

## Dual Role of the p75 Tumor Necrosis Factor (TNF) Receptor in TNF Cytotoxicity

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### Summary

Whereas there is ample evidence for involvement of the p55 tumor necrosis factor (TNF) receptor (p55-R) in the cytotoxic effect of TNF, the role of the p75 TNF receptor (p75-R) in this effect is a matter of debate. In this study, we probed the function of p75-R in cells sensitive to the cytotoxicity of TNF using a wide panel of antibodies (Abs) against the receptor's extracellular domain. Two distinct Ab effects were observed. The Abs triggered signaling for cytotoxicity. This effect: (a) was correlated with the extent of p75-R expression by the cells; (b) was dependent on receptor cross-linking by the Abs; (c) occurred in HeLa cells, but not in A9 cells transfected with human p75-R or in HeLa cells expressing cytoplasmically truncated p75-R mutants, indicating that it involves cell-specific activities of the intracellular domain of the receptor; (d) was synergistic with the cytotoxic effect of Abs against p55-R. Moreover, it seemed to reverse induced desensitization to the cytotoxic effect of anti p55-R Abs, suggesting that it involves mechanisms different from those of the signaling by the p55 TNF-R. In addition, the Abs affected the response to TNF in a way that does not involve the signaling activity of p75-R. These effects: (a) could be observed also in cells in which only p55-R signaled for the cytotoxic effect; (b) were not dependent on receptor cross-linking by the Abs; (c) varied according to the site at which the Abs bound to the receptor; and (d) were correlated inversely with the effects of the Abs on TNF binding to p75-R. That is, Abs binding to the membrane-distal part of the receptor's extracellular domain displaced TNF from the p75 receptor and enhanced cytotoxic effect, whereas Abs that bind to the membrane-proximal part of the extracellular domain — a region at which a conformational change seems to take place upon TNF binding — decreased the dissociation of TNF from p75-R and inhibited its cytotoxic effect. The above findings suggest that p75-R contributes to the cytotoxic effect of TNF both by its own signaling and by regulating the access of TNF to p55-R.

**T**NF initiates its multiple effects on cell function by binding to two distinct cell surface receptors (1–8). These two receptor species (p55-R<sup>1</sup> and p75-Rs) are expressed in many cell types, in various amounts and proportions. Both also exist in soluble forms, which are derived proteolytically from the extracellular domain of the corresponding cell surface form

(2, 9–11). The soluble TNF receptors (TNF-R) bind TNF in a reversible manner and can thus affect its access to the cell-surface receptors as well as its stability (12).

There is significant sequence homology between the extracellular domains of the two receptors. Both contain a four-fold repetition of a sequence module characterized by conserved location of several (usually six) cysteine residues, as well as of some other amino acid residues (4–8). Homologous repetitive cysteine-rich modules occur in the extracellular domains of a number of other receptors (the TNF/nerve growth factor [NGF] receptor family), including the low-affinity NGF receptor (13), the B cell antigen CD40 (14), the lymphoid activation antigen OX40 (15), the T cell antigens 4-1BB (16) and CD27 (17), the CD30 antigen (18), the Fas/APO-1 receptor (19, 20), and the T2 pox virus gene product (21). Significant structural homology among the

<sup>1</sup> Abbreviations used in this paper: A9p75WT, A9 cells expressing transfected wild type p75 TNF receptor; A9 p75CT, A9 cells expressing transfected cytoplasmically truncated p75 TNF receptor; ABTS, 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); CHI, cycloheximide; HeLa p75WT, HeLa cells expressing transfected wild-type p75 TNF receptor; HeLa p75CT, HeLa cells expressing transfected cytoplasmically truncated p75 TNF receptor; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; MBP, maltose binding protein; NGF, nerve growth factor; p55-R, p55 TNF receptor; p75-R, p75 TNF receptor; RIPA, Tris-HCl, NaCl, NP-40, deoxycholate, SDS, EDTA; TNF-R, TNF receptor.

ligands for several of these receptors suggests that they have evolved from a common ancestral receptor-ligand pair and that the conserved structural features of the repetitive cysteine-rich module underlie conserved common features of their action mechanisms (22–27).

Knowledge of the structural basis of TNF-R function is primarily confined to the p55-R species. X-ray analysis of co-crystals of its soluble form with TNF- $\beta$  has provided information on the way in which p55-R binds TNF (28). Detailed mutational studies of its intracellular and “spacer” regions (the region that links its cysteine-rich module to the transmembrane domain) have revealed motifs that are involved in its signaling and in its induced shedding (29–32, and Brakebusch C., E. Varfolomeev, M. Batkin, and D. Wallach, manuscript in preparation). Many of the known effects of TNF can be induced in cells by the use of Abs against the extracellular domain of p55-R, indicating that the signaling activity of this receptor species suffices for their induction (e.g. 33, 34). The efficacy of induction is correlated with the extent of receptor cross-linking by the Abs, suggesting that initiation of the signaling activity of p55-R involves receptor aggregation (33).

The nature of the signaling mechanisms of p55-R and p75-R is not known. However, the lack of structural similarity between their intracellular domains suggests that they provide distinct signals. Indeed, several studies indicate that the two TNF-Rs can induce different effects (35–38). Other studies suggest, however, that at least some of the effects of TNF induced by p55-R, for example its cytotoxic effect, are also affected by p75-R activity or activities (39–45). In some of these studies it was suggested that p75-R assists the induction of such TNF effects by enhancing the binding of TNF to p55-R (38), and in others that p75-R contributes via its own signaling activity (42, 44).

In this study, we explored the functional interactions of the two TNF-Rs by analyzing the effects of Abs against different regions in the extracellular domain of p75-R on TNF cytotoxicity. Our analysis confirmed that p75-R can participate in the cytotoxic effect and indicated that its contribution includes two distinct kinds of activities. One of them, signaling for the cytotoxic effect, known to be triggered by p55-R, was conclusively shown here to be triggered by p75-R as well. This signaling activity seems to emanate from the intracellular domain of p75-R and, like the signaling activity of p55-R, can be triggered by Ab-mediated receptor cross-linking. Second, by controlling the access of TNF to p55-R, p75-R appears to regulate p55-R-mediated cytotoxicity. This apparent activity of p75-R is also affected by Abs but is independent of receptor cross-linking. It appears to involve the extracellular domain of p75-R, not only in its membrane-distal part, to which TNF binds, but also in its membrane-proximal part, where a conformational change seems to take place upon TNF binding.

