Amplified Expression of Tumor Necrosis Factor Receptor in Cells Transfected with Epstein-Barr Virus Shuttle Vector cDNA Libraries*

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Tumor necrosis factor (TNF) is a 17-kDa polypeptide secreted by macrophages and a member of the immunoregulatory class of molecules termed cytokines that includes factors such as interleukins and interferons. Like other cytokines, TNF exerts a diversity of biological responses, including inflammation (1-3), endotoxin shock (4), cytotoxicity to certain tumors and tumor cell lines (5, 6), and the catabolic state termed cachexia associated with chronic disease (7, 8).

TNF interacts with a wide variety of cell types by binding to high affinity receptors present on the cell surface. The availability of radioiodinated derivatives of TNF has made it possible to measure the number of surface receptors on many different cell types. Scatchard analyses of the ligand-receptor binding data indicate that the number of receptors is small, usually in the range of 500 to 10,000 per cell (7, 9-12). Higher numbers (around 50,000 per cell) have been reported for HeLa-S3 (11) and a subcloned human histiocytic cell line U937 (13), although these have not been confirmed by others. There appears to be a single class of specific high affinity receptors with affinity constants for binding in the 0.1 to 10 nM range (11, 12, 14, 15). Ligand-receptor complexes are thought to be internalized, processed, and degraded in the lysosomes (11, 16-18). Interleukin-1 (19) and tumor promoters such as phorbol esters (19-22) down-regulate receptor numbers while interferon-γ enhances receptor expression (15, 17, 23). Although little is known about post-receptor events leading to the production of intracellular messengers, G-proteins have been implicated in the signal transduction pathway by the demonstration that pertussis toxin inhibits TNF-induced cytotoxicity (24). TNF also stimulates arachidonic acid release and subsequent production of prostaglandins (25).

One difficulty in the isolation of the receptor protein has been the low abundance of TNF receptors on cells. The protein has been partially purified from human histiocytic cells (U937) and estimated to have a size of 65 ± 8 kDa (19). However, extremely low recovery plus the presence of other proteins make this size estimate tentative. Covalent cross-linking of radioiodinated TNF to receptor with bis-sulfosuccinimidyl suberate generates products ranging from 74 to 105 kDa (11, 13, 16), but with 1,5-difluoro-2,4-dinitrobenzene (a different cross-linking reagent) two additional complexes of 54 and 138 kDa were reported (26). The size of the receptor itself from this ligand-receptor complex is difficult to derive because of the uncertainty of the form of TNF associated with the receptor. TNF self-associates predominantly as a trimer (27, 28), but its interaction with the receptor as a monomer, dimer, or as a higher order complex is not known. The receptor protein has a molecular mass of about 87 kDa and is thought to be internalized, processed, and degraded in the lysosomes (11, 16-18).

As an approach to isolate the cell-surface receptor for tumor necrosis factor (TNF), we have developed transfecants of human B-lymphoblastoid cells (UC cells) that overexpress the TNF receptor. These transfecants were isolated from UC cells transfected with cDNA libraries of HeLa or NG108 cells constructed in the mammalian expression vector EBO-pcD. This vector contains the Epstein-Barr virus origin of replication (ori-P) plus the EBNA-1 gene conferring replication function to ori-P and, therefore, the ability to replicate autonomously within the transfected cell (Margolskee, R. F., Kavathas, P., and Berg, P. (1988) Mol. Cell. Biol. 8, 2837-2947). Cells overexpressing the TNF receptor were identified and separated by the binding of fluoresceinated TNF and flow cytometric selection. Scatchard analysis of 125I-TNF binding data revealed a single class of high affinity receptors with a dissociation constant (Kd) of 0.2 to 2 nM and a receptor density of about 150,000 per cell, an increase of about 150-fold over UC cells. Cross-linking of receptor-ligand with bis-sulfosuccinimidyl suberate followed by polyacrylamide gel electrophoresis gave estimates of 87 and 104 kDa for the size of the complex. Based on its ability to bind TNF, a 68-kDa receptor protein was identified in cell extracts enriched for the receptor by using immobilized wheat germ agglutinin and TNF affinity chromatography. The difference in the molecular size of the receptor and the receptor-ligand complexes demonstrates that TNF binds to the receptor as a monomer or a dimer.

