The extracellular domain of the 55-kDa TNF receptor (rsTNFRβ) has been expressed as a secreted protein in baculovirus-infected insect cells and Chinese hamster ovary (CHO) dhfr- cells. A chimeric fusion protein (rsTNFRβ-hy3) constructed by inserting the extracellular part of the receptor in front of the hinge region of the human IgG Cy3 chain has been expressed in mouse myeloma cells. The recombinant receptor proteins were purified from transfected cell culture supernatants by TNFa- or protein G affinity chromatography and gel filtration. In a solid phase binding assay rsTNFRβ was found to bind TNFa with high affinity comparable with the membrane-bound full-length receptor. The affinity for TNFa was slightly impaired. However, the bivalent rsTNFRβ-hy3 fusion protein bound both ligands with a significantly higher affinity than monovalent rsTNFRβ reflecting most likely an increased avidity of the bivalent construct. A molecular mass of about 140 kDa for both rsTNFRβ-TNFa and rsTNFRβ-TNFβ complexes was determined in analytical ultracentrifugation studies strongly suggesting a stoichiometry of three rsTNFRβ molecules bound to one TNFa or TNFβ trimer. Sedimentation velocity and quasielastic light scattering measurements indicated an extended structure for rsTNFRβ and its TNFa and TNFβ complexes. Multiple receptor binding sites on TNFa trimers could also be demonstrated by a TNFa-induced agglutination of Latex beads coated with the rsTNFRβ-hy3 fusion protein. Both rsTNFRβ and rsTNFRβ-hy3 were found to inhibit binding of TNFa and TNFβ to native 55- and 75-kDa TNF receptors and to prevent TNFa and TNFβ bioactivity in a cellular cytotoxicity assay. Concentrations of rsTNFRβ-hy3 equimolar to TNFa were sufficient to neutralize TNF activity almost completely, whereas a 10-100-fold excess of rsTNFRβ was needed for similar inhibitory effects. In view of their potential TNF antagonizing activity, recombinant soluble TNF receptor fragments might be useful as therapeutic agents in TNF-mediated disorders.

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The abbreviations used are: TNF, tumor necrosis factor; TNFRα, 75-kDa TNF receptor; TNFRβ, 55-kDa TNF receptor; rsTNFRβ, recombinant soluble TNFRβ; rsTNFRβ-hy3, recombinant soluble TNFRβ-human IgG Cy3 fusion protein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary.
In the present work a recombinant soluble form of the 55-kDa TNF receptor (rsTNFR8) was produced in high yields in different eukaryotic expression systems. The rsTNFR8 was also expressed as a human IgG Cy3 fusion protein (rsTNFR8-hy3) in myeloma cells. The recombinant receptor molecules were found to bind stoichiometrically to TNFα and TNFβ trimers and to neutralize TNF bioactivity in different assay systems.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—The Spodoptera frugiperda (Sf9) cell line was obtained from American Type Culture Collection (ATCC CRL 1711). The baculovirus Autographa californica (AcNPV) virus was obtained from M. Summers, Texas A & M University, the Chinese hamster ovary (CHO)/dhfr cell line from P. Familletti, Hoffmann-LaRoche Ltd., Nutley, NJ, and the WEHI164 (clone 2A3) cell line from J. R. Frey (51). The mouse myeloma cell line J558L was kindly provided by A. Traunecker, Basel Institute of Immunology. The expression vector used to construct the rsTNFR8-hy3 fusion protein was modified from a CD4-immunoglobulin construct obtained from K. Karjalainen and A. Traunecker (44). Recombinant human TNFα and TNFβ and mouse TNFα produced in Escherichia coli were kindly provided by R. Famini and M. Caravatti (Hoffmann-LaRoche Ltd., Basel, Diagnostic Division).

**Construction of Vectors, Expression, and Purification**—The cDNA encoding the extracellular domain of TNFRα, including the signal peptide, was amplified by the polymerase chain reaction. Unique restriction sites were introduced at both ends of the fragment. In addition, a translational stop codon was introduced behind the last amino acid of the extracellular domain (Thr51*, numbering according to Ref. 27). The engineered fragment was cloned into an expression vector. The engineered construct was subjected to gel filtration chromatography on TSK3000SW or Superose 12 (Pharmacia) columns with PBS as solvent. The amount of protein was determined by amino acid analysis or BCA assay (Pierce). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (47) using the mini-gel system of Bio-Rad.

