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Role of 55- and 75-kDa Tumor Necrosis Factor Membrane Receptors in the Regulation of Intercellular Adhesion Molecules-1 Expression by HL-60 Human Promyelocytic Leukemia Cells in Vitro

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Abstract. Most human cells express two TNF and lymphotoxin (LT) membrane receptors (TNF-R), of 55 and 75 kDa. The regulatory effect of these two receptors on intercellular adhesion molecules (ICAM-1) expression was examined in various human cell lines in vitro, including human lymphokine-activated killer T cells (T-LAK) cells and HL-60 cells. Rabbit antihuman TNF-R antisera specific for each receptor were employed as probes to selectively stimulate 55- and 75-kDa TNF/LT membrane receptor production. These antisera compete with TNF/LT binding to each specific cell membrane receptor and have been found to bind to specific membrane receptors on various human cell lines in vitro. In the present study, we demonstrated biologic activity for anti-55-kDa TNF-R antiserum. For example, antibodies that bind to the 55-kDa TNF-R caused cytolysis of HeLa and ME-180 human cervical cancer cells and induced proliferation of MRC-5 human fibroblasts. In contrast, however, anti-75-kDa TNF-R antiserum demonstrated no bioactivity in these assays. In addition, no synergy or costimulation was observed when a combination of both anti-55- and anti-75-kDa TNF-R antisera were tested in these assay systems. Anti-55-kDa TNF-R antiserum up-regulated ICAM-1 expression on human HL-60, T-LAK, and THP-1 cells, whereas anti-75-kDa TNF-R antiserum had no effect. Unexpectedly, however, ICAM-1 expression was greatly enhanced by the addition of anti-75-kDa TNF-R to the anti-55-kDa TNF-R containing culture. This enhancing effect was also observed with human T-LAK cells and THP-1 monocytic leukemia cell, in vitro. *Journal of Immunology,* 1993, 150: 5070.

The human cytokines, LT/TNF-β[2] and TNF/TNF-α, exert their biologic effects through binding to two different membrane receptors (1). A TNF/LT receptor of 55 kDa was the first to be identified and cloned (2–4). The human 55-kDa receptor has a high affinity for both human rLT and rTNF. Subsequently, a second membrane receptor of 75 kDa was isolated from human continuous cell lines and cloned (5, 6). The 75-kDa membrane receptor also possesses high affinity for both TNF and LT (7, 8). These receptors have 40% AA sequence homology in their extracellular N terminal domains but show no AA sequence homology in their cytoplasmic domains. Identification of these two related, yet distinct, cell membrane TNF-R has raised questions as to their function and expression.

Antibodies that bind to each receptor and also mimic the effects of TNF or LT binding have been employed as specific probes to examine the role of each receptor in the biologic activity of these two cytokines. Monoclonal and polyclonal antibodies to the human 55-kDa TNF-R have been shown to have cytolytic activity against LT/TNF-

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[2] Abbreviations used in this paper: LT, lymphotoxin; R, membrane receptor; ICAM, intercellular adhesion molecules; FBS, fetal bovine serum; T-LAK, human lymphokine-activated killer T cells; NRS, normal rabbit serum; MC, median channel.

sensitive tumor cell lines in vitro (9–11). The cytolytic effect exerted by anti-55-kDa TNF-R antibody parallels that by human rLT/TNF, as indicated both by their similar dose-response relationships and their synergy with IFN-γ (10, 11). However, the respective Fab fragment pools of these same sera do not elicit the cytolytic effect (10), suggesting that cytolysis may require capping of 55-kDa TNF-R.

In contrast, other mAb to both human 55- and 75-kDa TNF-R will block cytolytic activity of human rTNF and rLT on cells in vitro (10, 12). Theoretically, these inhibitory antibodies act specifically by binding to the TNF-R and interfere with the binding of TNF and LT (13).

It has been reported that the cytolytic activity of LT and TNF on some human and mouse cell lines in vitro is mediated via the 55-kDa TNF-R (9–11). In fact, both TNF/LT receptors have demonstrated diverse biologic functions in previous work. Utilizing antireceptor antibodies as specific probes, several investigators recently have begun to investigate the roles of these two receptors in the variety of biologic effects induced by TNF and LT. For example, the intracellular expression of NF-kB in human promyelocytic leukemia HL-60 cells is up-regulated by antibodies that bind to the 55-kDa TNF-R (14, 15). Proliferation of human fibroblast (10), production of prostaglandin E_2 (10), and synthesis of Manganase-superoxide dismutage in cells can be induced by anti-55-kDa TNF-R antibodies (16). The 75-kDa receptor appears to be active in TNF-induced up-regulation of NF-kB expression (14), and killing activity of human LAK (13). In contrast, however, antiserum to the 75-kDa human TNF-R failed to elicit TNF/LT biologic activity in a variety of human cell lines that predominantly express the 75-kDa TNF-R (11).

It is now becomming apparent that the ICAM and lymphocyte function Ag are intimately involved in cell-to-cell contact during proliferation, differentiation, and target cell killing (17–19). Adhesion molecules also coordinate various types of interimmune cell communication, such as T cell-B cell and T cell-macrophage interaction (20). ICAM-1, in particular, plays a crucial role in inflammatory processes, Ag recognition, and lymphocyte-induced cell lysis (21, 22). ICAM-1 expression on endothelial cells and keratinocytes can be regulated by exposure to LT/TNF (23, 24).

