Efficient Purification of Recombinant Human Tumor Necrosis Factor β from Escherichia coli Yields Biologically Active Protein with a Trimeric Structure That Binds to Both Tumor Necrosis Factor Receptors*

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A fast and efficient method for medium scale purification of recombinant human tumor necrosis factor β (rTNF-β) from Escherichia coli cells is described. The purified rTNF-β displayed biological activity similar to rTNF-α in a WEHI 164 cell cytotoxicity assay. The titration curve of rTNF-β and elution profiles of rTNF-β in gel filtration experiments were different from those of rTNF-α. However, light scattering and ultracentrifugation studies showed that both cytokines have trimeric structures in solution at 0.5 mg/ml, with minor differences in the distribution of nontropic species. rTNF-β bound to purified 55- and 75-kDa TNF receptors with high affinity. The binding of rTNF-β to either receptor was analyzed on Scatchard plots and compared with that of rTNF-α.

Tumor necrosis factor β (TNF-β) or lymphotoxin is produced by lymphocytes and has an amino acid sequence that is about 30% homologous to tumor necrosis factor α (TNF-α or cachectin) (1, 2). Both factors have been cloned and expressed in Escherichia coli (1, 3). The molecular masses, as calculated from the primary amino acid sequences, are 17.4 kDa for TNF-α and 18.7 kDa for TNF-β. Natural TNF-α is not glycosylated and contains one internal disulfide bridge. Biologically active rTNF-β can be purified from E. coli (4) or from yeast using a high level expression system (5). Natural TNF-β is glycosylated, and its primary sequence contains no cysteines (2, 3). Its purification from natural sources requires several chromatographic steps and preparative SDS-PAGE and yields only low amounts of protein (2, 6).

The quaternary structure of TNF-α was extensively studied by gel filtration (7, 8), cross-linking (9), analytical ultracentrifugation (10, 11), laser light scattering (8), and x-ray diffraction (12-14). In most studies, TNF-α was found to be trimeric in solution; x-ray scattering also revealed a trimeric association in crystals. The biologically active form of TNF-α is considered to be either the trimer (15) or the dimer (16).

Far less data are available concerning the quaternary structure of TNF-β. A trimeric structure for TNF-β was postulated based on atomic coordinates of the TNF-α structure and by comparing stretches of regions of homologous sequence (14, 17). Data obtained from gel filtration experiments also suggested a trimeric structure (6). However, the molecular mass is only one of several parameters that influences the retention of a protein on a gel filtration column, and therefore, validation by independent methods is needed.

The biological functions of TNFs are mediated by specific receptors on the cell surface. The purification of a 55- and a 75-kDa TNF receptor from HL-60 cells has been published (18). Aggarwal et al. (19) showed that the binding of labeled TNF-α to receptors on human cervical carcinoma cells can be competed with unlabeled TNF-α as well as with unlabeled TNF-β. They concluded that either both factors bind to the same receptor site or that they bind to different sites in close proximity in a way that the binding of one factor is hindered when the second factor is present (20). To date, it is unclear whether the two ligands, which are quite different with respect to their amino acid sequences, have a similar quaternary structure and whether they bind to the same site or sites on the cell surface.

Here we describe an efficient method for medium scale purification of biologically active rTNF-β from E. coli cells. The quaternary structure of the purified rTNF-β in solution was compared with that of rTNF-α, and the binding of rTNF-β to purified 55- and 75-kDa TNF receptors was evaluated and quantitatively analyzed.