**Binding Assay and Scatchard Analysis**—A 96-well microtiter plate coated with the TNFRβ-specific non-neutralizing monoclonal antibody htr-20 (25) was incubated with 10 ng/ml rsTNFR or rsTNFR-hy3 in 1% defatted milk powder for 3 h at room temperature. Under these conditions only about 10% of the total binding sites were occupied by the receptor protein as determined from a tritation curve (low density packing). In some experiments the antibody-coated plate was incubated with 3 µg/ml soluble receptor to saturate ad receptor binding sites (high or maximum density packing). After washing with PBS the wells were incubated with different concentrations of radiolabeled TNFα or TNFβ (1-250 ng/ml) in the presence or absence of a 200-fold excess unlabeled ligand for 16 h at 4 °C. The radioactivity bound to single wells was directly counted in a γ-counter. Nonspecific binding was subtracted. Kd values were determined from Scatchard plots.

**Quasielastic Light Scattering and Ultracentrifugation Analysis**—Quasielastic light scattering experiments were performed with the system ALV-300 (ALV Laservertriebs m.b.H., Langen, Germany). Samples of 300 µl were filtered through 0.2-µm filters in closed cylindrical quartz cells. The protein concentration was 0.5-1 mg/ml. For aggregation studies with the program CONTIN (48) that yields a distribution of relaxations. Mean values for the diffusion coefficient D were calculated assuming either an extended, i.e. rodlike (0 moment of the observed distribution) or roughly spherical structure (3rd moment of the particles).

**Competitive Inhibition of Ligand Binding to Native TNFα and TNFRα Holoreceptors**—1-2 ng of native TNFα and TNFRα purified from HL60 cells (26) were spotted to wetted nitrocellulose membranes. After blocking with a solution of 1% defatted milk powder, the membrane was incubated with human radiolabeled TNFα or TNFβ (1 pmol/ml) in the presence of different concentrations of rsTNFRα or rsTNFRβ-hy3 for 2 h at room temperature. The membrane was then thoroughly rinsed with PBS and counted in a γ-counter.

**RESULTS**

**Expression, Purification, and Ligand Binding Affinities of rsTNFRα/Sf9 insect cells infected with the recombinant baculovirus secreted 5-10 µg/ml of soluble receptor into the culture supernatant.**
pressed and secreted in mouse myeloma cells with a yield of concentrations. The TNFRP-hr3 fusion protein was expressed and secreted in mouse myeloma cells with a yield of about 0.5–1 μg/ml.

The recombinant soluble TNF receptors were purified by TNFα or protein G affinity chromatography and gel filtration. SDS-PAGE analysis revealed for the baculovirus expressed protein three to four discrete bands between 21 and 25 kDa. The TNFP-hr3 fusion protein was ex-amplification in the presence of increasing methotrexate concentrations. The TNFRP-hr3 fusion protein was expressed in CHO/dhfr− cells yielding two bands migrating on SDS gels at around 28 and 32 kDa. Sequence analysis of the glycosylated baculovirus-produced material revealed a single sequence starting with the N terminus of the naturally occurring TNFRP fragment reacting band in a ligand blot experiment (not shown). N-terminal sequence analysis of the glycosylated baculovirus-produced material revealed a single sequence starting at Asp+1 of the mature TNFRβ (not shown). rsTNFRβ produced in CHO/dhfr− cells yielded two bands migrating on SDS gels at around 28 and 32 kDa. Sequence analysis of this material confirmed the expected N terminus, but a second N-terminal sequence starting at Asp+12 was also present in a roughly 1:1 ratio. Interestingly, Asp+12 has previously been found to be the N terminus of the naturally occurring TNFRβ fragment (36). The TNFRβ-hr3 fusion protein was expressed as a disulfide-linked homodimer indicating an antibody-like structure of this molecule. As shown in Fig. 1 reduced samples of baculovirus- or CHO/dhfr− derived rsTNFRβ migrated at a slightly lower rate on SDS gels. This is most likely due to the high content of cysteines in these proteins. A similar observation has been made earlier with the native 55-kDa TNFRβ purified from HL60 cells (26).