Analysis of cDNA sequences conferring receptor amplification in transfecants revealed that plasmid DNA was present at 30 or more copies per cell, most likely integrated into the genomic DNA or organized into autonomously replicating units could not be recovered. Therefore, while this vector was useful in generating stable receptors, it was not maintained as a recoverable episome.
dimer, or trimer has not been established. A full understanding of TNF-receptor interaction requires the isolation and identification of the receptor, characterization of its ligand binding properties, and elucidation of the intracellular mechanisms by which it asserts its cytotoxic action and other biological responses.

As an approach to the isolation of the receptor, we report the development and use of human B-cell lines that overexpress TNF receptor (the receptor is defined here as a cell-surface protein that binds TNF with high affinity). These cells were isolated from a pool of B-cells transfected with cDNA libraries, derived from HeLa or NG108 cells, constructed in the vector EBO-pcD. This vector permits expression of insert cDNA in mammalian cells, transforms human cells with high efficiency, and has the potential to exist as an autonomous plasmid at 2-10 copies within the transfected cell. Recovery of introduced cDNA clones from mammalian cells should therefore be facilitated (29). However, in this case the EBO-pcD vector appears either to integrate into cellular genomic DNA or to exist as a high molecular weight concatamer not recoverable as plasmids. We have nevertheless utilized these cell lines to identify the TNF receptor in detergent extracts of cells after enrichment for the receptor using affinity chromatography procedures. Based on the size of the receptor and the receptor-ligand complexes we conclude that TNF associates with the receptor primarily as a monomer and to a lesser extent as a dimer.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free Na\(^{125}\)I was purchased from Du Pont-New England Nuclear; IODO-GEN, bis-sulfosuccinimidyl suberate, NHS-LC-biotin, and Triton X-100 were from Pierce Chemical Co.; penicillin, streptomycin, RPMI 1640, Dulbecco's modified Eagle's medium, and fetal calf serum were from Gibco; complete RPMI 1640 and RPMI 1640 without phenol red or biotin was from Irvine Scientific, Santa Ana, CA. All restriction enzymes and hygromycin B were purchased from Boehringer Mannheim; fluorescein isothiocyanate (FITC) dissolved in sodium bicarbonate buffer, pH 9.5, was conjugated to fluorescein isothiocyanate (FITC) dissolved in dimethyl sulfoxide. A 290 μg aliquot of TNF was added to 66 μg of FITC in a total volume of 35 μl, and the vial containing the reagents was wrapped in aluminum foil and rocked gently at room temperature for 2-3 h. A PD-10 column was equilibrated with PBS buffer and the FITC-TNF solution was brought to 100 μl with PBS buffer was loaded onto the column. The column was washed with 1.9 ml of buffer and the flow-through discarded. FITC-TNF was collected with the next 1.5 ml of buffer eluate. NHS-LC-biotin (10 μg/ml) in dimethyl sulfoxide was reacted with TNF (1 μg/ml) in 0.1 m bicine buffer, pH 9.5, at a 30:1 molar ratio at room temperature for 2 h. Dimethyl sulfoxide in the reaction mixture was less than 10% of the total reaction volume. Biotinylated TNF was recovered by gel filtration as described above for FITC-TNF.

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Transfection of Human Lymphoblastoid Cells—UC cells (1 x 10\(^6\)) were electroporated separately with 50 μg of DNA from each library and 20 μg of EBO-pcD Leu 2. Conditions for cell preparation, electrophoresis, and selection of hygromycin-resistant populations were as described previously (29).