## Materials and Methods

### *Abs and Their Characterization*

A panel of 23 mAbs against human p75 TNF-R was established by immunizing mice with the soluble urinary receptor, as described

for the development of Abs against p55-R (33). Cross-competition analysis of the binding of these Abs to the soluble receptor (33) showed that they bind to five epitopes, denoted here as epitope A (14 Abs), B (Abs number 31 and 41), C (36 and 62), D (67 and 81), and E (32, 57, and 70).

Monovalent Fab fragments of the mAbs were produced either by cleavage with papain, as described previously (33), or with pepsin, followed by reduction, alkylation, and purification on a protein G-Sepharose column (Pharmacia, Uppsala, Sweden) (46).

Rabbit polyclonal Abs against the spacer region in p75-R, which extends between the transmembrane domain and the COOH-terminal cysteine in the extracellular domain, were raised against a recombinant protein consisting of amino acids 181–235 of p75-R (numbered according to reference 47), fused to the maltose-binding protein (MBP). This fusion protein, produced using the pMal-cRI vector (New England Biolabs Inc., Beverly, MA), was expressed in protease-deficient BL21 bacteria and purified on an amylose column according to the manufacturer's instructions. The Abs were purified by ammonium sulfate precipitation and Abs against MBP were then removed by their adsorption on an irrelevant MBP fusion protein. Flow cytometry demonstrated their binding to the native receptor expressed by HeLa cells. Concentrations of these Abs are specified in terms of dilution, compared to their initial concentration in the serum.

Mouse monoclonal and rabbit polyclonal Abs against the soluble form of human p55-R were produced as described (2, 33). Rabbit polyclonal Abs against mouse p55-R were raised by immunizing rabbits with recombinant extracellular domain of mouse p55-R fused to MBP, produced using the pMal-p vector. The monoclonal anti-IL-6 Ab (number 34.1), used here as an isotype-matched negative control for the flow cytometry experiments, was a gift from Interlab Laboratories (Ness-Ziona, Israel). FITC-labeled goat Ab against the Fab fragment of mouse IgG was obtained from Biomakor (Rehovot, Israel). Goat Ab against mouse IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Affinity-purified goat anti-rabbit Ig, goat anti-rabbit Igs linked to horseradish peroxidase, and goat anti-mouse Igs linked to horseradish peroxidase were obtained from Biomakor.

### *Establishment of HeLa and A9 Cell Transfectants that Overexpress the Wild-type or Cytoplasmically Truncated p75-R*

Human HeLa (48) and mouse A9 (49) cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. The cDNA for human p75-R was obtained by screening a  $\lambda$ gt11 cDNA library of U937 cells (Clontech, Palo Alto, CA) with oligonucleotides corresponding to the published sequence of the receptor (7). The 5' and 3' untranslated regions were deleted (upstream from nucleotide 90 and downstream from nucleotide 1476, according to the numbering of reference 7), and the receptor was expressed in the pMPSVEH expression vector, under control of the myeloproliferative sarcoma virus promoter (50), either fully or after cytoplasmic deletion, downstream from Gln 264 or from Gln 273. The receptor constructs, together with the pSV2neo plasmid conferring resistance to neomycin, were transfected to the A9 and HeLa cells by the calcium phosphate precipitation method. Expression of the transfected receptor in clones resistant to G418 (500  $\mu$ g/ml; Sigma Chemical Co., St. Louis, MO) was assessed by determining both the binding of radiolabeled TNF and that of monoclonal anti p75-R Abs to the cells. Levels of p75-R were quantified by measuring the binding of radiolabeled TNF at saturating concentrations to the cells in the presence of an Ab against p55-R (number 18). In all experiments, testing of cells of several clones overexpressing p75-R yielded qualitatively similar results.

The presented data concerning the HeLa cells that overexpress wild-type p75-R (HeLa p75 WT) refer to a specific clone that expressed about 72,000 TNF-R per cell. The effect of cytoplasmic truncation on p75-R function was tested in A9 cells by expressing the receptor truncated downstream from Gln 264. In HeLa cells, examination of mutants truncated downstream from Gln 264 and from Gln 273 revealed the same phenotype in both.

#### *Assay of Cytocidal Activity of TNF and Abs against the Receptors*

Recombinant human TNF- $\alpha$  ( $6 \times 10^7$  U/mg protein) was used, except in the indicated cases, where recombinant mouse TNF- $\alpha$  ( $4 \times 10^7$  U/mg protein) was employed. Both were produced by Genentech, Inc. (South San Francisco, CA) and kindly provided by Dr. G. Adolf (Boehringer Institute, Vienna, Austria). Unless otherwise stated, the cytotoxic effects of TNF and of anti TNF-R Abs in the HeLa cells were assessed by applying TNF or the Abs to the cells for 10 h, in the presence of cycloheximide (CHI, 25  $\mu$ g/ml). The A9 cells were treated with TNF or the Abs for 12 h, in the presence of 50  $\mu$ g/ml CHI. Cell viability was determined by the neutral-red uptake method, as described elsewhere (51). The cytotoxic effects of Abs against p75-R and of their Fab fragments after cross-linking with anti-Ig Abs, were assessed as described for Abs against p55-R (33). All tests were performed in triplicate. Viability of treated cells is presented as a percentage of the viable cells in cultures incubated with CHI alone  $\pm$  standard deviation. Results of the cytotoxicity tests are representative of sets of at least three independent experiments that yielded similar results.

#### *Quantification of TNF Binding to Cells and of its Modulation by Anti-p75 Abs*

TNF was labeled with  $^{125}$ I by the chloramine-T method, as previously described (52), to a specific radioactivity of 1,500 Ci/mmol. Specific binding of the radiolabeled TNF applied to the cells for 2 h at 4°C at a concentration of 0.5 nM, in the presence or absence of Abs against TNF-R, was determined as described (53).