The soluble receptor fragments produced in either expression system showed a high affinity for TNFα and a slightly lower affinity for TNFβ (Fig. 2). The difference in the apparent Kd values of rsTNFRβ for TNFα and TNFβ was most prominent with the CHO/dhfr− derived material. This finding is in contrast to the native cell surface-bound 55-kDa TNFRβ, which has been shown to bind both TNFα and TNFβ with about the same affinity, i.e. Kd values of 326 and 351 pm, respectively (24, 52)). Interestingly, fully deglycosylated rsTNFRβ as expressed in baculovirus-infected Sf9 cells in the presence of tunicamycin displayed similar binding characteristics as the glycosylated form (data not shown), confirming that the carbohydrate moieties are not essential for ligand binding (24, 26). The apparent affinity of the bivalent rsTNFRβ-hr3 fusion protein for TNFα and TNFβ was found to be significantly higher than the affinity of baculovirus- or CHO/dhfr− derived monovalent rsTNFRβ (Fig. 2). It is interesting to note that Kd values determined in the solid phase assay under low density packing conditions (see “Experimental Procedures”). The Kd values were determined from Scatchard analysis of the binding curves as indicated.

binding (24, 26). The apparent affinity of the bivalent rsTNFRβ-hr3 fusion protein for TNFα and TNFβ was found to be significantly higher than the affinity of baculovirus- or CHO/dhfr− derived monovalent rsTNFRβ (Fig. 2). It is interesting to note that Kd values determined in the solid phase assay under high receptor density conditions (see “Experimental Procedures”) were generally higher and did not show a marked difference in the apparent affinities between the fusion protein and rsTNFRβ (data not shown). It therefore appears that at maximum dense packing of the solid phase some interactions of receptor molecules leading to multiple valency and/or steric constrains cannot be excluded.

Stoichiometry of rsTNFRβ-TNFα and rsTNFRβ-TNFβ Complexes—rsTNFRβ purified from CHO/dhfr− cell culture medium was incubated with TNFα or TNFβ at different receptor to ligand molar ratios and fractionated according to size by gel filtration chromatography. The chromatographic conditions chosen allowed to separate receptor-ligand complexes from free receptor and free ligand. As shown in Fig. 3, at an approximate 1:1 molar ratio neither free receptor nor free TNFα or TNFβ could be detected in the elution profiles indicating that under these conditions complete complex formation had occurred. Amino acid composition analysis of the separated complexes evaluated by a recently described computer program (53) confirmed the 1:1 stoichiometry (not

![Fig. 1. SDS-PAGE analysis of purified rsTNFRβ and rsTNFRβ-hr3. Purified rsTNFRβ and rsTNFRβ-hr3 produced in different expression systems were separated by nonreducing and reducing SDS-PAGE and stained with Serva blue R. Expression systems: Bac., baculovirus-infected insect Sf9 cells; Bac.+Tun., baculovirus-infected insect cells grown in the presence of tunicamycin; CHO, CHO/dhfr− cells; J558L, mouse myeloma cells (expressing rsTNFRβ-hr3).](image-url)
shown). When the amount of TNFα added was gradually increased, a transition of the TNFRβ-TNFα complex toward a slightly lower molecular mass was observed in the elution profile (Fig. 3, left panel). In contrast, adding increasing amounts of TNFβ did not affect the elution behavior of the TNFRβ-TNFβ complex (Fig. 3, right panel).

To obtain a more accurate molecular mass estimate of rsTNFRβ and its complexes with TNFα and TNFβ, quasielastic light scattering and analytical ultracentrifugation studies were performed. The results are summarized in Table I. For rsTNFRβ a monomeric structure with a molecular mass of 25 kDa was found by sedimentation equilibrium analysis. The theoretical molecular mass for rsTNFRβ in its unglycosylated form is 20,467. Analysis of rsTNFRβ complexed to TNFα or TNFβ under conditions of complete complex formation (see above) yielded for both complexes a molecular mass of about 140 kDa. If a stoichiometry of three rsTNFRβ molecules bound to one 49-kDa TNFα or 57-kDa TNFβ trimers (9) is assumed, theoretical molecular masses of 124 and 132 kDa, respectively, are calculated which are in approximate agreement with the observed values. Sedimentation velocity analysis combined with quasielastic light scattering data confirmed the molecular masses observed in the equilibrium runs and were, in addition, indicative for a rather extended, i.e. rod-like structure of rsTNFRβ and its TNFα and TNFβ complexes.

**Inhibition of TNFα and TNFβ Binding by rsTNFRβ and rsTNFRβ-hy3**—rsTNFRβ and rsTNFRβ-hy3 were tested for their ability to competitively inhibit binding of TNFα and TNFβ to native TNFα and TNFRβ purified from HL60 cells. In this assay native highly purified receptors were spotted onto nitrocellulose membranes and incubated with 125I-TNFα or 125I-TNFβ in the presence of different concentrations of rsTNFRβ or rsTNFRβ-hy3. As shown in Fig. 4, A and C, binding of 125I-TNFα to both TNF receptors was blocked by rsTNFRβ and rsTNFRβ-hy3 in a concentration-dependent manner. It is interesting to note that a roughly equimolar concentration of the fusion protein was sufficient to prevent TNFα binding almost completely. rsTNFRβ was about 10–100 × less potent in inhibiting the binding. The binding of 125I-TNFβ was also inhibited (Fig. 4, B and D), but higher concentrations of rsTNFRβ and rsTNFRβ-hy3 were needed to achieve inhibitory effects comparable to TNFα. The 10–15% residual binding seen with iodinated TNFβ at high soluble receptor concentrations is due to nonspecific binding of radioactivity to the nitrocellulose filter.