Flow Cytometry and Sorting—Transfected UC cells (2 x 10\(^6\)) were prepared for flow cytometry by staining with FITC-TNF at a concentration of 180 μg/ml and 200 μg/ml of FITC-TNF in PBS containing 10% fetal calf serum and 2% hygromycin B. Propidium iodide (Calbiochem) was added at a final concentration of 5 μg/ml prior to analysis to label dead cells. Cells were analyzed and sorted on a FACStar Plus sorter (FACS II, Becton Dickinson, San Jose, CA) by illuminating them with 500 milliwatts of 488 nm laser light and measuring 530 nm FITC fluorescence, and propidium iodide fluorescence greater than 630 nm. Sort windows were generated to eliminate debris and to sort the brightest 1% of FITC fluorescence distribution which was propidium iodide-negative. Early selection was accomplished using Becton-Dickinson's "enrich" mode, which accepts coincident particles in positive sort envelopes. Approximately 50,000 cells were grown in culture to 1-2 x 10\(^6\) cells in the presence of 200 μg/ml hygromycin, and this cycle of selection and expansion was repeated as described. Cross-linking of \(^{125}\)I-TNF to Receptors—Cells (1-2 x 10\(^6\)) were incubated for 2 h at 4 °C in RPMI 1640 plus 10% fetal calf serum (culture medium) containing 300 μg/ml of \(^{125}\)I-TNF with or without 100-fold excess of unlabeled TNF. After incubation, the cells were washed three times by centrifugation through ice-cold culture medium, and the receptor and the receptor-ligand complexes were concluded that TNF associates with the receptor primarily as a monomer and to a lesser extent as a dimer.
solubilized in 200 µl of cold extraction buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, and 10 µg/ml each of peptatin, leupeptin, and aprotinin. The insoluble material was removed by centrifugation at 100,000 × g for 1 h, and the extract was enriched for the receptor by using two sequential affinity chromatography steps. The extract was first added to a 60-ml column of wheat germ agglutinin (WGA) coupled to Sepharose 4B (3.5 mg of WGA per gram of Sepharose 4B purchased from Pharmacia LKB Biotechnology Inc.) and prepared according to directions provided by the vendor. The column was washed with 10 column volumes of column buffer containing 0.2% Triton, 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl plus protease inhibitors described above. The column was eluted with 3 column volumes of the column buffer containing 0.6 M N-acetylgalactosamine. The eluates were dialyzed against cold PBS containing 0.2% Triton and added to a 5-ml column of TNF coupled to Sepharose 4B (3.5 µg of TNF per g of Sepharose 4B). The column was washed sequentially with 10 column volumes of column buffer and then 0.2 M glycine HCl, pH 4.0, plus 0.2% Triton and protease inhibitors. The receptor was eluted with glycine HCl, pH 2.5, containing 0.2% Triton and protease inhibitors. Eluates were concentrated with a Centricon filter (Amicon, Danvers, MA) and analyzed for receptor activity and protein content.

Ligand Blot Analysis of Protein with TNF Receptor Activity—Specific amounts of protein were resolved by electrophoresis in a 10% polyacrylamide gel run at 4°C according to the procedure of Laemmli (33) except that no sulfhydryl reducing agents were added and samples were electrophoresed in 6.8% agarose. The electrophoresed samples were electrophoretically transferred to nitrocellulose membrane in buffer systems described by Burnette (32). The nitrocellulose membrane was incubated in 8 M urea, 0.2% Triton X-100, 50 mM Heps, pH 7.4, for 1 h, blocked with 5% non-fat dry milk/PBS solution, and incubated with 125I-TNF, washed and analyzed as described for the soluble receptor assay.

Isolation of Episomal DNA from Transfected Cells—Episomal DNA from UC cell transfectants was isolated from 2 × 107 cells according to the method of Hirt (33). Cells were lysed in 0.6% SDS, 1 M NaCl, 10 mM Tris, pH 7.5, and 10 mM EDTA and kept at 4°C overnight. High molecular weight DNA was removed by centrifugation at 16,000 × g for 30 min at 4°C. The supernatant was treated with proteinase K for 1 h at 65°C, extracted with phenol/CHCl3 (1:1), and the DNA precipitated by addition of 2.5 M ammonium acetate plus 2 volumes of absolute ethanol. After overnight storage at −20°C, the DNA was pelleted by centrifugation at 16,000 × g for 30 min at 4°C, washed with 80% ethanol, dried, and resuspended in 20 µl of 10 mM Tris, pH 7.5, and 10 mM EDTA. Aliquots of this DNA were used to transform competent Escherichia coli cells (DH5) purchased from Bethesda Research Laboratories, according to instructions provided by the vendor. Bacterial colonies (100 µl) and DNA were incubated for 30 min on ice, heat-shocked for 45 s at 42°C, and cooled on ice for 2 min. SOC medium (0.3 ml) (SOC medium: LB plus 2.5 mM KCl, 10 mM MgSO4, 20 mM glucose) was added to this mixture, and cells were shaken at 225 rpm for 1 h at 37°C. Cells were plated on LB medium plates containing 50 µg/ml ampicillin, and transformants were recovered after an overnight incubation at 37°C. Individual bacterial colonies were grown in 2-ml cultures in LB plus ampicillin. Plasmid DNA was prepared according to the method of Holmes and Quigley (34) and the extracted DNA analyzed by restriction endonuclease digestion and agarose gel electrophoresis.