The effects of anti p75-R Abs and of their Fab monovalent fragments on the rate of TNF dissociation from the receptors were assessed as follows: radiolabeled TNF was applied to the cells in PBS containing 154 mM NaCl, 10 mM sodium phosphate, pH 7.4, at a concentration of 1 nM, in the presence or absence of 10  $\mu$ g/ml of the Abs. After incubation for 8 h, the cells were rinsed twice with ice-cold PBS to remove unbound TNF, and incubated further with PBS containing the Ab (10  $\mu$ g/ml), 0.5% BSA, 0.02% sodium azide, and unlabeled TNF (0.1  $\mu$ M), for the indicated time periods. They were then rinsed once with cold PBS, and the cell-bound radioactivity was determined. The data presented are average values for quadruplicate samples.

#### *Quantification of Ab Binding to p75-R and of Its Modulation by Pretreatment with TNF*

The extent and efficacy of binding of the various Abs to p75-R were assessed by the following three procedures:

**Flow Cytometry.** After treatment with TNF, nearly confluent HeLa p75WT cells were detached in PBS containing 5 mM EDTA and then incubated sequentially with anti p75-R Abs at various concentrations, and with FITC-labeled goat Ab against the Fab fragment of mouse IgG at a dilution of 1:20. Each incubation was carried out for 30 min at 4°C. The fluorescence intensity of the cells was analyzed using a FACScan<sup>®</sup> flow cytometer (Becton Dickinson & Co., Mountain View, CA).

**Immunoprecipitation of the Receptor.** Nearly confluent HeLa

p75WT cells were treated with TNF and then rinsed with cold PBS containing 0.5% BSA. They were then incubated for 1.5 h on ice in the same buffer containing the indicated monoclonal anti p75-R Abs at a concentration of 10  $\mu$ g/ml, or polyclonal Abs against p75-R spacer region at a dilution of 1:100. The cells were then washed, lysed, and extracted with RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, and 1 mM EDTA), supplemented with 1 mM PMSF and 10 mM benzamide HCl. Insoluble material was pelleted by centrifugation for 30 min at 30,000 g. Ab-receptor complexes were adsorbed on protein G-Sepharose beads for 4 h at 4°C. The beads were washed thoroughly with RIPA buffer and PBS, resuspended, and boiled in SDS sample buffer containing mercaptoethanol. After SDS-PAGE (10% acrylamide), the proteins were Western blotted on nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) and probed with rabbit polyclonal Abs against the soluble form of p75-R, or (when immunoprecipitation was performed with antispace Abs) with mAb number 32. The amount of Ab bound to the blots was determined by incubating the blots with goat anti-rabbit or goat anti-mouse Ab conjugated to horseradish peroxidase, and measuring the amount of bound conjugated Ab after further incubation with diaminobenzidine (Sigma Chemical Co.) and hydrogen peroxide.

**Cell-ELISA of Abs Bound to the Cell in the Presence of Detergent.** Confluent monolayers of HeLa p75WT cells in 96-microwell plates were treated with TNF, thoroughly rinsed with ice-cold PBS containing 1 mM calcium chloride and 1 mM magnesium chloride, and fixed with glutaraldehyde (0.1% in PBS) for 40 min at 4°C. Free aldehyde groups were blocked by incubation of the cells, first for 30 min at 37°C with 100 mM glycine in PBS, and then for 2 h with PBS, 0.5% BSA, 0.05% Tween-20, and 0.05% Na<sub>3</sub>N. The plates were rinsed with PBS containing 0.05% Tween-20 and incubated in the same buffer for 2 h at 4°C, with the indicated concentrations of the Abs. The cells were rinsed with RIPA buffer, and the amounts of Ab bound to the cells were determined by incubating the cells with goat anti-mouse or goat anti-rabbit Ab conjugated to horseradish peroxidase followed by further incubation with 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma Chemical Co.) and hydrogen peroxide. Alternatively, Ab binding was quantified using preparations of Abs radiolabeled by the chloramine-T method (52). After binding of these Abs to the glutaraldehyde-fixed cell monolayers, the wells were rinsed with RIPA buffer, and Abs bound to the cells were detached in 2% SDS solution and quantified using a  $\gamma$ -counter.

**Mapping of Epitope E.** DNA sequences encoding different overlapping parts of the extracellular domain of p75-R were produced by PCR; using the full-length cDNA as template. The antisense primers used for the PCR reaction contained a stop codon. The DNA sequences were introduced into the pET8c vector (54) or were expressed in fusion with MBP by introduction into the pMalcR1 vector (New England Biolabs, Inc.). Bacteria (BL21) expressing these proteins upon induction with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; Pharmacia) were resuspended in SDS sample buffer containing  $\beta$ -ME, denatured by boiling, and subjected to SDS-PAGE (10 or 12% acrylamide) followed by Western blotting. The blots were incubated sequentially with a blocking solution containing 154 mM NaCl, 10 mM sodium phosphate, pH 7.4, 0.05% Tween-20, 10% (vol/vol) bovine milk and 0.25% (vol/vol) normal goat serum, with an Ab against epitope E and with radiolabeled goat Ab to mouse IgG, and were then autoradiographed. Alternatively, binding of Abs to the blots was probed using goat anti-mouse Ab linked to horseradish peroxidase, as described above. The identity of the recombinant fragments of the

receptors was confirmed by determination of the molecular size of the IPTG-induced protein in the bacterial extracts by SDS-PAGE, and by interaction of the Western blot with polyclonal antiserum against soluble p75-R.

Involvement of the cysteine residues within epitope E in its recognition by Abs was explored in two ways: first, by determining whether the Abs interact with a recombinant protein corresponding to amino acids 3-181 of p75-R, where cysteine 179 was replaced by alanine. This protein (3-181 C → A) was produced and examined as described above, except that the antisense primer for PCR amplification contained the mutation (5' CTC GGA TCC TCA CGT GGA CGT [GGC] GAC TGC ATC CAT 3'). Second, the effect of alkylation of cysteine residues was assessed before and after reduction. A recombinant fusion protein, consisting of MBP fused to amino acids 125-192 of p75-R, was purified by adsorption on an amylose column and alkylated with iodoacetamide (75 mM for 1 h) before or after treatment with 100 mM dithiothreitol. It was then subjected to Western blotting as described above.

