Use of a Sensitive Receptor Binding Assay to Discriminate between Full-length and Truncated Human Recombinant Tumor Necrosis Factor Proteins*

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A radioreceptor assay (RRA) capable of detecting picomolar concentrations of human recombinant tumor necrosis factor (TNF) was used to compare the relative binding affinities of genetically engineered full-length and truncated TNF proteins. The specific cell-surface receptors for TNF present on the human cervical carcinoma cell line ME-180 were characterized as having a K_d of 0.2 nM and a density of 2700 sites/cell. Conditions were then defined for an RRA that maximized the specific binding of ^125 I-TNF to this adherent cell line. Incubation of ME-180 cells with ^125 I-TNF at 37°C in the presence of 0.02% sodium azide resulted in a 4-fold increase in assay sensitivity and a doubling of specific counts bound, as compared to binding done at 4°C with or without sodium azide. Inhibition of receptor-ligand internalization under these conditions was a likely reason for the increases. This system was utilized to compare low concentrations of the full-length TNF protein and a genetically altered TNF protein (mutein) which lacks the 10 N-terminal amino acids and contains an N-terminal methionine. Previous studies showing the truncated TNF to be lower in cytotoxic activity on a variety of tumor cell lines were corroborated by our findings that the mutein was also three and one-half times lower in relative affinity for the TNF receptor on ME-180 cells. These results suggest a possible role for these residues in receptor binding and illustrate the use of a highly sensitive RRA for the evaluation of TNF molecules altered by recombinant DNA technology.

Tumor necrosis factor (TNF) was identified by Carswell et al. (1) in the serum of mice infected with bacillus Calmette-Guerin and subsequently treated with endotoxin. This substance was found to cause hemorrhagic necrosis of the Meth A sarcoma and other transplantable tumors without destroys normal tissues (1, 2). This prompted efforts toward purification of TNF from several species (3-6), culminating in the cloning and expression in Escherichia coli of human recombinant TNF (6-8). Further studies have shown the availability of the highly purified, cloned TNF to have confirmed its in vitro cytostatic and cytotoxic effects (6, 9). In addition, studies such as stimulation of eosinophil toxicity against parasites (10), induction of HLA expression in endothelial cells and fibroblasts (11), and suppression of lipoprotein lipase activity in 3T3-L1 cells (12) suggest other possible roles of TNF.

The recent availability of deletion mutants of TNF has allowed investigation of the region(s) of the molecule that may be important for cytotoxic activity (13). We report here the development of a radioreceptor assay (RRA) for human recombinant TNF based on the competitive binding of the ligand to the human cervical carcinoma cell line ME-180. This highly sensitive RRA was used to determine the relative binding affinities of genetically engineered full-length and truncated TNF proteins and to illustrate a correlation between the cytotoxic activity and binding affinity of these two proteins.

MATERIALS AND METHODS

Cell Culture—The cervical carcinoma cell line ME-180 was maintained in subconfluent cultures in 175 cm² tissue culture flasks. The cells were grown in McCoy's 5A medium containing 15% fetal bovine serum, 2 mM glutamine, and 1% Fungi-Bact (Irvine Scientific). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. To whom correspondence should be addressed: Cetus Corp., Dept. of Cell Biology, 1400 Fifty-third St., Emeryville, CA 94608.

The abbreviations used are: TNF, recombinant human tumor necrosis factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DPBS, Dulbecco's phosphate-buffered saline; RRA, radioreceptor assay; EGF, epidermal growth factor; NaN₃, sodium azide.

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Receptor Binding Assay for TNF Muteins

125I-TNF in 0.5 ml was added to each well, and the plates were incubated an additional 2 h. The cell monolayers were washed three times with binding buffer, solubilized with DPBS containing 1 N NaOH and 1% SDS, and bound radioactivity was determined in a γ counter. Duplicate determinations varied by less than 10%.

The amount of bound 125I-TNF sensitive to acid treatment was determined by acid treatment of 1 ml/well of 0.05 M glycine, 0.15 M NaCl, adjusted to pH 3.0 with HCl (15). After 5 min at 4 °C, the solution was removed and replaced with another 1 ml of solution for 3 min. The cells were then washed, and bound radioactivity was determined.

RESULTS

Protein Sequencing—The first 16 amino acids of the -10 mutein were found to be as predicted by the DNA sequence of the full-length protein (Fig. 1), except for the presence of an N-terminal methionine. Removal of the methionine during bacterial processing of the protein is mediated by the enzyme methionine aminopeptidase, the activity of which is dependent upon the residue following that methionine. The removal of methionine in the full-length TNF where the penultimate residue is valine and the presence of methionine in the truncated TNF where the penultimate residue is lysine are consistent with the findings of Tsumasawa et al. (16).