Southern Blot Analyses—Episomal DNA and genomic DNA prepared from transfected UC cells were analyzed according to published procedures (35). After digestion with restriction endonucleases, DNA was electrophoresed in 6.8% agarose gels and transferred to nitrocellulose membranes (Schleicher & Schuell). These membranes were hybridized with 32P labeled, random hexamer primed probes (36), and the hybridized bands were observed by autoradiography. Recovery of episomes from transfected cells proved to be possible. Possible rearrangements that could be observed are the formation of concatamers from introduced vector-cDNA sequences, rearrangements between introduced and host DNA, and/or integration into genomic DNA. A loss of one S140 probe plus introduction of Alu repeated sequences (36) were some of the rearrangements noted in genomic DNA preparations analyzed from the transfectants (data not shown).

RESULTS
By using 125I-TNF we found that a variety of cell lines (e.g. HeLa and NG108) contain approximately 3000 TNF receptors per cell whereas the UC, B-lymphoblastoid line, contains about 1000 receptors per cell (Fig. 1). In each case a single class of high affinity receptors was identified with a Kd for TNF between 0.1 and 1.0 nM. Attempts to isolate a functional cDNA encoding the TNF receptor were initiated based on the notion that UC cells expressing an additional receptor from an exogenously introduced cDNA could be identified by one of several techniques. Consequently, we prepared cDNA expression libraries in the Epstein-Barr virus shuttle vector, EBO-pcD (Fig. 2), using RNA from HeLa and NG108 cells (29). The HeLa libraries were made from five different sizes of cDNA in which the cDNAs inserts were size 1, <0.4 kb; size 2, 0.4-0.8 kb; size 3, 0.8-1.6 kb; size 4, 1.6-2.9 kb; and size 5 with greater than 2.9 kb cDNA inserts. Fractions 2 through 5 alone or a pool of these four fractions referred to as HeLa 2-5 or the NG108 total cDNA library were introduced separately into UC cells by electroporation of 1 × 107 cells with 50 µg of library DNA. EBO-pcD Leu 2, harboring a cDNA encoding a human T-cell surface marker, was transfected separately into a population of UC cells to monitor the efficiency of electroporation, hygromycin selection, and flow cytometric analysis (29). Transformed cells were recovered by their resistance to hygromycin and required approximately 2 weeks of growth in drug-containing medium for selection. The expression of TNF receptor on these cells was measured by analyzing the binding of fluorescein-conjugated TNF in a flow cytometer.

FIG. 1. Specific binding of 125I-TNF to HeLa and NG108 (A) and UC cells (B). One × 106 cells were incubated at 4°C for 2 h with increasing amounts of 125I-TNF in the absence or presence of 100-fold excess unlabeled TNF. Cells were then washed three times and the amount of bound ligand determined by counting cell-associated radioactivity. The estimated number of receptors and the dissociation constants (Kd) calculated from Scatchard analyses (inset) are: UC cells 1300 receptors/cell, Kd = 0.9 × 10−9 M; HeLa cells, 2900 receptors/cell, Kd = 2 × 10−4 M; and NG108 cells, 3400 receptors/cell.
Selection of UC Transfectants Expressing Elevated Levels of TNF Receptor—In order to detect the rare cells expressing higher receptor number, two derivatives of TNF were prepared for fluorescent staining of cells for FACS analysis and tested for their specificity of staining. These derivatives were biotinylated TNF for use with avidin-phycocyanin for detection of cell surface receptors. Class A reagents yielded nonspecific binding, which was 5-fold higher than untransfected UC cells and therefore could not be used to measure specific binding of TNF (data not shown). However, FITC-TNF showed little or undetectable levels of nonspecific binding and was useful in detecting receptors on HeLa cells (Fig. 3). Furthermore, FITC-TNF binding was completely displaced by a 100-fold excess of TNF to the level of untransfected cells as measured by fluorescence intensity. The fluorescence profile obtained with UC cells (not shown), stained or unstained, was very similar to that of HeLa cells, showing essentially that these cells had very little autofluorescence or nonspecific binding with FITC-TNF. In tests of bioactivity FITC-TNF was comparable with TNF in its cytotoxicity to L929 cells (data not shown), suggesting that little or no loss of TNF function had occurred due to modification with FITC.