The mapping of the epitope was further confirmed by assessing the interaction of Abs against epitope E with synthetic peptides produced by t-Boc chemistry on a peptide synthesizer (model 430 A; Applied Biosystems, Foster City, CA). ELISA plates were coated with a fusion protein consisting of MBP fused to amino acids 125-192 in p75-R, produced as described above, and purified by adsorption on amylose. The protein, at a concentration of 1 μg/ml in PBS/0.02% sodium azide, was applied to the plates for 2 h. After further incubation of the plates for 2 h with a solution containing PBS, 0.5% BSA, and 0.02% sodium azide (blocking solution), an Ab against epitope E was applied for 2 h to the plates at a concentration of 1 μg/ml, with or without one of the synthetic peptides. The amounts of Ab bound to the protein-coated plate were determined by the use of goat anti-mouse Ig Ab conjugated to horseradish peroxidase and ABTS, as described above.

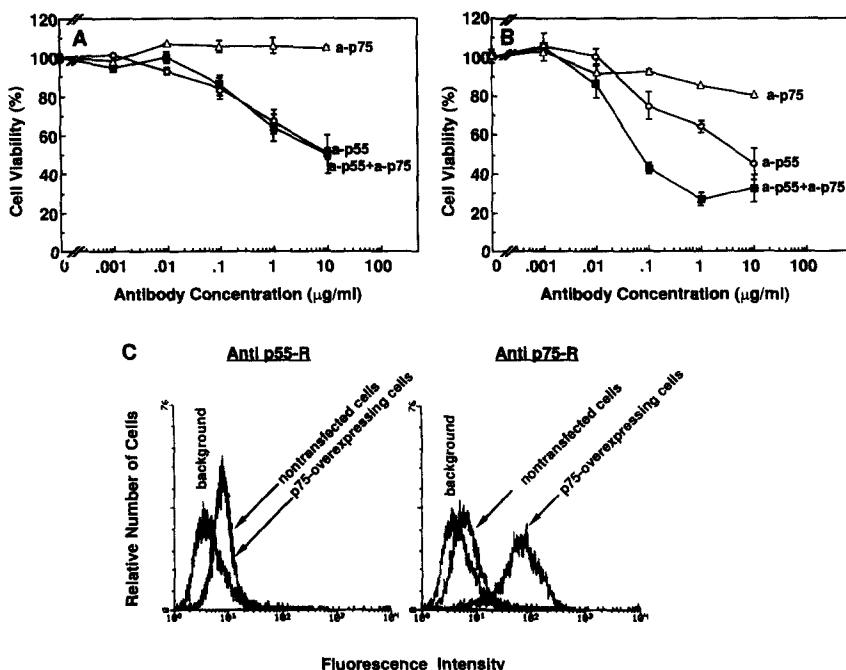
## Results

*Abs against p75-TNF-R Can Trigger Signaling for a Cytocidal Effect.* HeLa cells predominantly express p55-R and are

killed by agonistic Abs against this receptor species, but not by Abs against p75-R (Fig. 1 A). To examine the involvement of p75-R in the cytotoxic function of TNF, we transfected HeLa cells with p75-R cDNA and isolated clones of cells expressing large amounts of the receptor (HeLa p75WT, Fig. 1 C). In these cells, Abs against p75-R triggered a mild cytotoxic effect. Moreover, they potentiated the cytotoxic activity of simultaneously applied Abs against p55-R (Fig. 1 B). The intensity of these effects varied among different transfected clones, roughly in proportion to their p75-R levels.

To investigate the mechanism whereby anti p75-R Abs exert their own cytotoxic effect, we compared the ability of various individual Abs, or Ab combinations, to trigger such an effect. We employed 23 different mAbs raised against the soluble form of p75-R and antiserum raised against the spacer region in the receptor (a region not included in the soluble form of the receptor, extending between the cysteine-rich module in the extracellular domain and the transmembrane domain). Cross-competition analysis of their binding to soluble p75-R showed that the mAbs bind to five epitopes, denoted A to E. Table 1 presents a summary of the observed effects of the different Abs on HeLa p75WT cells, as well as on the other cells used in this study.

As shown in Fig. 2 A, Abs against p75-R were most effective when applied in combinations that bind to different epitopes. This increased efficacy was observed both when the anti p75-R Ab combinations were applied alone to HeLa p75WT cells and when applied in the presence of Abs against p55-R. Indeed, when applied alone, the anti p75-R Abs showed significant cytotoxic activity only in such combinations. Abs against p55-R greatly potentiated the cytotoxic effect of the anti p75-R Abs and in their presence the individual Abs and the combinations of Abs that bind to the same epitope also had some effect. The magnitude of the effect varied, depending on the epitope to which the Abs bound;



**Figure 1.** Additive cytotoxic effects of p75-R and p55-R in HeLa cells transfected with p75-R. Comparison of the cytotoxic effects of anti TNF-R Abs in (A) normal HeLa cells (expressing about 500 p75-Rs per cell) and (B) a clone of HeLa cells transfected with an expression vector containing p75-R cDNA (HeLa p75WT cells) and therefore expressing increased amounts of the receptor (about 72,000 p75-Rs per cell). The cytotoxic effects of Abs against p55-R (O, mixture of Abs 18 and 20, which recognize distinct epitopes [33]), of Abs against p75-R (Δ, mixture of Abs 13, 36, and 41, which recognize distinct epitopes), and of their combination (■), applied at the indicated concentrations, were assessed as described in Materials and Methods. (C) Flow cytometric analysis of expression of p55 (left) and p75 (right) TNF-R in the normal and transfected HeLa cells examined in A and B. Cells were incubated consecutively with mAbs against p55-R (number 20) or p75-R (number 14), or with isotype-matched control anti-IL-6 Abs (background), all at a concentration of 20 μg/ml, and then with FITC-conjugated goat anti-mouse Ig Ab (see Materials and Methods for details).

