Heterotrimers Formed by Tumor Necrosis Factors of Different Species or Muteins*

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Incubation of murine tumor necrosis factor (mTNF) at subnanomolar concentrations results in partial dissociation of the trimers, coinciding with a decrease in bioactivity. Using size-exclusion chromatography, we observed that the conversion of labeled mTNF to monomers is not only prevented by coincubation with an excess of unlabeled mTNF but also with unlabeled human TNF (hTNF). Moreover, after coincubation of mTNF and hTNF four different TNF complexes were revealed by native polyacrylamide gel electrophoresis, viz. homotrimeric mTNF and hTNF, as well as two complexes with an intermediate migration pattern. Analytical gel filtration in combination with native polyacrylamide gel electrophoresis and Western blot immunodetection indicated that these new complexes consisted of heterotrimeric TNF molecules. We conclude that an exchange of monomers takes place during coincubation of two different species of TNF, which results in homotrimeric and heterotrimeric TNF. To assess receptor interaction in vitro, TNF heterotrimeric molecules were used as obtained after incubation of mTNF with labeled hTNF (which only binds to mTNF receptor I) or with labeled mutein mTNF75 (specific for mTNF receptor II). These heterotrimers were retained by both mTNF receptors, which means that the mTNF subunits incorporated in heterotrimeric complexes still can bind to both types of TNF receptor. In addition, the gradual decrease in mTNF bioactivity during preincubation at subnanomolar concentrations was prevented by the presence of mutein mTNF75, which is inactive in an L929 cytotoxicity assay, indicating that heterotrimerization can influence the overall bioactivity.

Tumor necrosis factor (TNF) is a potent cytokine that exerts a wide range of activities in inflammatory and immune reactions and is an important mediator in many diseases (1–3). TNF exists both as a transmembrane and as a circulating, inactive TNF-R. TNF is an important mediator in many diseases (1–3). It is involved in many diseases and is an important mediator in many diseases. In addition, TNF also interacts with the other TNF-R, which means that the mTNF subunits incorporated in heterotrimeric complexes still can bind to both types of TNF receptor. In addition, the gradual decrease in mTNF bioactivity during preincubation at subnanomolar concentrations was prevented by the presence of mutein mTNF75, which is inactive in an L929 cytotoxicity assay, indicating that heterotrimerization can influence the overall bioactivity.

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§ The abbreviations used are: TNF, tumor necrosis factor; BSA, bovine serum albumin; h, human; LT, lymphotoxin; m, murine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RIA, radioimmunoassay; TNF-R, TNF receptor; smTNF-R, soluble murine TNF-R.

and the p75 TNF receptor (TNF-RII; CD120b). One TNF trimer can bind three TNF-R molecules, and this receptor clustering initiates the intracellular signaling cascade (4, 5). Human lymphotoxin (hLT)-α is another member of the TNF superfamily that also forms a homotrimeric complex and signals through both TNF-Rs. Its interaction with the solubilized, extracellular part of hTNF-RI has been studied by x-ray diffraction (6). The results showed in detail how the soluble receptors were bound in the three grooves, formed between neighboring LT-α subunits. In agreement with these data, receptor-specific muteins of hTNF were obtained by mutating amino acids bordering these grooves (7–10). During incubation at subnanomolar concentrations, TNF trimers dissociate to biologically inactive monomers. Treatment with particular reagents promotes the dissociation of TNF, which can be reversed by increasing the TNF concentration or by removing the chemical agent (11–14).

Murine TNF (mTNF) is 79% identical in amino acid sequence to hTNF. The three-dimensional structure of mTNF and hTNF is similar, diverging mainly in regions that are flexible and/or involved in crystal packing (15, 16). Moreover, TNF chimeric proteins, constructed by exchanging homologous regions between hTNF and mTNF DNA, are able to associate in trimeric, bioactive molecules (17). As we observed, by size-exclusion chromatography, that mTNF and hTNF prevent the conversion of labeled mTNF to monomers, we hypothesized that TNF subunits can be exchanged during coincubation of mTNF and hTNF, resulting in both heterotrimeric and homotrimeric TNF molecules. Experiments with monoclonal antibodies specific for mTNF and hTNF confirmed that such heterotrimeric TNF molecules are formed during coincubation. To study the effect of subunit exchange on the interaction with mTNF-Rs, we used heterotrimers containing, in addition to mTNF subunits, hTNF that interacts only with mTNF-RI, and mutein mTNF75 that interacts specifically with mTNF-RII.