The inhibition of TNF cytotoxicity by rsTNFRβ and rsTNFRβ-hy3 was tested in a cellular cytotoxicity assay using the 2A3 subclone of the murine fibrosarcoma cell line WEHI164 (51). As expected from the binding studies, rsTNFRβ-hy3 very efficiently inhibited TNF activity; at a concentration of 0.1 pmol/ml, i.e. equimolar to the TNFα concentration used in the assay, rsTNFRβ-hy3 prevented TNFα-induced cytolysis very efficiently (Fig. 5A). rsTNFRβ also had inhibitory activity but a concentration about 100-fold in excess of TNFα was needed for complete inhibition. TNFβ-induced cytotoxicity was also inhibited by the fusion protein, albeit not at equimolar concentrations. The protective effects of rsTNFRβ in these cytotoxicity assays were only evident at rather high concentrations (Fig. 5B).

**TNFα-induced Agglutination of rsTNFRβ-hy3-coated Latex Beads**—In view of the trimeric structure of TNFα and TNFβ, each capable of binding three recombinant soluble receptor molecules, it is very likely that these cytokines aggregate TNF receptors on the cell surface into microclusters which may be a necessary step in signal transduction. To mimic cell sur-

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**Table I**

Molecular mass determination of rsTNFRβ and its complexes with TNFα and TNFβ

| Molecular mass (kDa) | Sedimentation equilibrium | Sedimentation velocity | Putative stoichiometry |
|---------------------|---------------------------|------------------------|------------------------|
| rsTNFRβ             | 25                        | 20; 32                 | Monomer                |
| rsTNFRβ-TNFα complex| 140                       | 115; 156               | [TNFα]3 [TNFRβ]3       |
| rsTNFRβ-TNFβ complex| 140                       | 102; 139               | [TNFβ]3 [TNFRβ]3       |

*The 3rd and 0 moments of D were used in the calculation yielding molecular masses for a roughly spherical (listed first) and an extended (listed second) structure, respectively.*

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**Fig. 3.** Analysis of rsTNFRβ and its TNFα and TNFβ complexes by gel filtration chromatography. 1 nmol of rsTNFRβ purified from CHO/dhfr- cells was mixed with 0.5, 1, 1.5, 2, 3, and 5 nmol of TNFα or TNFβ in 0.1 ml of PBS. The amount of TNFα and TNFβ was calculated for the 17-kDa monomeric unit. The mixtures with the various receptor:ligand (RL) molar ratios were fractionated on a Superose 12 column (Pharmacia) in PBS. Numbers on top indicate the positions of molecular weight marker proteins (Bio-Rad). Left panel, TNFα complexes; right panel, TNFβ complexes.
A purified from HL60 cells was measured in a dot blot assay as described under "Experimental Procedures." The concentration of radiolabeled presence of increasing concentrations of rsTNFRB; closed circles, TNFRO-hy3. (The concentration of the rsTNFR0-hy3 homodimer hy3. to TNFRO; ligand binding in the presence of increasing concentrations of rs-

FIG. 4. Binding of 125I-TNFa and 125I-TNFβ to native TNFRβ and TNFRO: inhibition by rsTNFRβ and rsTNFRβ-hy3. Binding inhibition to native full-length TNFRβ and TNFRO purified from HL60 cells was measured in a dot blot assay as described under “Experimental Procedures.” The concentration of radiolabeled ligand in the assay was 1 pmol/ml. Open circles, ligand binding in the presence of increasing concentrations of rsTNFRB; closed circles, ligand binding in the presence of increasing concentrations of rsTNFRβ-hy3. (The concentration of the rsTNFRβ-hy3 homodimer was calculated for the 66-kDa monomer unit). A, 125I-TNFa binding to TNFRβ; B, 125I-TNFβ binding to TNFRβ; C, 125I-TNFa binding to TNFRO; D, 125I-TNFβ binding to TNFRO.