The human promyelocytic leukemia cell line, HL-60 can differentiate into macrophages/monocytes or into granulocytes in vitro (25). When HL-60 cells are stimulated by agents such as PMA or TNF, they differentiate into monocyte/macrophages. During this transformation, there is an up-regulation of adhesion molecules such as ICAM-1 and lymphocyte function Ag-1 (25, 26). The present studies were conducted to further examine the individual and combined role of the 55- and 75-kDa receptors in TNF/LT-induced cytolysis, proliferation, and control of ICAM-1 expression on cells in vitro.

Materials and Methods

Reagents

Forms of the extracellular domain of the human 55- and 75-kDa rTNF-R proteins were generous gifts of Dr. T. Kohno (Synergen Inc., Boulder, CO). Human rLT and rTNF were donated by Genentech Inc. (South San Francisco, CA). Human rIL-2, specific activity 1.8×10^7 U/mg, was a gift from Hoffmann-La Roche (Nutley, NJ). IFN-γ was purchased from Genzyme Corp. (Cambridge, MA). Actinomycin D (ActD) and Phytohemagglutinin-P (PHA-P) were obtained from Sigma Chemical Co. (St Louis).

Antisera

Anti-55- and anti-75-kDa TNF-R antisera were obtained by intradermal immunization of New Zealand white rabbits with 100 μg of recombinant receptor protein emulsified in Freund's complete adjuvant, by the method of Yamamoto et al (27). The specificities of these antisera were confirmed by the establishment of an ELISA for each receptor as described previously (28). No cross-reactivity was observed when antisera were tested against each TNF-R, and no reactivity was observed when antisera were tested against human recombinant forms of LT, TNF, IFN-γ, IL-1β, IL-2, IL-4, and IL-6. The IgG fraction of antisera was prepared by passing sera over a Protein G Sepharose 4B column as described by Pharmacia LKB (Uppsala, Sweden). The $F(ab')_2$ fraction of IgG was prepared by 2% pepsin (Sigma) digestion for 18 h at 37°C. For FACS analysis, anti-55- and anti-75-kDa TNF-R rabbit $F(ab')_2$ fragments were further affinity-purified using 55- or 75-kDa rTNF-R proteins coupled with CNBr-activated Sepharose 4B. $F(ab')_2$ fragments of rabbit IgG from nonimmunized animals were also prepared. Phycoerythrin-conjugated anti-CD54 (ICAM-1: Leu-54, IgG_{2b}) was purchased from Becton-Dickinson (Mountain View, CA).

Cells

The following human cell lines were obtained from the American Type Culture Collection (Rockville, MD): HeLa and ME-180 (cervical cancer), HL-60 (promyelocytic leukemia), K562 (myelocytic leukemia), THP-1 (monocytic leukemia), U937 (histiocytic leukemia), and MRC-5 (lung fibroblasts). All cell lines were cultivated in RPMI 1640 supplemented with 10% FBS (Irvine Scientific, Irvine, CA).

Human PBMC were prepared from blood obtained from healthy normal donors by density gradient centrifugation using Histopaque 1077 (Sigma). Human polymorphonuclear leukocytes were prepared by density gradient centrifugation of peripheral blood using Histopaque 1077 followed by hypotonic lysis of red blood cells.

Human T-LAK cells were generated by coculture with IL-2 as previously reported (29). Briefly, PBMC were cultivated in AIM-V (GIBCO, Grand Island, NY) supplemented with 2% FBS and 400 IU/ml of IL-2, 0.4 μg/ml of PHA-P at a cell density of 2×10^6/ml. On the third day of culture, cells were passed to 0.5×10^6/ml using AIM-V, 2% FBS, and 400 IU/ml of IL-2 (LAK media). T-LAK cells were passed using LAK media at 48-h intervals thereafter and employed after 7 days.

TNF/LT in vitro cytolytic assay

The cytolytic assay was conducted in microplates employing ActD (0.5 μg/ml)-treated cells as described previously (2). Briefly, HeLa cells, 100 μl at 8×10^6 cells/ml/well were precultured for 5 h in 96-well microtiter plates to allow firm adherence. Then, 50 μl ActD 2-μg/ml and 50-μl samples were added to wells, and cultures were incubated for 20 h at 37°C in an atmosphere of 5% CO_2. After aspirating culture supernatants, 50 μl 1% crystal violet solution was added, and cultures were incubated for 15 min at room temperature. Subsequently, wells were washed with tap water and allowed to air dry. Stains were then lysed by 100 μl methanol, 150 mM HCl, and the OD at 580 nm was calculated using an EAR 400T plate reader (SLT Lab Instruments, Austria).

Cell proliferation assay

The MRC-5 fibroblast cell proliferation assay was conducted using 96-well microtiter plates. 2×10^4 cells in 100 μl were co-incubated in each well with 100 μl of each sample to be tested. After incubation for 72 h at 37°C in an atmosphere containing 5% CO_2, culture media was aspirated, and adherent cells were stained by 1% crystal violet. After incubation for 15 min at room temperature, plates were washed with tap water and then air dried. Stained cells were lysed by 100 μl methanol, 150 mM HCl, and the OD at 580 nm was measured using an EAR 400T ELISA plate reader.