**Table 1.** Effects of Abs Against p75-R on HeLa and A9 Cell Expressing Wild-type and Cytoplasmically Truncated p75-R

EPITOPE:	CYSTEINE-RICH MODULE			SPACER REGION
	A, B, C (18 Abs) Intact Fab	D (2 Abs) Intact Fab	E (3 Abs) Intact Fab	Intact Fab
<b>Ab EFFECTS INDICATING SIGNALING BY THE p75-R</b>				
ON CYTOTOXICITY OF ANTI p55-R Abs				
IN HELA CELLS EXPRESSING WT p75-R	▲ —	▲ —	▲ —	▲ —
IN HELA CELLS EXPRESSING CT p75-R & IN A9 CELLS	— —	— —	— —	— —
<b>INFERRED MODE OF Ab ACTION</b>	<b>EFFECTIVE TRIGGERING</b>	<b>EFFECTIVE TRIGGERING</b>	<b>WEAK TRIGGERING</b>	<b>WEAK TRIGGERING</b>
<b>Ab EFFECTS INDICATING CONTROL BY THE p75-R OF TNF AVAILABILITY TO THE p55-R</b>				
ON CYTOTOXICITY OF TNF				
IN HELA CELLS EXPRESSING WT p75-R	▲ —	▲ ↓	↓ ↓	↓ ↓
IN HELA CELLS EXPRESSING CT p75-R & IN A9 CELLS	▲ ▲	↓ ↓	↓ ↓	↓ ↓
<b>Ab EFFECT ON TNF BINDING</b>	↓ ↓	▲ ND	▲ ▲	▲ ND
<b>TNF EFFECT ON Ab BINDING</b>	— —	▲ ▲	▲ ▲	▲ ▲
<b>INFERRED MODE OF Ab ACTION</b>	<b>INCREASED AVAILABILITY OF TNF</b>	<b>DECREASED AVAILABILITY OF TNF</b>	<b>DECREASED AVAILABILITY OF TNF</b>	<b>DECREASED AVAILABILITY OF TNF</b>

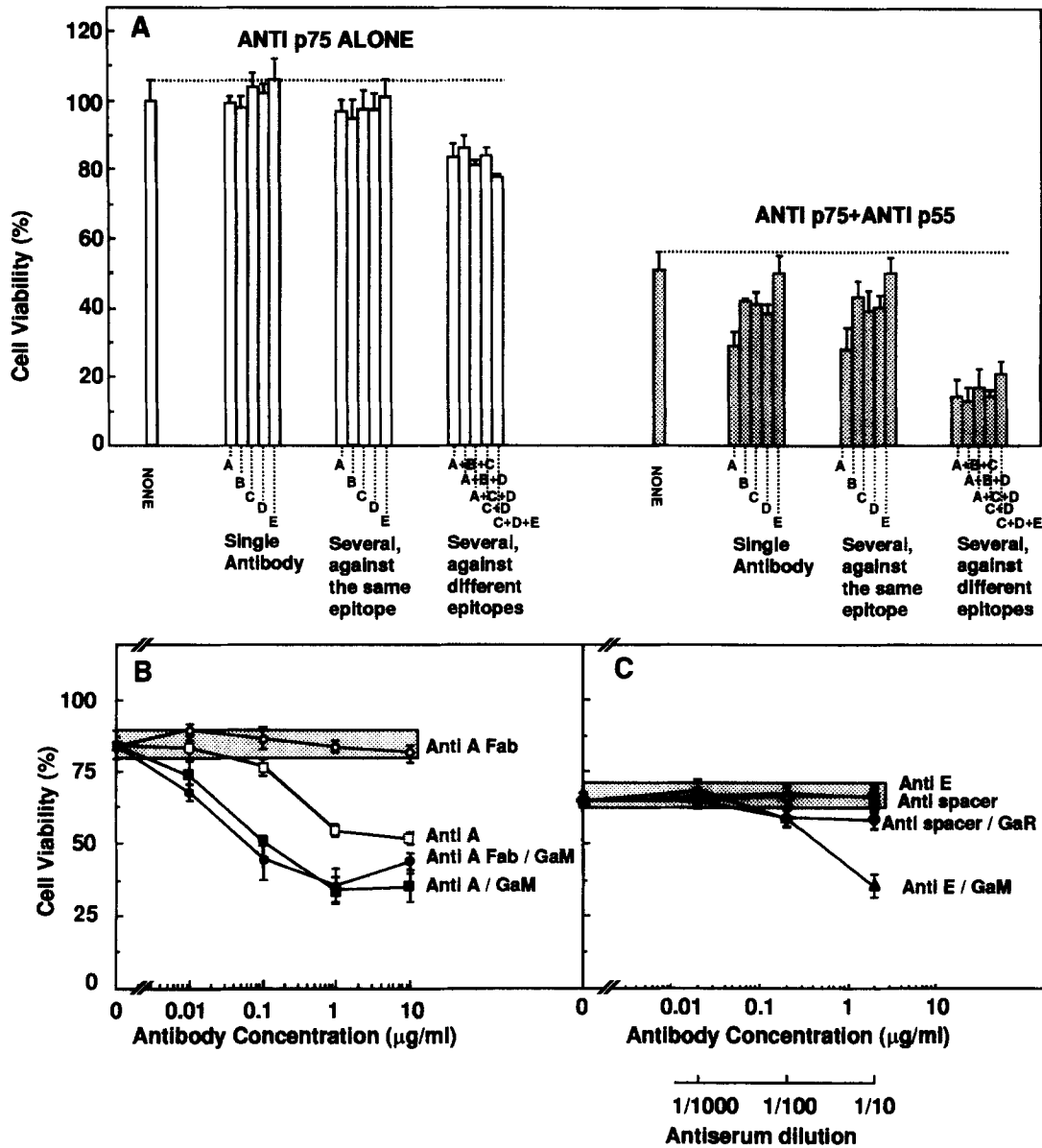
▲, stimulation; ▲, weak stimulation; ↓, inhibition; —, No effect; ND, Not determined.

it was lowest, indeed barely detectable, with the Abs against epitope E and against the spacer region (Fig. 2, A and C).