UC cell transfectants resistant to 200 μg/ml hygromycin were stained with FITC-TNF and analyzed flow cytometrically. Initially, the spectrum of fluorescence intensity in a population of FITC-TNF-stained UC transfectants compared with nontransfected cells was indistinguishable. Nevertheless, about 0.05% of the most intensely staining UC transfectants (about 50,000 cells) were collected aseptically and cultured to grow to a population of 1–2 × 10⁶ cells. These cells were restained with FITC-TNF and resorted. By the end of the fifth such selection, a population of cells with distinctly higher FITC-TNF binding was seen in transfectants from the entire HeLa library cDNA, and in HeLa size cut 4 cDNA (representing the 1.6- to 2.9-kb size), and in transfectants from the NG108 cDNA library. HeLa cDNA in the size range of 0.4–0.8, 0.8–1.6, or larger than 2.9 kb showed little enhancement of TNF receptor expression as did the EBO-pcD-Leu 2 plasmid DNA after equivalent repeated rounds of cell sorting (Fig. 4).

The transfectants were characterized further by several independent methods to verify the increase in receptor expression. Measurements made by ¹²⁵I-TNF binding confirmed flow cytometric observations (Fig. 5). Scatchard plots of the steady-state binding of ¹²⁵I-TNF to the three positive cell lines revealed the presence of approximately 150,000 TNF receptors with Kᵦ in the 0.2 nM range. These receptors per untransfected UC cell. ¹²⁵I-TNF binding to the negative transfectants UC/HeLa 5, UC/HeLa 3, or UC/Leu 2 was not significantly different from that of untransfected UC cells.

Size of Cross-Linked Receptor-Ligand Complexes—An estimate of the molecular size of the TNF receptor complex in amplified receptor cell lines and the parental UC cells was made by cross-linking cell-bound ¹²⁵I-TNF to its receptors with the reagent bis-sulfo-succinimidyl suberate. The cross-linked receptor-ligand complex was solubilized with Nonidet-P-40 and analyzed by SDS-PAGE. Preparations of receptor amplified cells showed the complex as two bands on autoradiographs of the gel: a dense band at 87 kDa and a fainter band at 104 kDa when cells were incubated with ¹²⁵I-TNF alone, but not when excess unlabeled TNF was included during the binding reaction (Fig. 7). The difference in size between the two bands is approximately 17 kDa and is due...
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**Fig. 4** Flow cytofluorometric analysis of UC cell transfectants. Unselected B-lymphoblastoid UC cells (UC/unsorted) or UC transfectants obtained by electroporation with DNA from with either EBO pE6 HeLa size selected libraries UC/HeLa 2 with 0.4–0.8 kb cDNA; UC/HeLa 3 with 0.8–1.6 kb cDNA; UC/HeLa 4 with 1.6–2.9 kb cDNA; UC/HeLa 5 containing greater than 2.9 kb cDNA; or a pool of all four sizes referred to as UC/2.5; or the UC/NG108 cDNA library were selected for hygromycin resistance and stained with 0.8 µg/ml FITC-TNF and analyzed. Upper left panel, UC/HeLa 4 transfectants stained with 0.8 pg/ml FITC-TNF in the absence or presence of 8 µg of TNF to demonstrate detectable TNF receptor after three rounds of flow selection. Upper right panel, UC/uns (control) plus UC transfectants after six repeated rounds of sorting to show amplifications of TNF-R determined by increase in fluorescence intensity due to increased FITC-TNF binding. These are UC/HeLa 4, UC/HeLa 2-5, and UC/NG108. Lower left panel, UC/uns (control) plus UC transfectants UC/HeLa 3 and UC/HeLa 5 showing very small increases in TNF-R after six selection cycles. Lower right panel, UC transfectants derived from NG108 cDNA library after three, five, six or nine repeated rounds of sorting to show the progression in TNF-R amplification. au. denotes arbitrary units.

**Fig. 5** Equilibrium specific binding of *^125*I-TNF to UC cell transfectants. Binding to UC/HeLa 2-5 (2-5), UC/HeLa 4 (4), UC/NG108 (NG), UC/uns control cells (Uns), and UC/HeLa 3 (3), UC/HeLa 5 (5), and UC/Leu 2 (Leu 2) was carried out in duplicate using *^125*I-TNF and 2 × 10^6 cells in a total volume of 200 µl for 2 h at 4 °C in the absence (total) or presence (nonspecific) of 18 nM unlabeled TNF after 6 cycles of sorting. Cells were washed three times with cold RPMI 10% fetal calf serum and specific binding determined as the difference between mean total and mean nonspecific radioactivity associated with the cells. Results confirm observations made with FITC-TNF that TNF-R was amplified in UC/HeLa 2-5, UC/HeLa 4, and UC/NG108 transfectants. Compared with controls UC/uns, no significant change had occurred in UC/HeLa 3, UC/HeLa 5, and UC/Leu 2 transfectants.