FACS analysis

Cells were analyzed using FACScan (Becton-Dickinson) with or without pretreatment by anti-receptor antisera or cytokines. Cells were established in 24-well plates, and incubated for 18 h at 37°C in an atmosphere containing 5% CO_2 with either human rTNF (20 ng/ml) and LT (20 ng/ml), NRS (0.5% (v/v) of final concentration), anti-55-kDa (0.5%) or anti-75-kDa (0.5%) TNF-R rabbit antisera. For the pulse stimulation study, HL-60 cells were incubated with antisera for 1 h at 37°C in an atmosphere containing 5 CO_2. After washing with PBS, cells were reincubated for 18 h in culture media without antisera. After incubation, cells were washed with PBS containing 1% FBS, 0.1% NaN_3 (PBS-FBS). Then, 5 to 10×10^5 cells were incubated with phycoerythrin-conjugated mouse monoclonal anti-CD54 for 30 min at 4°C. Rabbit antibodies to human TNF were isolated by affinity chromatography over a TNF-sepharose column by the method of Gatanaga et al. (2), and $F(Ab')_2$ fragments of the IgG fraction were prepared by the method of Ey et al. (30).

When analyzing TNF-R expression, cells were incubated for 1 h on ice with 20 ng of affinity-prepared anti-TNF-R rabbit $F(ab')_2$ fragment or natural rabbit $F(ab')_2$ fragment, then stained with anti-rabbit IgG goat $F(ab')_2$ adsorbed by human serum conjugated with FITC (Tago, Burlingame, CA) on ice for 30 min. After incubation, cells were washed with PBS-FBS, and then fixed with 1% paraformaldehyde in PBS at 4°C for 18 h. After resuspension in FBS-PBS, cells were then subjected to FACS analysis. The FACS machine was calibrated in each series of studies using FACS Calibration Beads (Becton-Dickinson) and the AUTOCOMP program (Becton-Dickinson). Data were collected using the FACScan program (Becton-Dickinson), and antibody reactivities were analyzed by median fluorescence channels. For the analysis of macrophage/monocyte or lymphocyte populations in the PBMC fraction, cells were stained without separation. All data were analyzed by using morphologic gates set by forward scatter and side scatter for peripheral blood macrophage/monocyte and lymphocyte cell populations, respectively.

Binding assay

Human rLT was ^{125}I-labeled by the chloramine T method (31). Briefly, 500 μCi $Na^{125}I$ and 25 μl of 2 mg/ml chloramine T were added to 5 μg rLT. After incubation for 60 sec at room temperature, 50 μl stop buffer (2.4 mg/ml sodium metabisulfate, saturated tyrosine, 0.1% xylene cylanol, in PBS) were added, and the solution was mixed well. Then, labeled protein was separated from unbound ^{125}I by passing the reaction solution over an NAP-5 column (Pharmacia LKB), and the ^{125}I-bound fraction was then collected. The concentrations of ^{125}-I-labeled LT were determined by specific ELISA kits (R&D Systems, Minneapolis, MN). The specific radioactivity was 100 μCi/μg protein.

HeLa, ME-180, and MRC-5 cells were established as monolayers in 12-well plates. Human U937 and HL-60 cells were maintained in 150-cm^2 flasks, and established at 2×10^6 cells/ml in 15-ml centrifuge tubes (Corning Glass, Corning, NY). After washing with PBS, cells were incubated with 0.5% anti-55- and 0.5% anti-75-kDa TNF-R rabbit antisera (0.25% each, where antisera were tested in combination), or 0.5% NRS, in RPMI 1640 supplemented with 10% FBS for 2 h at 37°C, 5% CO_2. After washing with ice-cold PBS, cells were incubated with 1 nM ^{125}I-LT in RPMI 1640 supplemented with 10% FBS, 0.1% NaN_3 1 mM HEPES (binding buffer) for 2 h at 4°C. For controls estimating the degree of nonspecific binding, 100 times

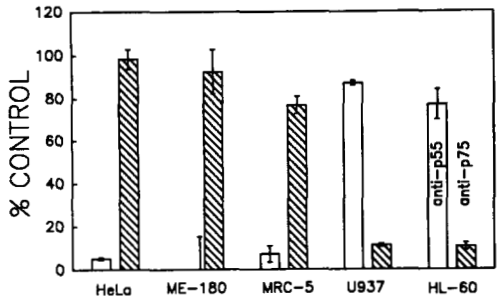

FIGURE 1. Expression of 55- and 75-kDa TNF-R as determined by the blocking effect of anti-55- and anti-75-kDa TNF-R antisera on the binding of ^{125}I-LT on various cells. Cells were incubated with each anti-TNF-R antiserum (0.5% serum) for 2 h at 37°C, 5% CO_2. After washing by PBS, cells were incubated with 10 nM of ^{125}I-LT in binding buffer for 2 h at 4°C. Finally, cells were washed with cold PBS; cellular radioactivity was determined as described in *Materials and Methods*. Data are expressed in cpm as percentage of NRS control.

excess rLT was added. After washing twice with ice-cold PBS, cells were lysed by 1 ml of 1% SDS, 1 N NaCl, and radioactivity was determined using a Clinigamma counter (Pharmacia LKB). Results were triplicate-averaged specific counts of each anti-TNF-R antiserum tested, expressed as percentage of control (assays using NRS).

To determine the effects of anti-TNF/LT antisera on TNF-R expression, HL-60 cells were pretreated with anti-TNF-R antisera or NRS (0.5%) for 16 h at 37°C, 5% CO_2. After discarding culture media, (2×10^6 cells) were subjected to the binding assay employing the addition of specific anti-TNF-R antisera, as described above, to discriminate specific TNF-R expression. Again, results were triplicate-averaged specific counts of each anti-TNF antiserum tested, expressed as % control.