MATERIALS AND METHODS

Cytokines and Antisera—Purified Escherichia coli-derived mTNF, hTNF, and hLT-α were produced in our laboratory; they had a specific biological activity of 2.1 × 10⁹, 8.4 × 10⁹, and 4.0 × 10⁷ units/mg, respectively, in an L929 cytotoxicity assay. Mutein mTNF75 was created by site-specific mutagenesis of Arg-32 → Tyr and Ala-145 → Arg. This mutein binds only to mTNF-RII in an in vitro receptor binding assay. IF3F3, a monoclonal antibody to mTNF (18), was a kind gift from Dr. R. Lucas and Dr. P. De Baetselier (Free University of Brussels, Brussels, Belgium). 61E71, a monoclonal antibody to hTNF (19), TN3, a monoclonal antibody to mTNF (20), and polyclonal rabbit antiserum against mTNF and hTNF were kindly provided by Dr. W. A. Buurman (University of Limburg, Limburg, The Netherlands).

Radiolabeling of Ligands—mTNF and hTNF were radiolabeled using an iodogen iodination agent (Pierce Chemicals, Rockford, IL). Labeled proteins were separated from unincorporated radioactivity on a G-25 column (PD10; Amersham Pharmacia Biotech) and had a specific radioactivity of 10–50 Ci/μg.

Gel Filtration—Gel filtration of radiolabeled TNF was performed on
a Sephacryl S-100 column (Amersham Pharmacia Biotech). The column was equilibrated and eluted in phosphate-buffered saline (PBS) containing 0.02% bovine serum albumin (BSA) and 0.02% NaN₃ (PBS/BSA) at a flow rate of 0.4 ml/min. Fractions of 0.3 ml were collected and tested for radioactivity in a γ-counter. All gel filtration chromatographies were performed at 4 °C. Gel filtration of unlabeled TNF was performed on a Superdex-75 fast protein liquid chromatography column (1-cm internal diameter × 30 cm; Amersham Pharmacia Biotech) with PBS at a flow rate of 0.5 ml/min. Eluted protein was detected by UV absorbance at 280 nm and collected in 1-min fractions.

Native Polycrylamide Gel Electrophoresis (PAGE) of TNF—This procedure was performed according to the instructions of Bio-Rad. Proteins were dissolved in sample buffer (75 mM Tris-HCl, pH 6.8, 50% glycerol, 0.25% bromphenol blue) and applied to a separating 10% polyacrylamide gel (375 mM Tris-HCl, pH 8.8). Running buffer consisted of Tris-glycine (24.8 mM, 191.8 mM, pH 8.3). Proteins were detected by staining with Coomassie Brilliant Blue R-250 or by immunodetection with anti-TNF antibodies after Western blotting on nitrocellulose.

Western Blot Immunodetection of TNF—Proteins in native polyacrylamide gels were blotted onto nitrocellulose in a semi-dry blotting apparatus with a Tris-glycine buffer (48 mM, 39 mM) containing SDS (0.0375%) and methanol (20%). Blots were saturated with antibody to rabbit polyclonal anti-TNF or anti-hTNF serum. Subsequently the blots were incubated with anti-rabbit peroxidase-labeled conjugate (Pierce). Finally, blots were incubated with Renaissance chemiluminescence reagent (Du Pont); light emission was detected with autoradiography films.