MATERIALS AND METHODS

rTNF-α, purified from E. coli by DEAE-Sepharose chromatography and gel filtration chromatography, was kindly provided by Dr. E. Hochuli and U. Rütlin (both Microbiology Department, Hoffmann-La Roche, Basel). rTNFs were labeled with Na125I (Amersham Corp.) and Iodo-Gen (Pierce Chemical Co.) to a specific activity of 0.3-1.0 × 106 cpm/µg as described (21). Rabbit anti-human lymphotoxin antibody (code EP-600) was purchased from Genzyme Corp., Boston. Dialysis tubes were from Spectrum Medical, Los Angeles (Spectra Por 6; molecular weight cutoff, 1000), and standard proteins were from Bio-Rad. DNase I (code 18025) was from Serva, Heidelberg. Fetal bovine serum was obtained from Amimed, Basel, and RPMI 1640 medium, nonessential amino acids, penicillin, and streptomycin were from Gibco (Paisley, Scotland). The protease inhibitors benzamidine hydrochloride, o-phenanthroline, and phenylmethylsulfonyl fluoride were from Fluka AG, Buchs; Trasylol (aprotinin) was purchased from Bayer AG (Leverkusen, Germany). All chemicals were of analytical grade.

Expression of TNF-α and TNF-β in E. coli—A genomic DNA clone containing the human TNF-α and TNF-β loci (22) had been isolated for other purposes. The TNF-α and TNF-β expression cassettes were

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† The abbreviations used are: TNF, tumor necrosis factor; rTNF, recombinant human tumor necrosis factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

‡ M. Brockhaus and U. Rütlin, unpublished data.
obtained from this clone by a combination of site-directed mutagenesis (to remove intron 3) and DNA synthesis (to replace the coding sequence of the N-terminal fragment of the mature proteins).

For TNF-β, two BamHI restriction sites were introduced by site-directed mutagenesis at the exon-intron junctions flanking intron 3. Digestion with BamHI and religation removed intron 3 without a change in the amino acid sequence. The resulting fragment was then cleaved with PvuII, which cuts within codon 10 of the mature TNF-β coding sequence. A synthetic oligonucleotide encoding amino acids 1-9 of the mature protein preceded by an ATG initiation codon contained in an SpII fragment ligated to the PvuII site. The EcoRI site was introduced by transducing the entire fragment containing the entire mature TNF-β coding sequence plus 165-base pair 3'-nontranslated sequence was cloned into the expression vector pDS78/RBSII, SpII (23).

Similarly, for TNF-α, the intron 3 was removed by introduction of two NruI sites flanking the intron followed by digestion with NruI and religation. The N-terminal coding sequence was obtained by cleavage with AcOCl, which cuts between codons 7 and 8, and replacement of codons 1-7 with a double-stranded oligonucleotide that also provided the ATG initiation codon contained in an SpII fragment. The coding sequence of mature TNF-α plus 130-base pair 3'-nontranslated sequence was cloned into the expression vector pDS78/RBSII, SpII as above.

The coding sequences of both the TNF-α and the TNF-β expression cassettes were verified by sequence analysis. E. coli M15 host cells containing the lac repressor expression plasmid pDS78/RBSII were transformed with the described constructs and grown up in 100-liter fermenters using standard procedures.

Extraction of E. coli Cells—After expression of TNF-β, about 60 g of wet E. coli pellet obtained from 10-liter fermentation broth was dissolved by incubation in the desired buffer on ice. About 5 mg/ml were investigated with the laser light scattering system ALV-3000 (ALV Laservertriebsgesellschaft, Langen, FRG) after extensive dialysis against a buffer containing 10 mM NaCl, (pH 7.0), and 100 mM NaCl. Samples of 300 µl were filtered through 0.2 µm Nucleopore membrane filters (Sterico, Dietlikon, Switzerland) in a 2 ml polycarbonate cuvette. The light-scattering intensity was measured in a temperature-controlled cuvette bath (20.0 °C), and the scattering was observed at 90°. Correlation functions obtained with the ALV correlator/structuretator were acquired under computer control. Two methods of data analysis were used. The first, in the observed second order correlation function was fitted by nonlinear regression to one or two exponentials with free background adjustment. Solutions of rTNF-α were found to be satisfactorily fitted by a single exponential, directly yielding the diffusion coefficient and the hydrodynamic radius. For rTNF-β, fitting with two exponentials was necessary, yielding two diffusion coefficients and two radii corresponding to smaller and larger species of particulate matter. In the second method, the correlation function was analyzed with the program CONTIN (27). This analysis yields a distribution of diffusion coefficients and radii from distinct particle species in the sample.