Results

Specific inhibition of ^{125}I-labeled human TNF and LT binding to cells in vitro by anti-55 and anti-75-kDa antibodies

Adherent cell lines were analyzed for expression of 55- and 75-kDa TNF-R by measuring specific binding of ^{125}I-LT and competition of binding with antiserum against 55- and 75-kDa TNF-R (Fig. 1). When the adherent cell lines HeLa, ME-180, and MRC-5 fibroblasts were pretreated with anti-55-kDa TNF-R antiserum, specific ^{125}I-LT binding was blocked by 90% to 100% of NRS-treated controls. On the other hand, treatment of these same cells with anti-75-kDa TNF-R antiserum only affected specific LT binding by 5% to 20% of NRS-treated controls. The opposite pattern was observed for nonadherent human U937 and HL-60 cell lines. Specific ^{125}I-LT binding was blocked only by 10% of NRS-treated controls when U937 and HL-60 cells were pretreated with anti-55-kDa TNF-R antiserum. However, LT binding was blocked by 90% of NRS-treated control when these same cells were preincubated with anti-75-kDa TNF-R antiserum. Pretreatment of these same cell lines with a combination of both anti-55 and anti-75-kDa TNF-R antisera had similar effects on ^{125}I-TNF binding (data not shown). These antisera are specific, do not cross-react, and compete with cytokine binding to each specific receptor.

Table I
Expression of 55- and 75-kD TNF-R on cells as determined by FACS analysis[a] (median channel)

	CTRL[b]	anti-p55[c]	anti-p75[d]
HL-60	334	402	543
U937	422	471	577
T-LAK	282	309	582
PMN	252	247	369
PBL	92	93	244
Peripheral blood monocytes	155	156	259

[a] Cells were prepared and incubated with anti-55- or anti-75-kDa TNF-R rabbit F(ab')$_2$, followed by staining using FITC-conjugated anti-rabbit Ig. After washing, cells were subjected to analysis using FACS. Antibody immunoreactivities are expressed as median fluorescence channel.
[b] Samples stained with natural rabbit F(ab')$_2$.
[c] Samples stained with anti-55-kDa TNF-R antibody.
[d] Samples stained with anti-75-kDa TNF-R antibody.

FACS analysis of TNF-R expression and anti-TNF-R antisera binding

Suspended cells were also analyzed for expression of 55- and 75-kDa TNF-R by FACS analysis using F(ab)$_2$ fragments of IgG prepared from anti-receptor serum (Table I). T-LAK and HL-60 cells showed the highest MC shift, of 209 for HL-60 and of 300 for T-LAK, for 75-kDa TNF-R expression. This was followed by that for U937 cells and PBL, each with MC shifts of 150. Peripheral blood neutrophils and monocytes each demonstrated MC shifts of 100 when exposed to the same antisera. For all cells tested, 55-kDa TNF-R expression was observed to be lower than that of 75-kDa TNF-R. Specifically, HL-60 cells showed highest MC shift, of 70, followed by U937 cells (MC shift of 50) and T-LAK cells (MC shift of 30). We found that the shifts produced by each antisera were specifically blocked by the addition of purified human 55- or 75-kDa rTNF-R proteins to the reaction (data not shown). This latter result further supports the contention that these antisera bind specifically and directly to each TNF/LT membrane receptor.

Anti-55- and anti-75-kDa TNF-R antisera express biologic activity similar to that of TNF/LT

Antibodies to the 55-kDa TNF-R previously were reported to be lytically active when incubated with the LT/TNF-sensitive cell lines HeLa, MCF-7, and U937 in vitro. To establish biologic activity of our antisera, we tested the ability of rabbit anti-55- and anti-75-kDa TNF-R antisera to induce lysis of the LT-sensitive HeLa and ME-180 cell lines in vitro. The data in Figure 2*A* and *B* indicate that

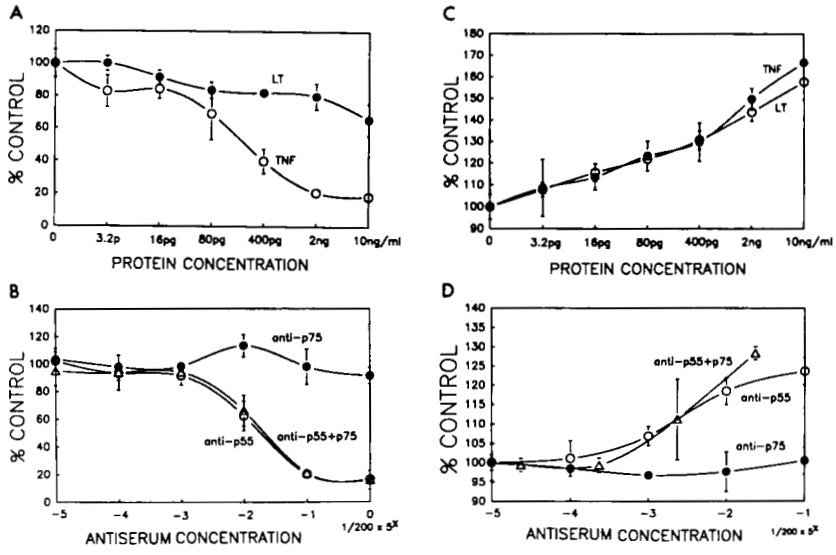

FIGURE 2. Cytolytic, and proliferation induced by LT/TNF and anti-55 and anti-75-kDa TNF-R antisera. LT/TNF or anti-TNF-R antiserum was assayed for cytolytic activity on HeLa (human cervical cancer) cells (*A* and *B*), and for a proliferative effect on MRC-5 (human fibroblast) cells (*C* and *D*) as described in *Materials and Methods*. *A*, cytolytic effect of LT (●) and TNF (○) on HeLa cells; *B*, cytolytic effect of anti-55-kDa TNF-R antiserum (○), anti-75-kDa TNF-R antiserum (●), and both anti-TNF-R antisera (△) on HeLa cells; *C*, proliferative effect of LT (○) and TNF (●) on MRC-5 cells; *D*, proliferative effect of anti-55-kDa TNF-R antiserum (○), anti-75-kDa TNF-R antiserum (●), and both anti-TNF-R antisera (△) on MRC-5 cells.