**Fig. 6** Specific binding of *^125*I-TNF to UC/HeLa 2-5, UC/HeLa 4, and UC/NG108 transfectants. The number of receptors per cell on the TNF-R amplified cells plus the affinity constants for receptor-ligand interaction were calculated from Scatchard analyses of the binding data. Equilibrium saturation binding to cells was carried out as described in Fig. 2. The values calculated for UC/HeLa 2-5 were 155,000 receptors/cell and Kd of 0.26 × 10^-9 M; UC/HeLa 4, 144,000 receptors/cell and a Kd of 0.22 × 10^-9 M; UC/NG108, 187,000 receptors/cell and a Kd of 0.28 × 10^-9 M.
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Identification and Size Determination of TNF Receptor by Ligand-blot Analysis—Detergent extracts of UC cells and UC/HeLa 2-5 receptor amplified cells were prepared and enriched for the receptor by sequential binding to lectin and TNF affinity columns. Fractions were tested for the presence of the soluble receptor using a filter binding assay (described under “Experimental Procedures”), and aliquots were simultaneously analyzed by nonreducing SDS-PAGE and ligand blotting to determine the size of the protein associated with receptor activity. An approximately 68-kDa band with a high level of [125I]TNF binding activity was recognized readily in detergent cell extracts and ligand blotting (Fig. 9A). A faint 68-kDa protein band was recognized only in receptor amplified cells and was not detectable under these conditions in UC cells. Furthermore, this band corresponded in size and mobility with the 68-kDa protein exhibiting high TNF-binding activity as assessed by ligand blotting. Based on this evidence we infer that the 68-kDa protein is the TNF receptor.

Identification of a 68-kDa TNF Receptor Protein Band—Parallel preparations from UC cells and UC/HeLa 2-5 cells were processed through the same affinity chromatography procedures, and the TNF affinity eluates were subjected to reducing and nonreducing SDS-PAGE; proteins were detected by silver staining (Fig. 9B) and also analyzed by ligand blotting (Fig. 9C). A 68-kDa protein band was recognized in receptor amplified cells and was not detectable under these conditions in UC cells. Furthermore, this band corresponded in size and mobility with the 68-kDa protein exhibiting high TNF-binding activity as assessed by ligand blotting.

EBO-pcD Plasmids in Receptor Amplified Cells and Their Rescue—Low molecular weight DNA was isolated by the method of Hirt (23) or its modification described previously (29) from cells enriched in TNF-receptor after four, five, or six serial flow selection cycles. No plasmids were recovered in E. coli from any of these cell lines after many repeated attempts. A conclusion to be drawn from these experiments was that the introduced plasmids no longer existed as recoverable episomes. The plasmids may have integrated or rearranged such that they could not be propagated in E. coli. Analyses of total genomic DNA from positive and negative cell lines were made after digestion with EcoRI (two sites within the vector) or BamHI (to release insert cDNA). Southern blots analyzed with radiolabeled probes containing EBO-pcD sequences of the EBO-pcD Leu 2 plasmid (Fig. 10) or pcD containing plasmids such as pcD-DHFR (dihydrofolate...
reduced cDNA inserted into the cloning site of the pcD vector (30), or only SV40 promoter sequences (data not presented) identified plasmid sequences. Different restriction patterns were obtained from the various transfectants indicating that the integration had occurred at different sites in the genome or that rearrangements and concatemeric forms were generated. The number of copies of integrated vector sequences in UC/HeLa 2-5 was estimated (by comparison of autoradiographic band intensity with known amounts of DNA) to be approximately 30 copies per cell.

One consideration for the absence of recoverable episomal DNA in transfected cells may be the duration of time between transfection and the selection of receptor enriched cells, normally a period of 6–8 weeks, during which time the plasmid could rearrange or integrate into genomic DNA. One way to reduce the probability of integration or recombination would be to decrease the time taken for selection. One such experiment is shown in Fig. 11. Here, 100 μg of EBO-pcD NG108 DNA were transfected into 1 × 10⁶ UC cells; the cells were immediately transferred to hygromycin-containing medium and analyzed in the FACS 4 days later to measure expression from the introduced plasmids. This 4-day period was selected arbitrarily to allow cells to recover from the shock of electroporation and is comparable with a transient expression assay.