Sedimentation velocity runs were carried out in a An-D rotor at 56,000 rpm, 21 °C using a 12-mm double sector Epon cell. For sedimentation equilibrium runs, the An-F rotor was used at 15,000 rpm, and the same cell was used. The results are given in Table II. The cells were filled with 0.11 ml of solution in each sector.

Relative mole masses were calculated from the observed sedimentation velocities and the diffusion constants, as obtained from light scattering, by the Svedberg equation, assuming a partial specific volume of 0.73 g/ml. Alternatively, the molecular masses were ob-

**Quaternary Structure of Recombinant TNF-β**

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RESULTS

Purification of Bioactive Recombinant TNF-β from E. coli Cells—E. coli cells were harvested and extracted in the presence or absence of different detergents at various pH (data not shown) to optimize extraction conditions. As tested by SDS-PAGE and Western blotting analyses (Fig. 1), undergraded rTNF-β was extractable by sonication of cells in Tris-HCl buffer (pH 8.5) in the absence of detergent. This extract competed with the binding of radiolabeled rTNF-α to a TNF receptor preparation semipurified from human placenta as shown in Fig. 2. After high speed centrifugation, the rTNF-β-containing supernatant was fractionated using a DEAE-Sepharose fast flow column. At pH 8.5, rTNF-β did not bind to this column but had a higher retention time than most nonadsorbed E. coli proteins. Therefore, it was enriched to a remarkable degree in a late peak of the isocratic elution (Fig. 3A). This peak was pooled and directly applied to an S-Sepharose fast flow column. rTNF-β eluted from this column around 200 mM NaCl and was only slightly contaminated with E. coli proteins as shown in Fig. 3B. rTNF-β was pooled and further fractionated by ammonium sulfate precipitation at 20% relative saturation. The final precipitate appeared homogeneous by SDS-PAGE (Fig. 4).

Ammonium sulfate-precipitated rTNF-β could be dissolved in 20 mM HEPES buffer (pH 7.2) on ice at concentrations of up to 10 mg/ml; higher concentrations of protein were not tested. It was found that the solubility of rTNF-β increased with decreasing temperature.

The procedure described above yielded about 10 mg of bioactive rTNF-β from 10 liters of E. coli fermentation broth. Amino acid analysis of the final product (data not shown) coincided with the sequence published by Nedwin et al. (29). The molar extinction coefficient, ε, for rTNF-β at 278 nm was determined as 2.76 × 10⁷ cm²/mol (rTNF-α, 2.41 × 10⁷ cm²/mol).

Table I shows a comparison of biological activities of rTNF-α and rTNF-β as determined in the WEHI 164 cell assay relative to a recombinant TNF-α reference preparation. It is evident that the specific biological activity of rTNF-β was similar to that of rTNF-α.

Titration Curves—Fig. 5A shows titration curves obtained with a mixture of rTNF-α and rTNF-β. For comparison, theoretical titration curves between pH 3 and 10 calculated from the known amino acid sequences are shown in Fig. 5B. From a separate isoelectric focusing experiment (data not shown), the apparent isoelectric point of rTNF-β was determined as 8.3.

Analytical Gel Filtration—rTNF-α was applied to a TSK G-2000 SW column at pH 7. In the concentration range between 0.01 and 0.5 mg/ml, a sharp peak was observed (Fig. 6A) with a retention time corresponding to a molecular mass of about 34 kDa, the theoretical molecular mass of the dimer. Under the same conditions, rTNF-β eluted as a broad peak with an unexpectedly high retention time, corresponding to a molecular mass lower than that of the monomer (the chromatogram is not shown).

From a Superose 12 column at pH 7, rTNF-α again eluted as a narrow peak corresponding to a molecular mass of 34 kDa. rTNF-β eluted as a broad peak at about 19 kDa (Fig. 6C). However, increasing the column pH from 7.0 to 8.5 or increasing the NaCl concentration from 100 to 200 mM resulted in peak sharpening and a slightly reduced retention time (data not shown).