HeLa cells, expressing predominantly 55-kDa TNF-R, are lysed by human rLT/TNF. Anti-55-kDa TNF-R antiserum exhibited cytolytic activity; however, anti-75-kDa TNF-R antiserum demonstrated no such activity. In addition, no antagonism or synergy of cytolytic activity was observed by co-incubation of HeLa cells with both anti-55- and anti-75-kDa TNF-R antisera (Fig. 2*B*). Similarly, ME-180 cells were lysed by anti-55-kDa but not by anti-75-kDa TNF-R antiserum (data not shown).

As is depicted in Figure 1, MRC-5 fibroblasts express predominantly the 55-kDa TNF-R. We found that the ability of LT/TNF to induce proliferation of MRC-55 cells could be reproduced by anti-55-kDa but not anti-75-kDa TNF-R antiserum (Fig. 2*D*). There was no additive effect when cells were cocultured with both anti-55- and anti-75-kDa TNF-R antisera (Fig. 2*D*).

Induction of ICAM-1 expression by culture of various human cell lines with anti-55 and anti-75-kDa TNF-R antisera

After exposure to either LT/TNF or anti-TNF-R antisera, suspended cell lines and normal human peripheral blood cells were FACS-analyzed for ICAM-1 expression (Table II). Human T-LAK cells incubated with LT/TNF for 18 h showed moderate up-regulation of ICAM-1 expression. ICAM-1 expression on human T-LAK cells was also up-regulated by incubation with either anti-55- or anti-75-kDa TNF-R. A similar effect was observed with HL-60, THP-1, and U937 cells, although U937 cells exhibited only a minimal response. In contrast, anti-TNF-R antisera had no observable effect on ICAM-1 expression on K562 cells, neutrophils, lymphocytes, or monocytes freshly isolated from peripheral blood.

Induction of ICAM-1 expression on HL-60 cells by LT/TNF and anti-TNF-R antisera

Up-regulation of ICAM-1 expression on HL-60 cells was further investigated (Fig. 3). As shown in Table II, ICAM-1 expression on HL-60 cell was greatly up-regulated by 18 h of incubation with LT/TNF. When HL-60 cells were incubated with anti-55-kDa antiserum, the median channel shifted from 290 (control) to 328, whereas incubation with anti-75-kDa TNF-R antiserum had no effect on the MC level. Strikingly, however, when HL-60 cells were co-incubated for 18 h with both anti-55- and anti-75-kDa TNF-R antisera, expression of ICAM-1 was greatly increased (MC = 577). As a control, the co-incubation of a mouse mAb recognizing nonhuman protein did not effect the MC of cells incubated with either LT/TNF or anti-TNF-R antisera (data not shown). Up-regulation of ICAM-1 expression on HL-60 cells by both anti-55- and 75-kDa TNF-R antisera was blocked by co-culture with human 55- and 75-kDa rTNF-R proteins (Fig. 3*C*). Additional studies revealed that incubation of HL-60 cells for 18 h with human interferon gamma (IFN-γ) further up-regulated TNF-induced or anti-TNF-R antisera-induced expression of ICAM-1 (Fig. 4*A* and 4*B*).

Dose-dependent effects of anti-TNF-R antisera and LT/TNF on ICAM-1 expression on HL-60 cells

We examined up-regulation of ICAM-1 expression by various concentrations of anti-55-kDa TNF-R antiserum in the presence of a fixed concentration (0.5%) of anti-75-kDa TNF-R antiserum (Fig. 5*A*). Maximum up-regulation was observed between 0.1% and 0.5% of the anti-55-kDa TNF-R antiserum, increasing in a dose-dependent manner.

Table II
Expression of ICAM-1 (CD54) on cells as determined by FACS analysis[a] (median channel)

	[b]	CTRL[c]	LT	TNF	NRS[d]	p55[e]	p75[f]	p55/p75[g]
T-LAK (A)	243	485	510	548	501	516	508	546
T-LAK (B)	232	506	545	529	540	535	591	617
PMN	214	329	331	324	358	360	361	356
PBL	143	376	382	366	377	382	372	378
Peripheral blood monocytes	212	596	603	589	601	607	595	607
K562	464	716	710	726	720	730	721	726
HL-60	290	298	613	736	290	328	292	577
THP-1	410	533	685	708	580	597	603	710
U937	386	726	724	772	756	747	743	772

[a] Cells were incubated with LT/TNF or anti-TNF-R antisera as described in *Materials and Methods*. After washing, cells were stained by phycoerythrin (PE)-conjugated anti-CD54 (ICAM-1) mouse monoclonal antibody, and analyzed using FACS. Immunoreactivities were expressed as median fluorescence channel.
[b] Unstained samples stained by using nonhuman protein-specific mouse mAb and PE-conjugated anti-mouse Ig.
[c] Unstimulated samples stained by anti-CD54 mouse monoclonal antibody.
[d] Normal rabbit serum (0.5%)-treated samples.
[e] Anti-55-kDa TNF-R antiserum (0.5%)-treated samples.
[f] Anti-75-kDa TNF-R antiserum (0.5%)-treated samples.
[g] Anti-55- and anti-75-kDa TNF-R antisera (0.5% each)-treated samples.