FIG. 5. Inhibition of TNFa- and TNFβ-induced cytotoxicity in WEHI164 cells. WEHI164 cells were cultured in the presence of 0.1 pmol/ml TNFa (A) or TNFβ (B) and different concentrations of rsTNFRβ (open circles) and rsTNFRβ-hy3 (closed circles). Cell viability was analyzed after 48 h at 37 °C.

face-bound TNF receptors, Latex beads were coated with rsTNFRβ-hy3 fusion protein and subsequently exposed to different concentrations of TNFa. TNFα induced an oligomerization of rsTNFRβ-hy3 as visualized by agglutination of the Latex beads (Fig. 6). A similar effect was seen with TNFβ, but agglutination was much less pronounced (results not shown).

DISCUSSION

In this study TNF binding and inhibiting properties of the extracellular region of the human TNFRβ were analyzed. The recombinant soluble receptors (rsTNFRβ and rsTNFRβ-hy3 fusion protein) expressed in different eukaryotic expression systems displayed high affinity binding to human TNFα similar to that of native cell surface-bound 55-kDa TNFRβ. In contrast, the binding affinity of rsTNFRβ for TNFβ was significantly decreased when compared with the native cell surface receptor. A similar observation, i.e. impaired neutralization of TNFβ versus TNFα, has also been made with a so-called TNF binding protein, which is a naturally occurring soluble receptor derived from TNFRβ (33, 36, 39). It therefore appears that with respect to ligand binding properties, rsTNFRβ closely resembles the natural TNF inhibitor. The apparent lower affinity of rsTNFRβ (and also of the detergent-solubilized holoreceptor (9)) for TNFβ might reflect a microenvironment of the ligand binding site which is slightly different from that of the cell surface-bound full-length TNF receptor. It is noteworthy that with respect to monovalent rsTNFRβ the rsTNFRβ-hy3 fusion protein binds both TNFα and TNFβ with a severalfold higher affinity when measured under appropriate assay conditions. This increase in affinity most probably reflects a higher avidity of the rsTNFRβ-hy3 construct due to its bivalency. Comparison of rsTNFRβ and the fusion protein to compete with native full-length TNF receptors for TNF binding and to protect WEHI 164 cells from TNF-induced cytotoxicity indeed confirmed the expected higher activity of the fusion protein.

The results from the ultracentrifugation analyses indicate that rsTNFRβ is monomeric in solution. The complexes of rsTNFRβ with TNFα or TNFβ both had a molecular mass of about 140 kDa which favors a stoichiometry of three rsTNFRβ monomers bound to one TNFα or TNFβ trimer. It has been proposed that the receptor binding site on the TNFα trimer is located at the boundary of two monomeric units near the base of the bell-shaped structure thus implying three potential receptor binding sites (7, 54). Such a model is fully compatible with the size of receptor-ligand complexes as determined in the present study. It is interesting to note that an intermediate lower molecular weight form of the rsTNFRβ-TNFα complex can be partially resolved by gel filtration when a slight excess of TNFα over rsTNFRβ is present. Most likely, this intermediate form represents TNFα trimers complexed to only one or two rsTNFRβ molecules. Such intermediate forms are not seen with rsTNFRβ-TNFβ complexes. Whether these distinct binding characteristics of TNFα and TNFβ are also true for cell surface-bound receptors remains to be elucidated.

The results of sedimentation velocity and quasielastic light scattering measurements indicate that rsTNFRβ and its TNFα and TNFβ complexes have a rather extended, i.e. rod-like structure. This conclusion is supported by the relative large apparent molecular masses of 62, 170, and 150 kDa for rsTNFRβ, rsTNFRβ-TNFα and rsTNFRβ-TNFβ complexes, respectively, determined by gel filtration chromatography. A similar relatively large apparent molecular mass (50 kDa) has been found for the natural soluble TNFRβ on sizing columns (42).
Soluble fragments of both TNFRβ and TNFRα are found in vivo. They are present at relatively high concentrations in normal human serum and urine but can be drastically increased in certain disease states. The cellular source and the mechanism of receptor shedding remain unclear. It has been speculated that soluble TNF receptor fragments might participate in the control of TNFα and/or TNFβ toxicity by neutralization and rapid clearance of systemic TNFα and TNFβ (33, 36, 37, 42). However, the fact that at least a 10-fold excess of the soluble receptor with respect to TNFα (and more than a 100-fold excess with respect to TNFβ) is needed to obtain a significant neutralization demonstrates that the neutralizing capacity of serum is restricted. The rsTNFRP-hy3 construct as described in this study, therefore, is a promising TNF antagonizing agent for neutralization of systemic TNF toxicity in certain disease states.

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