Laser Light Scattering and Ultracentrifugation—Measurements of the molecular masses of rTNF-α and -β by light scattering and ultracentrifugation gave similar results for both proteins and are presented in Table II. Based on sedimentation equilibrium, rTNF-α appears to have a molecular mass of 49 kDa, with an indication of the presence of some lower molecular mass material. This number lies between the theoretical trimer (52 kDa) and dimer (35 kDa) values and can

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*H. Loetscher, unpublished data.*
Fig. 3. Elution profiles and analytical SDS-PAGE of the DEAE-Sepharose step (A) and the S-Sepharose step (B). A, DEAE-Sepharose chromatography. The column was eluted isocratically as described under "Materials and Methods." The following samples were applied to the SDS gel: lane 1, mixture of standard proteins as listed in Fig. 1; lane 2, supernatant of E. coli extract (starting material); lanes 3-5, fractions as indicated in the chromatogram; lane 6, material bound to the DEAE column and eluted with 1 M NaCl. B, S-Sepharose chromatography. The column was eluted with a salt gradient as indicated. The following samples were applied to the SDS gel: lane 1, standard proteins as above; lane 2, pool as indicated in A; lane 3, flow-through of S-Sepharose column; lanes 4-7, fractions as indicated in the chromatogram.

Fig. 4. The pooled TNF-β fractions from the S-Sepharose column have been subjected to ammonium sulfate fractionation and then analyzed by SDS-PAGE. Lane 1, mixture of standard proteins (see Fig. 1); lane 2, rTNF-β pool from S-Sepharose column as indicated in Fig. 3B; lane 3, rTNF-β after ammonium sulfate precipitation at 20% relative saturation.

Table I
Comparison of specific bioactivities of various TNF preparations relative to a TNF-α reference preparation as described under "Materials and Methods"

| Preparation        | Specific activity relative to preparation 1
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>TNF-α (preparation 1)*</td>
<td>1.0</td>
</tr>
<tr>
<td>TNF-α (preparation 2)</td>
<td>0.7</td>
</tr>
<tr>
<td>TNF-α (preparation 3)</td>
<td>1.8</td>
</tr>
<tr>
<td>TNF-β (preparation 1)</td>
<td>0.7</td>
</tr>
<tr>
<td>TNF-β (preparation 2)</td>
<td>0.7</td>
</tr>
<tr>
<td>TNF-β (preparation 3)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Reference preparation.

be explained by the assumption of a mixture containing 80% trimer and 20% dimer. rTNF-β is found with 56 kDa and the manifest presence of lower molecular mass material. The 56-kDa value corresponds to the theoretical molecular mass of the trimer, but since the lower molecular mass material contributes more to the absorption profile, we conclude that there is less trimer in solution as compared to rTNF-α.

While the correlation functions for rTNF-α obtained from quasielastic light scattering experiments can be fitted satisfactorily to a single exponential, those of rTNF-β appeared to correspond to a more polydisperse sample and were therefore fitted to two exponentials. Combined with the observed sedimentation velocities (3.60 and 3.54 S) we obtained 48 and 46 kDa for the molecular masses of rTNF-α and -β, respectively. The necessity for a two-exponential fit in the case of rTNF-β seems to be due to the presence of some larger aggregates (about 80 kDa), possibly representing a minor fraction of unfolded protein.

As a control, the light scattering correlograms of both proteins were analyzed in terms of a continuous distribution of exponentials (program CONTIN). Due to the presence of minute quantities of heavier particles discussed above, these distributions extend to higher molecular masses than the trimer. In addition, the distribution of rTNF-β was broader than that of rTNF-α. The smallest particles detected by this analysis were the dimers for rTNF-α and the monomers for rTNF-β.