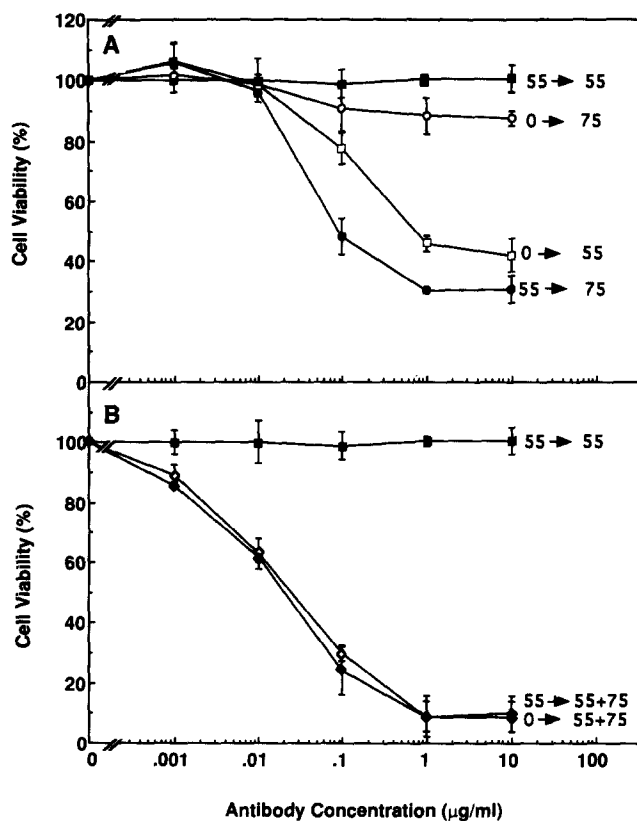
Combinations of Abs that bind to different epitopes can cause extensive aggregation of the antigen molecules to which they bind, whereas Abs that bind to a single epitope are capable, at most, of linking the antigen molecules in couples. The greater cytotoxic activity of combinations of Abs that bind to different epitopes in p75-R suggests that the receptors are triggered as a consequence of their cross-linking. This notion gained further support when we examined the cytotoxic activity of Fab monovalent fragments of an Ab against epitope A. In contrast to the intact Ab, its Fab fragments were without cytotoxic effect, even when applied in the presence of Abs against p55-R (Fig. 2 B). These Fab fragments did however have a cytotoxic effect when cross-linked with anti-Ig Abs. Moreover, anti-Ig Abs enhanced the cytotoxic activity of the intact Ab molecules (Fig. 2 B). Also, cross-

linking of the Abs against epitope E or the spacer region, which by themselves had almost no cytotoxic effects, resulted in some cytotoxicity (Fig. 2 C).

*Signaling by p75-R Appears to Interact with Signaling by p55-R.* The more than additive effect of Abs against the two TNF-Rs suggests that, even though they initiate the same effect, they do so by different signaling mechanisms. To explore further the relationship between the mechanisms of cytotoxicity induction by the two receptors, we examined whether triggering of one of them can cross-desensitize the cells to the effect of the other. The cytotoxic effect of Abs against p55-R was previously shown to be subject to homologous desensitization (33). As shown in Fig. 3, HeLa p75WT cells pretreated with anti p55-R Abs in the absence of a protein synthesis blocker (which in these cells is needed to elicit TNF cytotoxicity) showed almost no cytotoxic effect following a second exposure to these Abs in the presence of a protein



**Figure 2.** Cytocidal effects of Abs against p75-R in HeLa cells expressing wild-type p75-R reflect cross-linking of the receptors. (A) Cytocidal effects of individual Abs, combinations of Abs that bind to the same epitope, and combinations of Abs that bind to different epitopes in the absence (left) and presence (right) of Abs against p55-R. The cytocidal effects of the various anti-p75-R mAbs were assessed in HeLa p75WT cells, in the absence or presence of Abs against p55-R (numbers 18 and 20, both applied at a concentration of 1 µg/ml). Anti p75-R Abs were applied, each at a concentration of 2 µg/ml, individually (Single Ab: epitope A, number 14; epitope B, 41; epitope C, 36; epitope D, 67; epitope E, 32), in combinations of several Abs that bind to the same epitope (Several against the same epitope: epitope A, 14, 20, and 22; epitope B, 41, 47, and 82; epitope C, 36 and 62; epitope D, 67 and 81; and epitope E, 32, 57, and 70) or in combinations of Abs that bind to different epitopes (Several against different epitopes: A+B+C, 14, 41, and 36; A+B+D, 14, 41, and 67; A+C+D, 14, 36, and 67; C+D, 36 and 67; C+D+E, 36, 67, and 32). (B) Cytocidal effects on HeLa p75WT cells of an Ab against epitope A in p75-R (□, number 19) and of its Fab monovalent fragments (○), and their effects on these cells after their further cross-linking by goat anti-mouse Fab Abs (GaM, ●, ■; see Materials and Methods). The cytocidal effects were assessed in the presence of Abs against p55-R (rabbit polyclonal serum against the soluble form of the receptor (2), applied at a dilution of 1:500). (C) Cytocidal effects on HeLa p75WT cells of an Ab against epitope E in p75-R (Δ, number 32) and of antiserum against the spacer region in this receptor (◇), and effects on these cells after further cross-linking of the Abs with anti-Ig Abs (▲, ◆, goat anti-mouse Fab and goat anti-rabbit Ig Abs [GaM and GaR], respectively). The cytocidal effect of the mAb against epitope E was assessed in the presence of rabbit polyclonal Abs against p55-R (applied at a dilution of 1:250). The effect of the antiserum against the spacer region was determined in the presence of mouse mAbs against p55-R (numbers 18 and 20, both applied at concentration of 1 µg/ml). Dotted boxes in B and C indicate the range of the response observed when only the Abs against p55-R were applied.