Radioimmunoassay (RIA)—96-Well plates were incubated overnight at 4 °C with monoclonal antibody (2.5 μg/ml; 100 μl/well) in PBS. After coating, wells were saturated for 3 h at 26 °C with 150 μl/well of PBS containing 2% BSA. After washing with PBS, plates were incubated with samples containing radiolabeled TNF and diluted in PBS/BSA (100 μl/well) for 90 min at 26 °C. After washing with PBS, wells were separated and individually counted in a γ-counter.

Receptor Binding Assay—Soluble (extracellular) fragments of mTNF-R (smTNF-R) or smTNF-RII were produced with the baculovirus expression system in Sf9 insect cells and partially purified. Medium was dialyzed with samples containing radiolabeled TNF and diluted in PBS/BSA (100 μl/well) or smTNF-RII (100 μl/well) in PBS. After an 18-h incubation at 26 °C, the wells were washed with PBS/0.02% BSA, and bound radioactivity was measured in a γ-counter.

L929 Cytotoxicity Assay—L929 murine fibrosarcoma cells (Bega Institute, Leuven, Belgium) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), and L-glutamine (2 mM) (Dulbecco's modified Eagle's medium/fetal calf serum). Cells were seeded in 96-well microtiter plates at 30,000 cells/well. The next day, a serial dilution of TNF in Dulbecco's modified Eagle's medium/fetal calf serum with or without mTNF was applied to the wells in the presence of 0.02% bovine serum albumin (BSA) and 0.02% NaN₃ (PBS/BSA). After an 18-h incubation, the amount of surviving cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric method (21).

RESULTS

hTNF Keeps Subnanomolar Amounts of mTNF in a Trimeric Form—The stability of mTNF after incubation at subnanomolar concentrations was studied by size-exclusion chromatography on Sephacryl S100, which allows differentiation between trimeric and monomeric forms of TNF. Without prior incubation, iodinated mTNF (400 μCi) eluted in a single peak with a molecular mass of 49 kDa, whereas after overnight preincubation at 26 °C mTNF eluted in two peaks with a molecular mass of 49 and 11 kDa, corresponding to mTNF trimers and monomers, respectively (Fig. 1A). When unlabeled mTNF (4 nM) had been added during preincubation, almost all labeled mTNF remained in the trimer peak. When labeled mTNF (400 μCi) was preincubated for 16 h in the presence of hTNF (4 nM), all label remained in the trimer peak, indicating also that hTNF can keep labeled mTNF subunits in a trimeric form (Fig. 1B).

Similarly, the appearance of labeled hTNF monomers following preincubation at low concentrations was prevented by the addition of a higher concentration of hTNF or mTNF (data not shown). Coincubation with hLT-α (up to 10 nM), however, did not affect the elution profile of labeled mTNF or labeled hTNF (Fig. 1B). This argues against an aspecific effect because of the presence of higher levels of protein.

Native PAGE Reveals Complexes with Intermediate Migration Patterns following Coincubation of Different TNFs—Gel filtration experiments showed that mTNF and hTNF specifically influence each other's quaternary structure during prolonged coincubation; they do not, however, directly prove the formation of heterotrimeric complexes. As mTNF and hTNF have a nearly identical molecular mass, they cannot be discriminated by SDS-PAGE. However, mTNF migrates faster than hTNF during native PAGE because of differences in overall charge distribution (Fig. 2A). After coincubation of mTNF and hTNF (both at 3 μg/10 μl) for 16 h at 4 °C, two additional bands with a migration pattern intermediate between that of homogeneous mTNF and hTNF are revealed by native PAGE and staining with Coomassie Brilliant Blue (Fig. 2). These additional bands already become apparent after coincubation of only 4 h. Western blot immunodetection of these native gels showed that the two additional bands are recognized efficiently both by anti-hTNF and anti-mTNF antibodies (Fig. 3, A and B).

Heterotrimeric TNF Molecules Are Formed during Coincubation of mTNF and hTNF—To study the conformation of the complexes formed during coincubation of mTNF and hTNF (both 30 μg), we applied the mixture to a Superdex 75 fast protein liquid chromatography column. Calibration of this column revealed that mTNF (trimers) and hTNF (trimers) eluted after 20.7 and 21.2 min, corresponding with an apparent molecular mass of 49 and 46 kDa, respectively (Fig. 4B).