Binding of rTNF-α and -β to Isolated 55- and 75-kDa TNF Receptors—Two distinct receptors with molecular masses of 75 and 55 kDa were isolated from HL-60 cells (25). Aliquots of each receptor were blotted to nitrocellulose mem-
Our goal was the development of a purification method that
would not destroy the quaternary structure of TNF-β and
di rectly applied to the S-Sepharose column. The
crucible supernatant of the
easily upscaled. The
was applied to a DEAE-Sepharose column under
conditions where the partition coefficient of rTNF-β is between 0 and 1.
though excellent purification was achieved (data not shown).
Our results provide an efficient purification procedure that
uses only two ion exchange chromatographies and can be
easily upscaled. The crude supernatant of the E. coli extract
was applied to a DEAE-Sepharose column under conditions
where the partition coefficient of rTNF-β is between 0 and 1.
Therefore, rTNF-β was elutable with an isocratic buffer and
could be directly applied to the S-Sepharose column. The
resolution of the DEAE column was optimized by decreasing the
applied sample volume and increasing the ratio of column
length to diameter.

rTNF-β purified according to the described protocol was
not fragmented as verified by SDS-PAGE and amino acid
analysis, displayed similar cytotoxic activity as rTNF-α in the
WEHI 164 cell assay, and was highly soluble in different
nondenaturing buffers.

The difference in amino acid composition of both proteins
was reflected by distinct titration curves. Aggarwal et al. (6)
reported that native TNF-β purified from lymphoblastoid
were monitored after each purification step in the WEHI 164
cytotoxicity cell assay. In addition, we evaluated the capacity of
different rTNF-β preparations to compete with the binding of
rTNF-α to purified TNF receptors from human placenta
in a dot blot assay. Results from those analyses showed that
the native structure of rTNF-β was irreversibly destroyed by
reversed phase fast protein liquid chromatography, even
though excellent purification was achieved (data not shown).

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uses only two ion exchange chromatographies and can be
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where the partition coefficient of rTNF-β is between 0 and 1.
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**Table II**

<table>
<thead>
<tr>
<th>Technique</th>
<th>rTNF-α</th>
<th>rTNF-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation equilibrium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16,000 rpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mg/ml</td>
<td>49, 36*</td>
<td>57, 45*</td>
</tr>
<tr>
<td>0.2 mg/ml</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>24,000 rpm</td>
<td>48, 35</td>
<td>56, 36</td>
</tr>
<tr>
<td>0.5 mg/ml</td>
<td>ND</td>
<td>54, 48</td>
</tr>
<tr>
<td>13,000 rpm, 0.5 mg/ml</td>
<td>49</td>
<td>46, 79</td>
</tr>
<tr>
<td>Sedimentation velocity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56,000 rpm, 0.5 mg/ml</td>
<td>3.60</td>
<td>3.54</td>
</tr>
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</table>

**Distribution (CONTIN)***

<table>
<thead>
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<th></th>
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<tbody>
<tr>
<td>Dimer</td>
<td>23%</td>
</tr>
<tr>
<td>Trimer</td>
<td>39%</td>
</tr>
<tr>
<td>Tetramer</td>
<td>27%</td>
</tr>
<tr>
<td>Pentamer</td>
<td>9%</td>
</tr>
<tr>
<td>6-10-mer</td>
<td>2%</td>
</tr>
<tr>
<td>10-20-mer</td>
<td>1%</td>
</tr>
</tbody>
</table>

*For two components, the value of the main component is underlined. The second value corresponds to a minor correction necessary for curve fitting; the existence of corresponding particle species in the sample is not implied.

*ND, not done.

*R_h, hydrodynamic radius.

*Weight percentages for oligomers are calculated assuming that all molecules are globular.
cells binds to DEAE-cellulose at pH 7.8 and had an isoelectric point at pH 5.8. Hains and Aggarwal (34) observed an isoelectric point of 6.85 for rTNF-P expressed by a mammalian cell line. Our preparation of TNF-P from *E. coli* had an apparent isoelectric point of 8.3. This value approaches the theoretical value of 9.3 as calculated from the primary amino acid sequence (Fig. 5B). The higher isoelectric point, relative to isoelectric points of material expressed in eukaryotic systems, may be explained by the lack of some posttranslational modifications of the material expressed in *E. coli*.

In the second part of this study, we investigated whether the quaternary structures of rTNF-α and rTNF-β are as different as their electrochemical properties.