Higher concentrations of anti-55-kDa antiserum produced an inhibitory effect.

The converse experiments were also conducted, analyzing various concentrations of anti-75-kDa TNF-R antiserum in the presence of a fixed concentration (0.5%) of anti-55-kDa TNF-R antiserum (Fig. 5*B*). In contrast to the effect of high anti-55-kDa antiserum concentrations, high concentrations of anti-75-kDa antiserum did not inhibit ICAM-1 expression. In fact, no effect on ICAM-1 expression on HL-60 cell was observed for any concentration of anti-75-kDa TNF-R antiserum tested.

Finally, we examined the effect of varying doses of both human rTNF and rLT on ICAM-1 expression on HL-60 cells (Fig. 5*C*). When samples were corrected for total protein concentration, we found that human TNF is more effective in the induction of ICAM-1 expression on HL-60 cells than is LT.

The effect of pulse-chase exposure of HL-60 cells to antireceptor antisera on ICAM-1 expression in vitro

HL-60 cells were exposed to either anti-55- or anti-75-kDa TNF-R antiserum for a 1-h period, washed to remove free antiserum, and then further incubated for 18 h in normal media (Table III). When HL-60 cells were pulse-treated by anti-55-kDa TNF-R antiserum, expression of ICAM-1 was increased beyond that of the NRS control (MC increase = 20, p value <0.05). This up-regulation was accentuated by exposure to both anti-55- and anti-75-kDa TNF-R antisera (MC increase = 45, compared with that for anti-55-kDa antiserum alone, p value <0.05).

The effects of anti-TNF-R sera on TNF-R expression on HL-60 cells

HL-60 cells were preincubated with anti-55- or anti-75-kDa TNF-R antiserum for 18 h. Subsequently, 55- and 75-kDa TNF-R expression on cells was determined by the com-

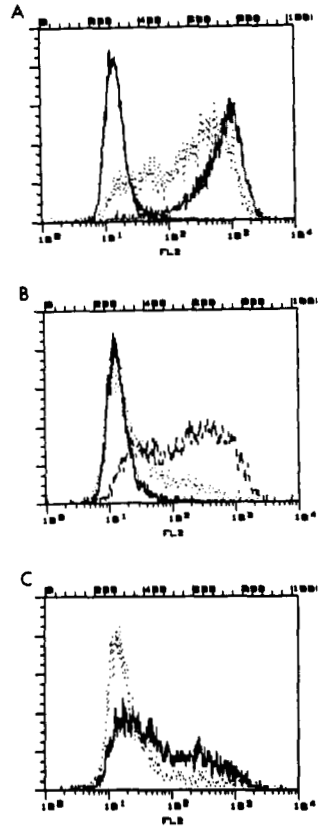

FIGURE 3. Induction of ICAM-1 expression on HL-60 cells by LT/TNF and anti-TNF-R antisera. HL-60 cells were treated with either LT/TNF or anti-TNF-R antisera for 18 h at 37°C, 5% CO_2, after which ICAM-1 expression was determined using FACS as previously described in *Materials and Methods*. *A*, treatment with 20 ng/ml of LT (– – –) and TNF (. . . .); *B*, treatment with 0.5% serum of anti-55-kDa TNF-R antiserum (– – –), anti-75-kDa TNF-R antiserum (. . . .), and by 0.5% each of both anti-TNF-R antisera (—); *C*, HL-60 cells were treated for 18 h with both anti-TNF-R antisera (0.5% each) with (. . . .) or without (—) the addition of 55-kDa and 75-kDa rTNF-R proteins as an adsorption test.

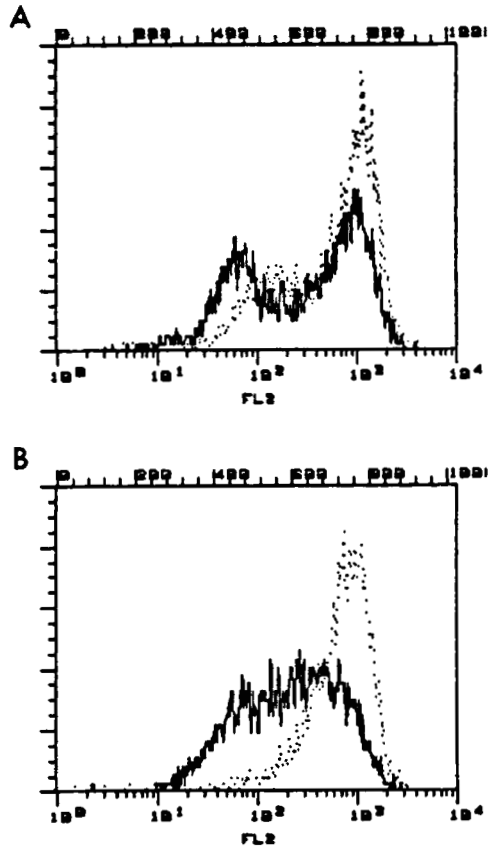

FIGURE 4. IFN-γ enhancement of TNF or anti-TNF-R antisera induce ICAM-1 expression. HL-60 cells were incubated with TNF (20 ng/ml) or both anti-55- and anti-75-kDa TNF-R antisera (0.5% each) in the presence or absence of IFN-γ (50 ng/ml), afterwhich ICAM-1 expression was determined by FACS as described in *Materials and Methods*. *A*, TNF effect on ICAM-1 expression in the presence of (– – –) absence of (—) IFN-γ; *B*, anti-TNF-R antisera effect on ICAM-1 expression in the presence (– – –) or absence (—) IFN-γ.