After coincubation, all TNF complexes eluted in a single peak be-
mTNF, there is increased competition for binding on the antibody between labeled heterotrimeric molecules and unlabeled homotrimeric mTNF. Formation of heterotrimeric TNF at 26 or 37 °C reached a maximum after 24 h but was already clearly detectable after 2 h. At 4 °C the formation of heterotrimeric TNF molecules was slower but reached an almost comparable level after prolonged coincubation (data not shown). Furthermore, we observed, using an 1F3F3-RIA, that natural mTNF derived from a murine macrophage cell line was as efficient as recombinant mTNF to form heterotrimeric complexes with labeled hTNF (data not shown).

Specificity of Different TNF Monoclonal Antibodies—To study the specificity of different monoclonal antibodies for monomeric and/or trimeric TNF forms, labeled TNF (1.6 nM) was dissociated by treatment with 3 M guanidinium chloride or 0.1% Triton X-100 (19, 20) and subsequently subjected to gel filtration. The TNF-containing fractions were then isolated and used in RIA with different monoclonal antibodies. As shown in Fig. 6, 1F3F3 trapped the label present in the peak with homotrimeric mTNF (maximum around fraction 40) more efficiently than that in the peak with monomeric mTNF (maximum around fraction 60). TN3, on the other hand, trapped the label in the peak with monomeric TNF better than that in the trimeric peak. It is also obvious that TN3 cannot bind heterotrimeric TNF (Table I). 61E71 bound trimeric hTNF more efficiently than monomeric hTNF (data not shown).

Interaction of Heterotrimeric Complexes with mTNF-Rs—As the interaction between TNF and TNF-Rs determines its biological function, we studied the binding of heterotrimeric TNF with both mTNF-Rs. As expected, labeled hTNF only interacted with smTNF-RI in an in vitro receptor binding assay; this binding could be blocked by competition with unlabeled hTNF or mTNF. However, after preincubation with mTNF, a significant amount of labeled hTNF was retained also on smTNF-RII-coated wells (Fig. 7). Similar observations were made with mTNF75, a mutein of mTNF, which binds strongly to smTNF-RII but not to smTNF-RI (Fig. 8). Labeled mTNF75 was retained on smTNF-RII-coated wells, and this binding was inhibitable by unlabeled mTNF, whereas the mutein did not bind to smTNF-RI-coated wells. However, after prior incubation of labeled mutein with unlabeled mTNF, allowing the formation of heterotrimers, part of the label was retained on wells coated with smTNF-RI.

The dissociation of mTNF at subnanomolar concentrations is accompanied by a strong decrease in cytotoxicity to L929 cells (Table II). This decrease in mTNF activity was partially prevented by mTNF75, a mutein that is inactive in this assay (Table II). The mTNF75 mutein did not affect the biological activity of mTNF when no preincubation took place. These experiments demonstrate that the apparent receptor specificity of a TNF species can be lost because of incorporation of heterologous TNF subunits.