Equilibrium Saturation Binding—The ME-180 cervical carcinoma cell line has previously been shown to be sensitive to the cytotoxic effects of TNF (9) and to possess high affinity TNF binding sites (17). To confirm these findings and to help ascertain the potential usefulness of this cell line in a RRA, saturation binding studies and Scatchard analysis were performed (Fig. 2). 125I-TNF bound to cells with specific activity, as seen by the displacement of up to 87% of the total counts by a 1000-fold excess of unlabeled ligand. 125I-TNF specific binding was also saturable, reaching a maximal level at 2.35 nM and a half-maximal level at approximately 0.24 nM. Kinetic equilibrium studies at 4 °C indicated maximal specific binding occurred with saturating concentrations of ligand in 1.5 to 2.5 h. Scatchard analysis of the data (Fig. 2, inset) revealed 2700 receptor sites/cell and a Kd of 0.2 nM. These values agree well with those found by a number of other groups working with various cell types (17–20), although lower Kd values have been reported (21). These differences may be technical in nature or related to species or cell line variations in receptor characteristics.

RRA Studies—Studies were then carried out to maximize the binding of TNF to ME-180 cells under the nonsaturating concentrations necessary for optimal assay sensitivity. Thus, the effects of temperature and the metabolic inhibitor sodium azide were investigated. TNF dilutions were prepared in binding buffer with or without 0.02% sodium azide, and binding was carried out at 37 or 4 °C as described under “Materials and Methods.” Fig. 3A illustrates the increase in assay sensitivity that resulted from the addition of sodium azide at 37 °C. There was a 4-fold decrease in the amount of unlabeled ligand required to displace 50% of the 125I-TNF specifically bound. The increase in sensitivity has been as much 7-fold in other experiments. Kinetic equilibrium studies have shown maximum specific binding to occur in 2 to 3 h at 37 °C with sodium azide under these conditions. Sodium azide had no effect on assay sensitivity at 4 °C.

There was also a 90% increase in the number of specific counts bound (total minus nonspecific) at 37 °C when sodium azide was present (4765 ± 402) versus when it was absent (2509 ± 65). In contrast, there was little difference at 4 °C when sodium azide was present (2556 ± 99) or absent (3122 ± 166).

To investigate the possible reason(s) for the increased binding and assay sensitivity in the presence of sodium azide, the effects of acid treatment on specifically bound counts were studied. ME-180 cells were incubated with 0 or 1000 ng/ml TNF (approximately 400-fold excess; “cold” ligand) with or without sodium azide at 37 or 4 °C for 2 h. Radiolabeled ligand was then added for an additional 2 h. Nonspecific counts were subtracted from the total to derive specific counts bound. The sensitivity of specifically bound 125I-TNF to removal by pH 3 treatment was then used to determine if the ligand was surface-associated (15). As seen in Fig. 3B, the percentage of acid-sensitive counts bound at 4 °C with and without sodium azide was 68 and 70%, respectively. This agrees well with the values obtained for acid-removable binding by other TNF-sensitive cell lines (18). Binding in the absence of sodium azide at 37 °C resulted in a marked decrease of acid-sensitive counts to 21%, suggesting internalization of the majority of specifically bound 125I-TNF. However, including azide at 37 °C prevented internalization and resulted in virtually the same percentage of 125I-TNF bound at the cell surface (65%) as at 4 °C.

Use of the RRA to Determine Relative Binding Affinities—This assay system was then utilized to compare the relative binding affinities of the full-length recombinant TNF used above, termed 711, and the novel mutant TNF protein shown in Fig. 1 that lacks the 10 N-terminal amino acids, termed 742. Previous comparisons of the biological activities of the two proteins had shown 742 to be up to three times less cytotoxic than 711 on ME-180 cells and eight other human cell lines (13). To determine if the binding affinity of the protein was also altered by the deletion of these 10 residues, the ability of the two proteins to compete with iodinated 711 protein was compared. In the experiment illustrated in Fig. 4, 711 displaced 50% of the specifically bound 125I-TNF at a concentration of 0.32 ng/ml. In contrast, 742 showed nearly a 5-fold lower ability to compete for the same binding sites on ME-180 cells, displacing 50% of the counts bound at a concentration of 1.5 ng/ml. In four experiments of this type, 50% displacement occurred at 0.33 ± 0.04 ng/ml (mean ± S.D.) of full-length TNF versus 1.16 ± 0.24 ng/ml of truncated TNF (p < 0.005), a difference of approximately 3.5-fold. In all experiments the linear range of the curve occurred over approximately a 15- to 30-fold concentration of competing TNF. Kinetic equilibrium studies showed that the apparent difference in the relative binding affinities of the TNF proteins was not due to a difference in their rate of binding. Also, in contrast to the results obtained with the -10 mutein, muteins lacking the first 4, 7, or 8 N-terminal residues were found to exhibit binding competition curves which were superimposable on that of the full-length protein.