**Figure 3.** Absence of cross-desensitization by the two TNF-Rs to each other's cytotoxic effects. HeLa p75WT cells were preincubated for 2 h, in the absence of CHI, with Abs against p55-R (numbers 18 and 20, each at a concentration of 1 µg/ml, closed symbols) or without anti-p55-R Abs (open symbols). The cytotoxic effects of anti-p55-R Abs (□, ■, numbers 18 and 20), of anti-p75-R Abs (○, ●, numbers 13, 36, and 41), and of their combination (◇, ◆), at the indicated concentrations, were subsequently assessed in the presence of 25 µg/ml CHI, as described in Materials and Methods.

synthesis blocker. They were not, however, desensitized to the effect of anti-p75-R Abs. On the contrary, p75 treatment of cells desensitized to the effect of anti-p55-R Abs resulted in a more than additive cytotoxic effect, much greater than that induced in HeLa p75WT cells by Abs against either of the receptors alone (Fig. 3 A). A similar synergy was observed when cells pretreated with anti-p75-R Abs were challenged with Abs against p55-R (data not shown). No desensitization was observed when cells pretreated with anti-p55-R Abs were challenged by Abs against both receptors (Fig. 3 B).

**Effects of Anti-p75-R Abs on the Cytotoxicity of TNF Point to an Additional Mode of Receptor Function.** Application of anti-p75-R Abs to HeLa p75WT cells in the presence of suboptimal TNF concentrations resulted, with most of the Abs, in enhancement of TNF cytotoxicity. This enhancement is consistent with the observed ability of the Abs to trigger a cytotoxic effect when applied alone. As in that case, the enhancement appeared to be a consequence of receptor cross-linking by the Abs: it was most pronounced when the Abs were applied in combinations that bind to different epitopes (Fig. 4 A). Moreover, enhancement of TNF cytotoxicity was

not observed with Fab monovalent fragments of the Abs, but appeared when these fragments were cross-linked with anti-Ig (Fig. 4 B).

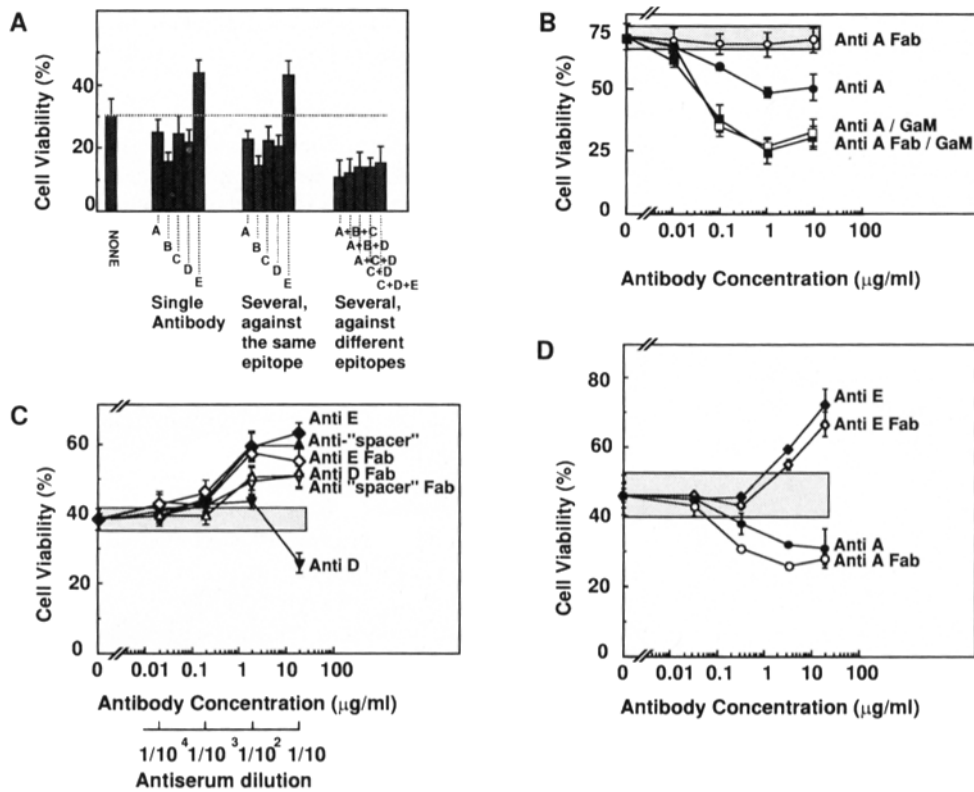
Unlike most of the other Abs, however, the three that bind to epitope E did not enhance the cytotoxic activity of TNF, but rather inhibited it (Fig. 4, A and C). Also, the Abs raised against the spacer region had a slight though significant inhibitory effect on TNF function (Fig. 4 C). These inhibitory effects, unlike the enhancement of TNF cytotoxicity by the other Abs, were also observed when monovalent Fab fragments of the Abs were applied. This suggests the involvement of mechanism(s) independent of receptor cross-linking (Fig. 4 C). Some inhibition of the cytotoxic effect of TNF was also observed in the presence of Fab monovalent fragments of the Abs against epitope D (Fig. 4 C), even though in the intact form these Abs enhanced TNF cytotoxicity (Fig. 4, A and C).

Whereas they inhibited the cytotoxic effect induced by TNF, Abs against epitope E and against the spacer region did not interfere with the induction of a cytotoxic effect by Abs against p55-R (Fig. 2 C), nor did they interfere with enhancement of the cytotoxic effects of anti-p55-R Abs or of TNF by other anti-p75-R Abs. (Compare the effect of the combination of Abs that bind to epitopes C, D, and E with that of the combination of Abs against C and D only, in Figs. 2 A and 4 A.)

**An Additional Mode of p75-R Function Is also Apparent in HeLa Cells Transfected with Cytoplasmically Truncated p75-R Mutants.** To examine the involvement of the intracellular domain in the activities of p75-R, we transfected HeLa cells with cDNAs encoding cytoplasmically truncated mutants of the receptor (HeLa p75CT). In contrast to cells expressing large amounts of the wild-type p75-R (HeLa p75WT), which responded normally to the cytotoxic effects of anti-p55-R Abs and of TNF, all clones expressing p75-R mutants (truncated below Gln 264 or Gln 273) were relatively resistant to such cytotoxicity. Anti-p75-R Abs had no effect at all when applied alone to cells expressing the receptor mutants, nor did they enhance the mild cytotoxicity of Abs against p55-R. Thus, as with p55-R (29–31), triggering of cytotoxicity by p75-R seems to depend on the integrity of the receptor's intracellular domain. A representative example of the data is presented in the legend to Fig. 4 D.