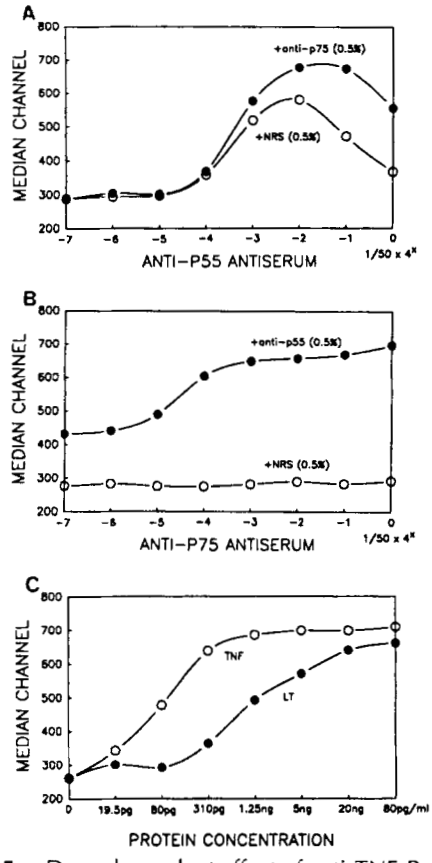

FIGURE 5. Dose-dependent effect of anti-TNF-R antiserum or LT/TNF on ICAM-1 expression on HL-60 cells. HL-60 cells were incubated with anti-TNF-R (*A* and *B*) and LT/TNF (*C*) for 18 h at 37°C, 5% CO_2, after which ICAM-1 expression was determined by FACS as described in *Materials and Methods*. *A*, cells were incubated with various concentrations of anti-55-kDa TNF-R antiserum in the presence of a fixed concentration (0.5% serum) of anti-75-kDa TNF-R antiserum (●) or NRS (○); *B*, cells were incubated with various concentrations of anti-75-kDa TNF-R antiserum in the presence of a fixed concentration (0.5%) of anti-55-kDa TNF-R antiserum (●) or NRS (○); *C*, cells were incubated with various concentrations of LT (●) or TNF (○).

petitive binding assay, using ^{125}I-LT and anti-TNF-R antisera (Table IV). Treatment of HL-60 cells with anti-75-kDa TNF-R resulted in a slight decrease in ^{125}I-LT binding attributable to the 55-kDa TNF-R. In contrast, pretreatment of HL-60 cells with anti-55-kDa TNF-R antiserum significantly up-regulated ^{125}I-LT binding attributable to the 75-kDa TNF-R.

Discussion

The functionally and structurally related cytokines, LT and TNF, exert multiple and diverse biologic effects on receptive cells and tissue (32). The initial step in inducing these effects is binding to specific 55- and 75-kDa membrane receptors. The receptor ligand binding signal is then transduced across the cell membrane to initiate a cellular response (1). Most mammalian cells thus far studied express both receptors; however, one class of receptor, 55- or 75-kDa, may predominate in a given cell type. We examined the role of 55- and 75-kDa TNF-R, both singly and in combination, in such processes as cytolysis, stimulation of effector cell proliferation, and induction of ICAM-1 expression in a diverse array of cell lines in vitro. These studies employed antisera that bind specifically with 55- and 75-kDa receptors and thereby mimic the activity of human rTNF/LT on these cells.

Antisera to human 55- and 75-kDa rLT/TNF-R were raised in rabbits. These antisera were shown to interfere specifically with the binding of radiolabeled TNF to each class of receptor, and FACS analysis verified the specificity of binding of each antibody type to its respective cell membrane receptor. Further experiments demonstrated that these antisera possess biologic activity strikingly similar to

Table III
Pulse stimulation study of HL-60 cells by anti-TNF-R antisera[a] (median channel)

NRS	anti-p55	anti-p75	anti-p55 + p75
349.7 ± 1.2+	370.0 ± 7.3+*	354.7 ± 4.5	93.7 ± 2.5*
			+,*: $p < 0.05$

[a] HL-60 cells were incubated with anti-TNF-R antisera for 1 h at 37°C, 5% CO_2 followed by washing with PBS (pulse stimulation). Then, cells were reincubated without stimulants for 18 h at 37°C, 5% CO_2. Cells were stained by PE-conjugated anti-ICAM-1 antibody, and analyzed using FACS. Immunoreactivities were expressed by median fluorescence channels (mean ± SD of triplicated samples).

Table IV
Regulation of expression of 55- and 75-kDa TNF-R on HL-60 cell by anti-TNF-R antisera[a] (cpm)

Pre-incubation[b]	Treatment[c]	
	anti-p55[d]	anti-p75[e]
NRS	31138 ± 1723*	5545 ± 252+
anti-p55	39732 ± 1557*	(1538 ± 106)
anti-p75	(15422 ± 1161)	4788 ± 189+
		p<0.05;+,N.S.

[a] HL-60 cells were preincubated with anti-TNF-R antisera for 18 h at 37°C, 5% CO_2. After washing with PBS, cells were treated by anti-TNF-R antisera to inhibit binding, as indicated, and analyzed for ^{125}I-LT binding capacity as an indication of each TNF-R expression.

[b] HL-60 cell were first incubated with anti-55- or anti-75-kDa TNF-R antisera (0.5%) for 18 h at 37°C, 5% CO_2.

[c] To discriminate each TNF-R expression, HL-60 cells were treated by these antisera for 2 h at 37°C, 5% CO_2 immediately prior to the binding assay.

[d] ^{125}I-LT binding capacity attributable to 75-kDa TNF-R expression.