Contrary to our experience with stably transformed pools of UC cells, a distinct population of cells with high TNF receptors was observed (Fig. 11B, arrow). These cells were recovered and are referred to as sort 1B (S1B). They were transferred to growth medium and expanded into a larger population. From these, 2 × 10⁶ cells were removed for the preparation of episomal DNA while 1 × 10⁶ cells were cycled through repeated flow selection of the brightest 1–2% of cells. These cells remained amplified through subsequent sorts (Fig. 11, S3B and S5B). S1B cells increased in receptors 30-fold over UC/ums cells and S3B and S5B another 3-fold amounting to a total 90-fold amplification. From 2 × 10⁶ cells of S1B low molecular weight DNA was recovered and used for bacterial transformation to yield about 1 × 10⁶ bacterial colonies. These plasmids were expected to represent a population enriched in genes conferring TNF receptor abundance. Plasmid DNA was prepared from a pool of these transformants, and 100 μg of DNA was transfected into a fresh population of 1 × 10⁶ UC cells which were flow cytometrically analyzed 4 days later. No increase in TNF receptor was seen in these cells after 4 days of recovery or in cells selected for high FITC-TNF binding even after seven cycles of sorting and expansion. Low molecular weight DNA was prepared from the selected cell populations and, except for S1C (see Fig. 9E) which yielded 20 transformants, no plasmids were recovered from DNA of any other sort upon repeated attempts. Restriction endonuclease digests of plasmid DNA from S1B and S1C with BamHI to release insert cDNA showed that the insert size was either very small (less than 500 base pairs) or absent. Transient expression assays have also been performed with other cells such as COS-7 and 293S as recipients for HeLa and NG108 cDNA libraries. In these experiments no receptor enriched cells were obtained upon initial transfection nor repeated flow selection of the brightest 2% of cells. Plasmid recovery was low and repeated transfections with rescued plasmid DNA failed to generate positive cells. Plasmids can be recovered from recipient cells but their abundance declines rapidly within 2–3 weeks after transfection, and, when plasmids are recovered, they have either very small cDNA inserts or have no detectable inserts (data not shown).

**DISCUSSION**

The diversity of biological effects induced by TNF is initiated by its interaction with cell surface receptors. The nature of the signals transduced by this interaction is not yet known, but, as a step toward understanding the signal transduction pathways, a knowledge of the receptor is required. Receptors for TNF are found on most cell types but their number is usually in the range of a few thousand per cell (7, 9–12). Because of its low abundance, purification of the receptor is a difficult task. To overcome this limitation we have combined the expression of transfected cDNAs with a powerful selection using the FACS to identify rare cells expressing high levels of receptor. Using this approach we have succeeded in isolating UC cell lines from both HeLa cDNA and NG108 cDNA transfected cells that express TNF receptor at levels 150-fold higher than untransfected UC cells. The results of the HeLa cDNA transfections were particularly interesting since only one size fraction of the cDNA library, that with 1.6–2.9-kb inserts, conferred increased receptor density in two separate sets of independent transfections. These observations suggest the presence of a specific cDNA sequence in the HeLa cell library responsible for the increase in receptors. However, the mechanism by which receptor numbers are increased is not clear. The selection pressures that were applied to select and maintain stable transfectants were maintenance on 200 μg/ml hygromycin and flow cytometric sorting for high TNF receptor levels. The cDNA responsible for conferring this phenotype could possibly be a full length sequence encoding the receptor, integration and/or amplification of which causes receptor numbers to increase. It could also be due to a partial cDNA sequence whose insertion into the resident TNF receptor gene followed by amplification could lead to higher levels...
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FIG. 10. Analysis of vector DNA in cell transfectants. Genomic DNA was prepared from different cell lines and digested with either BamHI (B) or EcoRI (R) endonucleases and separated by electrophoresis in 0.8% agarose gels, stained with ethidium bromide to visualize DNA, and then transferred to nitrocellulose and hybridized to radiolabeled probe. A, ethidium bromide-stained gel showing restriction endonuclease-digested DNA and in the left margin the positions of the size markers. B, corresponding Southern blot hybridized with EBO-pcD, specific DNA probe containing pBR, SV40, hygromycin, ori-P and EBNA-1 sequences minus a cDNA insert to locate sequences homologous to the Epstein-Barr virus or pcD-derived vector DNA. UC cells are EBV-transformed and contain unintegrated EBV-sequences in the genome.

of expression. Alternatively, we may not be selecting a receptor encoding gene but one whose protein product could up-regulate receptor numbers. Such a cellular product, e.g. interferon-γ, has been reported, although the magnitude of increase by it is a modest 2-3-fold (15, 17, 23).