**DISCUSSION**

Bioactive TNF trimers, capable of clustering three TNF-R molecules on the cell surface, dissociate to inactive monomers during incubation at subnanomolar concentrations or by treatment with particular reagents, such as Triton X-100 or guanidinium chloride. This dissociation was shown to be reversible by enhancing the TNF concentration or by removing the chemical agent (12, 14). In the present report we studied TNF stability during coincubation of different types of TNF and the influence of coincubation on the interaction with monoclonal antibodies and TNF-Rs. Using size-exclusion chromatography we showed that the appearance of labeled mTNF monomers was not only prevented by the presence of excess unlabeled mTNF but also by hTNF, whose amino acid sequence is 79%
identical to mTNF. It may be noted that hTNF and mTNF form trimeric complexes with a similar three-dimensional structure, although detailed differences have been observed (15, 16). Excess hLT-α, which is 32% identical to mTNF, did not mask this dissociation, indicating that the effect was specific and not because of a change in overall protein concentration. Intriguingly, coincubation of mTNF and hTNF at micromolar concentration resulted in the formation of two new TNF complexes. These could be discriminated from homotrimeric mTNF or hTNF by native PAGE and were recognized both with anti-mTNF and anti-hTNF antibodies. Finally, gel filtration clearly indicated that these complexes are trimeric. The experiments show that TNF trimer/monomer transitions occur both at subnanomolar and at micromolar concentrations. Because of these transitions TNF heterotrimeric molecules (A₂B and AB₂) are formed during coincubation of two different TNF species (A₃ and B₃). Undoubtedly, the relative abundance of the different forms depends on several parameters, such as the initial concentration of TNF species, reaction temperature, and coincubation time. RIA experiments with monoclonal antibodies specific for mTNF (1F3F3) or hTNF (61E71) yield evidence that these heterotrimeric TNF molecules still interact with specific anti-TNF antibodies, indicating that their (trimer) conformation is similar to that of homotrimeric TNF complexes.

To assess the binding of heterotrimeric TNF to mTNF-R, we preincubated unlabeled mTNF with labeled hTNF, which binds only to mTNF-RI, or with an mTNF75 mutein specific for mTNF-RII. We proceeded to measurement of the binding of label to immobilized smTNF-RI and smTNF-RII. In both situations label was retained by both types of receptor, indicating that the mTNF subunits present in heterotrimers still bind to both TNF-R types. These results imply that the receptor specificity and/or biological activity of a given mutein can be affected by coincubation with a different type of TNF. The resulting overall activity is then dependent on the specificities of the two partners. To rationalize this, we can consider the formation of TNF heterotrimers.

**Table I**

| Coating antibody | Labeled mTNF bound (% of total) | Labeled hTNF bound (% of total) |
|------------------|---------------------------------|---------------------------------|
| Labeled mTNF preincubated in PBS/BSA | With hTNF | With mTNF |
| TN3 | 46.5 ± 0.5 | 21.1 ± 0.1 |
| 1F3F3 | 41.1 ± 1.8 | 30.5 ± 0.4 |
| 61E71 | 0.3 ± 0.1 | 13.8 ± 2.6 |

1²⁵I-TNF (200 pM) was incubated for 24 h at 26 °C with or without unlabeled TNF (1 nM). Subsequently, the samples were diluted 1:5 in PBS/BSA and applied to wells coated with anti-mTNF monoclonal antibodies (TN3 and 1F3F3) and anti-hTNF antibody (61E71). After 90 min at 26 °C, the wells were washed with PBS/BSA, and the remaining label in the individual wells was counted in a γ-counter. Results (bound label/applied label × 100) are means ± S.D. of three experiments.

**Fig. 4.** Size-exclusion chromatography of TNF complexes after coincubation. A, mTNF and hTNF (both 20 μg/100 μl) were coincubated for 16 h at 4 °C and applied to a Superdex 75 fast protein liquid chromatography column. Protein elution was followed by measuring the absorption at 280 nm, and 1-min fractions were collected (white circles). B, the column was calibrated with the following protein standards (black circles): bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa; ribonuclease A, 13.7 kDa. The elution time for blue dextran 2000, viz. 2,000 kDa, is indicated by a black diamond. Elution of mTNF (white triangle) and hTNF (upside down white triangle) is also indicated. C, aliquots (10 μl) of peak fractions 19–23 (from A) were subjected to native 10% PAGE followed by Western blot immunodetection with anti-hTNF antibodies. TNF complexes are as follows: h, homotrimeric hTNF; hm and mh, heterotrimeric TNF.