DISCUSSION

The measurement of cytokines and cytokotoxins such as TNF has typically begun with bioassays and then progressed to more specific radioimmunoassays and radioreceptor assays as the molecule becomes more well defined. We report here a competitive binding assay for TNF that provides greater specificity than cytotoxicity assays that in contrast cannot discriminate between TNF and other cytokotic factors that may bind to distinct receptor sites. Since the RRA specificity is dependent upon receptor binding, we were also able to detect purified native TNF-β (lymphotoxin), in agreement with a recent report which indicates that these two factors share a common receptor (17). The TNF RRA using ME-180 cells does not require lengthy periods for cell growth and differentiation after plating, as with a receptor-binding assay using murine 3T3-L1 cells (22). Also, as opposed to the murine

3 Data not shown.
system, the TNF RRA described here is designed to measure recombinant human TNF in a species-homologous system. The sensitivity of this RRA was equal to or greater than that possible with the L929 cytotoxicity assay, in which 1 unit/ml is the operational limitation. The initiation of human trials of TNF as a therapeutic agent and the possible clinical significance of measuring TNF production by cancer patients (23) suggest potential applications of a sensitive RRA for TNF.

The density of TNF receptors on ME-180 cells is comparable to that of other murine and human adherent cell lines tested (18, 21); however, this is a relatively low number of sites/cell in comparison to other ligands such as colony-stimulating factor-1 and epidermal growth factor (EGF), for which RRAs have been developed (24, 25). To overcome this inherent limitation on the assay signal, specific binding was increased, along with assay sensitivity, by incubating the cells at 37 °C in the presence of sodium azide during binding. This technique has also proven useful in detecting low numbers of receptors (<500/cell) for colony-stimulating factor-2 (26). The increases seen here were most likely related to the inhibition of receptor-ligand internalization. This is supported by the lack of an effect by sodium azide at 4 °C, when internalization is already arrested. In addition, 125I-TNF bound at 37 °C was found to be acid-sensitive only when sodium azide was present. Internalization of TNF has previously been noted to occur in other cell types (18, 19, 27).

The TNF RRA was applied as a means of measuring the relative binding affinity of genetically altered TNF proteins. The bioactivity of a molecule may be correlated to its receptor binding ability, as seen here. The 3.5-fold difference in binding affinity between the full-length and mutant deletion proteins is in close agreement with the 2- to 3-fold difference in bioactivity seen previously. Our interpretation of the data as representing a difference in relative binding affinity assumes that equivalent proportions of both muteins are capable of binding. In a similar study of insulin, deletions, substitutions, or both resulted in decreased as well as increased binding and bioactivity (28). The results seen here suggest part or all of the 10 N-terminal amino acids play a role in the binding of TNF to its receptor, or possibly the methionine present in the -10 mutein may adversely affect protein binding. Interestingly, binding competition studies of muteins lacking the first 4, 7, or 8 N-terminal amino acids showed them to have
relative binding affinities that are indistinguishable from that of the full-length protein.

Our data suggest that the residues in this N-terminal region may, for example, be directly involved in receptor binding, may be important for maintaining the tertiary structure of a separate receptor binding domain, or may be involved in stabilizing the receptor-ligand complex. Further studies are necessary to determine the full significance of this region in receptor binding and whether it may be possible to generate TNF molecules with higher relative affinities.

The availability of genetically altered proteins, as well as proteins altered by other physical or chemical means, provides unique opportunities to investigate structure-function relationships. This approach was applied in a recent report which indicated that mutant calmodulin proteins can differentially activate various target enzymes (29). Similar studies may help in developing agonists and antagonists to the various biological activities of TNF and in determining if these activities are the result of common or distinct mechanisms. A highly sensitive RRA, therefore, will be a useful method for the characterization of the properties and mechanisms of TNF.

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FIG. 4. Inhibition of binding of 125I-TNF to ME-180 cells by different TNF preparations. The indicated concentrations of TNF preparations 711 (○) and 742 (□) were determined by absorbance at 280 nm and competed as described in Fig. 3a with iodinated 711 at 37°C in the presence of 0.02% sodium azide. The noncompeted control (●) is also shown. Values are the mean of duplicate determinations.