Scatchard analysis of steady-state receptor binding with 125I-TNF shows that indeed the dissociation constant of the over-expressed receptor is characteristic of high affinity receptors and similar to that of HeLa and UC cells with a Kd in the range of 0.9-2 x 10^-9 M. Cross-linking studies were performed with the receptor-enriched transfectants using 125I-TNF and the cross-linking reagent bis-sulfosuccinimidyl suberate. Cross-linked complexes were observed when 125I-TNF was incubated with the cells in the absence of cold TNF and followed by treatment with the cross-linking reagent. Two protein bands were observed in gels at positions corresponding in size to 87 and 104 kDa consistent with previously observed bands (11, 13, 16, 23). Neither band was seen if cross-linking was performed in the presence of cold TNF. It appears that the molecular mass of the receptor observed in our studies is approximately 68-70 kDa if these complexes represent the binding of TNF monomer and dimer, respectively. However, if the complexes represent binding of a dimer and timer of TNF (27, 28) then the receptor itself may be 50-55 kDa.

Given the high concentration of the receptor in amplified cells we have undertaken to characterize its size and ligand binding properties in order to eventually purify the protein to homogeneity. An assay developed to measure 125I-TNF binding activity. Nonreducing and reducing SDS-polyacrylamide gels (stained for protein and silver) showed a 68-kDa band in enriched cell extracts of amplified cells. This band was difficult to find in comparable preparations from UC cells. Therefore, our identification of the 68-kDa protein as the receptor is based on the use of the ligand binding functional assay and the presence of a protein band coincident with the activity. The usefulness of the immunoaffinity procedures employed here brings into question an earlier published report (13) describing the failure of TNF receptor to bind to TNF immobilized to agarose and its poor recovery and lack of enrichment from lectin-affinity resins. Possibly, the rich source of receptor in the amplified cells plus the development of a solubilized receptor assay to trace the receptor has helped us to optimize the use of these affinity chromatography procedures.

The sizes of the TNF-receptor complexes are approximately 87 and 105 kDa as determined by cross-linking 125I-TNF to the receptor on intact cells. The estimated size of the TNF receptor deduced from the ligand blot is 68 kDa. The subtracted difference approximates the sizes of the TNF monomer (17 kDa) and dimer (34 kDa). Although TNF reportedly self-associates into a trimer (27, 28), there has been considerable discussion as to the preferred size of the TNF molecule that associates with the receptor. Since the intensity of the 87-kDa receptor-ligand complex is greater than the 105-kDa band (Fig. 7), our data reveal for the first time that TNF binds to its receptor predominantly as a monomer and to a lesser extent as a dimer.

An important aspect of the approach we employed was the use of the EBO-pcD vector for the construction of the libraries. These libraries were initially prepared in the pcD vector containing the SV40 early region promoter and the late splic-
Fig. 11. Flow cytofluorometric analysis of UC cell transfectants obtained with DNA from NG108 cDNA library or plasmids rescued from TNF-R amplified cells. Panel A, control UC/uns cells stained with FITC-TNF prior to transfection to profile level of receptor expression. Panel B, sort 1B from 2 x 10^6 UC cell transfectants generated by electroporation of 100 μg of DNA from NG108 cDNA library into 1 x 10^6 cells after 4 days of growth in hygromycin-containing medium to reveal a distinct population of TNF-R amplified cells (arrow). Five x 10^6 cells from this population were grown for isolation of episomal DNA and for subsequent rounds of sorting. Panels C and D, sorts 3B and 5B are from two subsequent sorts to show retention of receptor amplified phenotype. Panel E, sort 1C are UC cells electroporated with 100 μg of plasmid DNA obtained from 1000 amp' bacterial colonies transformed with episomal DNA of S1B cells marked by the arrow in panel B. Panels F and G, sorts 3C and 5C, two subsequent sorts of the top 5% of cells from sort 1C to show lack of receptor enriched cells. a.u. denotes arbitrary units.

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Addendum—After this manuscript was submitted for publication, Smith and Baglioni (45) using ligand blotting reported 60- and 70-kDa receptors on HeLa cells. These results are partially consistent with our estimates of a 68-kDa receptor.

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