**Fig. 5.** Effect of TNF concentration on the formation of heterotrimeric TNF. 1²⁵I-hTNF (200 pM) was preincubated with different concentrations of unlabeled mTNF (squares) or hTNF (circles) for 24 h at 26 °C. The samples were diluted 1:5 and incubated for 90 min on wells coated with anti-hTNF antibody 61E71 (black symbols) or anti-mTNF antibody 1F3F3 (white symbols). The wells were washed, and bound labeled TNF was counted (RIA).
of heterotrimers between mTNF and mTNF75 (the latter does not bind to mTNF-RI and is therefore inactive in L929 assays). As the two mutations in mutein mTNF75 are oriented toward the same intersubunit groove, the heterotrimERIC molecule (mTNF75/mTNF) should have two wild-type intersubunit grooves and one mutated groove. It may be assumed that such a heterotrimeric molecule can bind three TNF-RII molecules or two TNF-RI molecules. Transfection studies with chimeric erythropoietin receptor (extracellular)/TNF-R (intracellular) constructs, which become dimerized after binding of erythropoietin, indicated that dimerization is sufficient to induce signal transduction (22). However, studies with chimeric erythropoietin receptor/TNF-RII constructs showed that signaling after dimerization of TNF-RII (intracellular) is less efficient than after trimerization (23). The fact that mTNF75 only partially restored mTNF bioactivity might have two explanations. First, part of the heterotrimers will have the mTNF75/mTNF conformation and hence be unable to induce clustering of TNF-RI. Second, it can be assumed that clustering/dimerization of TNF-RI after binding of an mTNF75/mTNF2 molecule will be less efficient in signal induction than trimerization with mTNF. The observation that mTNF75 partially prevents the decrease in mTNF bioactivity after preincubation confirms that heterotrimerization with this mutein can affect the overall bioactivity. This conclusion is also relevant to some in vivo experiments. Because endogenous TNF is induced after a single administration to monkeys of a TNF-RI-specific hTNF mutein (24), endogenous TNF might also be induced by repeated administration of hTNF needed to obtain tolerization or to treat tumor-bearing mice (25, 26). It is likely that in such

| mTNF | mTNF75 | Preincubation | Biological activity |
|------|--------|---------------|---------------------|
| 180  | –      | –             | 11550 ± 610         |
| 180  | 200    | –             | 11100 ± 300         |
| 180  | 600    | –             | 11850 ± 210         |
| 180  | 200    | 24            | 210 ± 10            |
| 180  | 600    | 24            | 550 ± 60            |
| 180  | 600    | 24            | 800 ± 280           |

mTNF was serially diluted in Dulbecco’s modified Eagle’s medium/ FCS, with or without mTNF75, and directly or after a 24-h preincubation at 37 °C, 100 μl was added to wells containing L929 cells. After an 18-h incubation in the presence of actinomycin D, surviving cells were measured by MTT staining. The biological activity of the samples is represented in units/ml, 1 unit/ml being the TNF concentration at which half of the cells are killed. Results are means ± S.D. of three experiments.

FIG. 6. Specificity of anti-mTNF monoclonal antibodies. 125I-mTNF (1.6 nM) was chromatographed on a Sephacryl S100 column as such (black circles) after overnight incubation in 0.1% Triton X-100 (white circles) or after 1-h incubation in 3 M guanidinium chloride (black triangles). Fractions were counted in a γ-counter (A), and 100 μl of each fraction was tested in IF3F3-RIA (B) or TN3-RIA (C).

FIG. 7. Binding of labeled hTNF to smTNF-Rs. 125I-hTNF (400 pM) was incubated with unlabeled mTNF (black symbols) or hTNF (white symbols) for 0 (circles) or 24 h (triangles) and was subsequently allowed to bind on wells coated with smTNF-RI (A) or smTNF-RII (B). Bound label was measured in a γ-counter.

FIG. 8. Binding of labeled mTNF75 mutein to smTNF-Rs. 125I-mTNF75 (400 pM) was incubated with mTNF for 24 h and subsequently applied to wells coated with smTNF-RI (white circles) or smTNF-RII (black triangles). Bound label was measured in a γ-counter.
vivo situations monomer exchange occurs between administered and endogenous TNF. The effect of such exchanges on TNF pharmacokinetics or overall biological activity deserves further study.

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