DMSO correlated with O-TNF levels (16% of untreated controls).

(b) Oligomeric and monomeric TNF were prepared by partial dissociation of TNF with 10% DMSO and gel-filtration chromatography, as described above. The cytolytic activity of fractions was then tested on L-M cells after 20-fold dilution. As Fig. 9 shows, only fractions containing oligomeric TNF were bioactive, whereas fractions containing monomeric TNF were inactive even at a concentration as high as 600 pg/ml (by OM-e.l.i.s.a.). In this bioassay, 50% cytotoxicity is usually achieved with 150 pg of recombinant TNF/ml; thus TNF monomers, though able to re-associate at higher concentrations, are not, or remarkably less, active on L-M cells than oligomers.

In conclusion, these results support the concept that dissociation of oligomeric TNF is accompanied by loss of cytotoxicity.

DISCUSSION

The stability of oligomeric and monomeric TNF at low concentrations has been studied by means of two new sandwich ELISAs, namely O- and OM-e.l.i.s.a.

Whereas OM-e.l.i.s.a. is able to detect both oligomeric and monomeric TNF (OM-TNF), O-e.l.i.s.a. is able to detect only the oligomeric TNF (O-TNF), with detection limits of 0.1 and 1 ng/ml respectively. Experiments carried out with oligomers and monomers, prepared by gel-filtration chromatography of TNF partially dissociated with DMSO, have shown that the ratio between [O-TNF] and [OM-TNF] is dependent on the proportion of oligomeric TNF over the total antigenic TNF in solution. Thus subunit dissociation-re-association reactions can

be easily monitored, even at low concentrations, by measuring the [O-TNF]/[OM-TNF] ratio.

This analytical approach offers the advantage over previous studies on the molecular form of TNF at low concentrations, which were based on radioactively labelled and cross-linked TNF [13,17] in that TNF can be studied in its native form. This should prevent artifacts that may arise from chemical modification and labelling reactions. Recombinant TNF used in the present study was almost totally oligomeric in stock solutions, as evidenced by gel-filtration chromatography. Moreover, SDS/PAGE analysis of the protein after cross-linking with disuccinimidyl suberate indicated that more than 90% of the product was trimeric in stock solution (results not shown).

When recombinant TNF was diluted to low concentrations with physiological buffer, human serum and cell-culture media, evidence for instability of the oligomeric structure were obtained by O- and OM-e.l.i.s.a. In particular, only a half of TNF antigen was recovered in the oligomeric form after 72 h incubation (37 °C) at 0.12 nM, whereas no apparent dissociation was detected at 4 nM. The structural changes occurring at 0.12 nM were rapidly reversed by ultrafiltration of TNF to about 2 nM, indicating that the observed [O-TNF]/[OM-TNF] changes were related to subunit dissociation and re-association and not to proteolytic degradation of the product.

This suggests that recombinant TNF exists in solution with quaternary structures that differ according to the storage concentration of TNF and the period of incubation. These results and those of previous studies conducted under different conditions allow a more comprehensive view of TNF quaternary structure. For instance, it has been found that cross-linked TNF is solely oligomeric (trimeric) at concentrations as low as 0.8 ng/ml [13], whereas ¹²⁵I-TNF at a similar concentration (25 pM) was a mixture of oligomers and monomers after incubation for 1 day at 4 °C [17]. The lack of monomers in the cross-linked TNF experiments may be easily explained by the slow kinetics of TNF dissociation; since dissociation takes several hours or days to reach equilibrium, a considerable proportion of TNF molecule is likely to be found in the oligomeric form when TNF is prepared by diluting concentrated stock solutions and rapidly cross-linked. In general this may have important implications on experimental work carried out with diluted TNF solutions, such as cell supernatants and sera, or with recombinant TNF solutions freshly prepared from stock solutions, since different molecular forms are very likely to be present.

The instability of oligomeric TNF correlates with the loss of cytolytic activity when studied on a heterologous system such as mouse L-M cells, a murine cell line frequently used in TNF bioassays. The correlation has been strengthened by direct testing of isolated monomers obtained by dissociation of oligomers with DMSO followed by gel-filtration chromatography. Monomers appear to be non-cytotoxic, confirming previous findings on a human cell line [16].

The observation we have made on TNF subunit dissociation-re-association reactions *in vitro*, probably also applies *in vivo*, since monomers and oligomers have been found, by gel-filtration chromatography [22], in various proportions in the spinal fluid of patients with meningitis.

The O-e.l.i.s.a. method, able to detect only bioactive oligomeric TNF, could be conveniently used to complement conventional immunoassays (for total antigen) in TNF detection in biological fluids under physiopathological conditions.

Moreover, the analytical approach described here could probably be extended to structure-function studies of other oligomeric cytokines, e.g. TNF- β [23], interferon- γ , interleukin-5 [24] and interleukin-6 [25].

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