First we compared the retention times of both proteins on a silica-based (TSK G-2000 SW) and a polymer-based (Superose 12) gel filtration column. On both columns, rTNF-α eluted at retention times as expected for its dimeric form. However, the retention of rTNF-β was dependent on the type of column and the pH. Other laboratories have reported varying molecular masses for TNF-α in solution as determined by gel filtration chromatography. Davis et al. (4) found elution at 46 kDa on a Sephadex G-75 column at pH 8.5. Smith and Baglioni (15) reported a 55-kDa (trimer) and a minor 17-kDa (monomer) peak using Sephadex G-75 chromatography in phosphate-buffered saline, whereas Petersen et al. (16) observed a major 33-kDa (dimer) peak from a Sephadex G-200 column. Narhi and Arakawa (7) described pH- and concentration-dependent elution profiles for rTNF-α on a Bio-Sil TSK 250 column. We interpret these distinct observations as indicating that pH-dependent hydrophobic or ionic interactions with the different column supports have a major influence on elution profiles of rTNFs. As a consequence, it is not possible to calculate realistic molecular masses for TNF-β from such gel filtration experiments. However, our comparative studies show that there are also significant differences in the elution profiles of rTNF-α and rTNF-β, even when chromatographed under identical conditions. These differences might reflect distinct hydrophobic or ionic properties of the proteins' surfaces, molecular shapes, or degrees of association.

Laser light scattering and ultracentrifugation measurements clearly indicated similar molecular masses for rTNF-α and -β as shown in Table II. At the concentration used in
these experiments (0.5 mg/ml) the main species in solution was the trimer, coexisting with about 20% dimers in the case of rTNF-α and about 50% dimers and monomers for rTNF-β. This result may imply that trimer formation is a dynamic process and, with decreasing protein concentration, the trimer decays to dimers and monomers, with a corresponding shift of the mean molecular mass. The concentration dependency was not studied by light scattering. However, sedimentation equilibrium measurements were performed for two concentrations of rTNF-β (0.5 and 0.2 mg/ml, Table II), and no putative shift of the mean molecular mass was detected.

Recently, the purification of two different TNF receptors from HL-60 cells was described (25). Monoclonal antibodies against both types of TNF receptors inhibit the binding of rTNF-α and rTNF-β to the cell surface to similar extents (35). We asked whether one or the other receptor type would preferentially bind rTNF-α or rTNF-β. This does not appear to be the case. Thus, both ligands competed with similar efficiency to either purified receptor (see Fig. 7). Dissociation constants between $1 \times 10^{11}$ and $16 \times 10^{11}$ mol/liter were obtained from saturation experiments with all four ligand and receptor combinations. Despite the fact that our $K_d$ values were derived from purified receptors immobilized on nitrocellulose, they are in good agreement with data obtained from TNF-binding studies on intact cells (19, 36–40). This indicates that the conformations of the binding sites were well preserved during the isolation and purification of the receptors from the cell membranes. Linear Scatchard plots suggested a one-site binding model in all four cases. Measurements at low ligand concentrations (<20 pmol/liter; 0.4 mg/ml) revealed in three cases (rTNF-α with 75-kDa receptor and rTNF-β with both receptors) systematically lower binding of radiolabeled ligand than theoretical values calculated from our dissociation constants (data not shown). This could be explained by the occupation of binding sites with monomers at these concentrations.

Recently, COS cells expressing a recombinant TNF receptor that is identical with the 75-kDa receptor with respect to its amino acid sequence (41) were reported to have two binding sites for each TNF (42). These data, however, can be explained by the presence of the endogenous COS cell TNF receptor of the 55-kDa type (43).

In conclusion, our studies revealed no major differences between rTNF-α and rTNF-β with respect to biological activity, self-association, and receptor binding despite different physicochemical properties. However, these similarities do not exclude the possibility that postreceptor events of signal transduction are specific for one or the other ligand.

Acknowledgments—We are grateful to Dr. B. Wipf for providing E. coli fermentation broth, to Dr. E. Hochuli and U. Ettlin for supplying recombinant TNF-α, to Dr. M. Brockhaus for radiolabeling of rTNF-α and rTNF-β, and to Drs. M. Brockhaus, H. Ettinger, W. Lesslauer, and M. Steinmetz for careful reading of the manuscript and for helpful discussions.