[e] ^{125}I-LT binding capacity attributable to 55-kDa TNF-R expression.

that of TNF/LT, both for TNF/LT-mediated cytolysis and for growth stimulation of various human cell lines in vitro. The definitive evidence that antisera binding and activity were specific was the finding that antisera effects could be inhibited by incubation with corresponding free human recombinant receptors.

The 55-kDa TNF/LT receptor is primarily involved in cytolysis of human HeLa and ME-180 cells in vitro. Antisera to 55-kDa but not the 75-kDa receptor induced cytolysis of these two cell lines, with a dose-response curve similar to that observed with TNF itself. In addition, enhancement of the cytolytic activity of 55-kDa TNF-R antiserum was observed on U937 cells by addition of human rIFN-γ; the same effect is observed when these cells are exposed to TNF and IFN-γ (11). It was interesting that no cooperative effect was observed by using both anti-TNF-R antisera in the cytolytic assay. Anti-75-kDa TNF-R antisera has been reported to block TNF/LT-induced cytolysis of human U937 cells, which express mainly the 75-kDa TNF-R species (12). We obtained similar results using rabbit anti-75-kDa TNF-R serum and F(ab')$_2$ fragments of IgG alone (unpublished data).

The 55-kDa TNF receptor may also be the predominant receptor in TNF/LT-induced proliferation of human fibroblasts in vitro (10). Our studies revealed that anti-55-kDa TNF-R antiserum is as biologically active as human LT/TNF. This similarity was clearly seen in a dose-dependent manner when anti-55-kDa TNF-R was compared with both TNF and LT. In contrast, anti-75-kDa TNF-R antiserum did not show any such activity. In addition, no cooperative effect was observed when cells were exposed to both anti-TNF-R antisera. It is interesting that proliferation of mouse thymocytes and a mouse CTL cell line, both of which express predominantly the 75-kDa TNF-R, was induced by antimouse 75-kDa but not anti-55-kDa TNF-R polyclonal antibody (4). One can hypothesize then that the 75-kDa TNF-R may mediate proliferation signalling in lymphoid cells that express predominantly the 75-kDa TNF-R.

It appears that the 55-kDa TNF/LT receptor is the primary membrane receptor involved in TNF/LT-induced ICAM-1 up-regulation on human HL-60 cells in vitro; antiserum to the 55-kDa receptor induced up-regulation of ICAM-1 expression, whereas anti-75-kDa TNF-R antiserum did not show any effect. This finding is especially interesting because the 75-kDa receptor is the predominant membrane form in this cell line (11, 15) and suggests that factors other than cell surface receptor concentration may be involved in controlling the magnitude of cellular response. Furthermore, pulse-chase data indicate that the effects on ICAM-1 expression by anti-55-kDa and anti-75-kDa TNF-R antisera are present with even short durations of exposure. The interpretation of these relationships is further complicated by the observation that ICAM-1 expression was enhanced by simultaneous exposure of HL-60 cells to both antisera. This costimulation effect was also observed with T-LAK cells, which also express predominantly the 75-kDa TNF-R. Trefzer et al. (24) reported that ICAM-1 expression is up-regulated on human keratinocytes (which express predominantly 55-kDa TNF-R) in vitro, by exposure to mAb to the 55-kDa TNF-R but not by anti-75-kDa TNF-R. Unfortunately, however, the combined effect of anti-55- and anti-75-kDa TNF-R antibodies on keratinocytes was not examined; such data certainly would help to elucidate the role of individual receptors on ICAM-1 expression.

Although cells express both TNF membrane receptors, studies have shown that the 55-kDa form is responsible for initiating many of the biologic effects attributed to TNF. NF-kB activation in HL-60 cells is induced by anti-55-kDa TNF-R (15). Cytolysis of cancer cells (9–11), proliferation of fibroblasts (10), and induction of ICAM-1 expression (22) are also induced by anti-55-kDa but not anti-75-kDa TNF-R antibody. In contrast, anti-75-kDa TNF-R antibodies appear to be more involved in blocking the effects of TNF/LT on cells in vitro. Murine mAb against human 75-kDa TNF-R demonstrated inhibitory activity on TNF-induced U937 cell cytolysis (12), T-LAK cell cytolytic activity (13), and HL-60 expression of NF-kB (14). However, selective stimulation of the 75-kDa receptor did stimulate

cell division in certain cell lines. These apparently conflicting data indicate that the two receptors may mediate different biologic effects, depending on the type of effector cell.

The 55-kDa TNF/LT-R appears to be the more important of the two TNF/LT-R in induction of ICAM-1 expression in the cell lines we studied. ICAM-1 expression was up-regulated by exposure of human HL-60, THP-1 and T-LAK cells to the anti-55-kDa serum but not to anti-75-kDa serum. However, these two receptors may act cooperatively, because the induction of ICAM-1 expression by human HL-60 cells was further up-regulated when both receptors were stimulated in vitro. The mechanism of cooperativity is not clear. We found that anti-75-kDa TNF-R antiserum did not up-regulate of 55-kDa TNF-R expression, whereas anti-55-kDa antiserum moderately up-regulated 75-kDa TNF-R expression. It does not appear likely, therefore, that regulation of 55-kDa TNF-R expression by anti-75-kDa TNF-R antiserum is the mechanism responsible for ICAM up-regulation. It is interesting that a short-duration stimulation of the 75-kDa-R can potentiate 55-kDa TNF-R-mediated up-regulation of ICAM-1 expression on HL-60 cells. Future studies will further explore the molecular basis for differential function of each TNF-R.

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