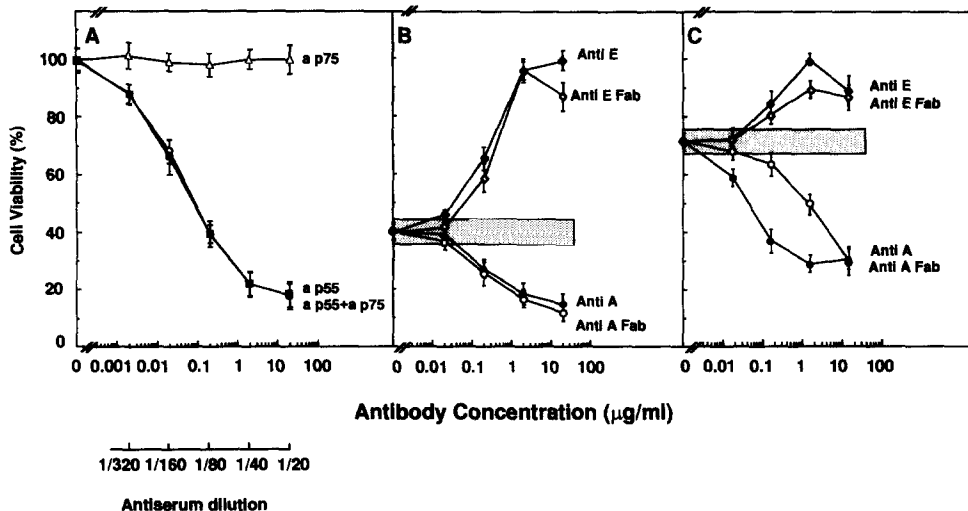
Although unable to initiate signaling for the cytotoxic effect in HeLa p75CT cells, anti-p75-R Abs did affect the extent of cell killing by simultaneously applied TNF. Abs against epitope E or their Fab monovalent fragments inhibited the cytotoxic effect of TNF, whereas Abs against epitope A enhanced it to a small but significant extent (Fig. 4 D). In contrast to the case where the Abs were applied together with TNF to HeLa p75WT cells, this latter enhancement could also be observed with Fab monovalent fragments of the Abs (Fig. 4 D); it therefore seems to involve not triggering of the signaling activity of p75-R, but some other mechanism(s), possibly related to the ability of the anti-A Abs to displace TNF from p75-R (see below).

**Effects of Anti-p75-R Abs in A9 Cells Transfected with the Human Receptor Support a "Non-signaling" Role of p75-R in TNF Function.** To determine whether the effects of anti-p75-R



**Figure 4.** Enhancement and inhibition of the cytotoxic effect of TNF by anti-p75-R Abs in HeLa cells expressing wild-type p75-R or a cytoplasmically truncated p75-R. (A) Effects on TNF cytotoxicity of individual Abs, combinations of Abs that bind to the same epitope, and combinations of Abs that bind to different epitopes. The cytotoxic effects of the various monoclonal anti-p75-R Abs on HeLa p75WT cells were assessed in the presence of TNF (50 U/ml). The Abs and their concentrations are as in Fig. 2 A. (B) Effects of an Ab against epitope A (●, ■, number 19) and of its Fab monovalent fragments (○, □), with or without their cross-linking by goat anti-mouse Fab Abs (GaM), on the cytotoxic effect of TNF (20 U/ml) in HeLa p75WT cells. (C) Effects of Abs against epitope D (▼, number 67) and E (◆, number 32), antiserum against the spacer region in p75-R (▲) and Fab monovalent fragments of these Abs (▽, ◇, △) on the cytotoxic effect of TNF (50 U/ml) in HeLa p75WT cells. (D) Effects of an Ab against epitope A (●, number 13) and of its Fab monovalent fragments (○)

and effects of an Ab against epitope E (◆, number 32) and of its Fab monovalent fragments (◇) on the cytotoxic effect of TNF (1,000 U/ml) in HeLa cells expressing cytoplasmically truncated p75-R (HeLa p75CT, about 140,000 receptors per cell). When applied alone to the HeLa p75CT cells, anti-p75-R Abs (numbers 14, 62, and 82, each at a concentration of 20 μg/ml) had no effect on TNF cytotoxicity, nor did they enhance the cytotoxicity induced by anti-p55-R Abs (numbers 18 and 20, each applied at a concentration of 20 μg/ml, which caused killing of  $17 \pm 3\%$  of the cells in this experiment). Dotted boxes in B-D indicate the range of the response observed when TNF was applied alone to the cells.



**Figure 5.** Anti-p75-R Abs have no cytotoxic effect in A9 cells expressing high levels of p75-R but do modulate the cytotoxic effect of TNF on these cells. (A) Lack of a cytotoxic effect of anti-p75-R Abs (Δ, mixture of Abs 13, 36, and 41) on A9 cells expressing large amounts of full-length p75-R (A9 p75WT, about 90,000 receptors per cell), in contrast to the marked cytotoxicity of an antiserum against the murine p55-R (○). Anti-p75-R Abs also fail to enhance the cytotoxicity of the anti murine p55-R antiserum (■). (B and C) Enhancement of the cytotoxic effect of TNF (1,000 U/ml) by an Ab against epitope A (●, number 13) and by its Fab monovalent fragments (○), and inhibition of the effect by an Ab

against epitope E (◆, number 32) and by its Fab monovalent fragments (◇) in A9 cells expressing wild-type or cytoplasmically truncated human p75-R. (B) A test performed with the same A9 p75WT clone that was tested in A. (C) A test performed with A9 p75CT (~110,000 receptors per cell). Abs against epitope B and, to a lesser extent, Abs against epitope C also enhanced the cytotoxic effect of TNF in A9 p75WT or A9 p75CT cells, whereas Abs against epitope D and against the spacer region had inhibitory effects similar to those of the Abs against epitope E. The Abs had the same effects when mouse rather than human TNF was used. Dotted boxes in B and C indicate the range of the response observed when TNF was applied alone to the cells.

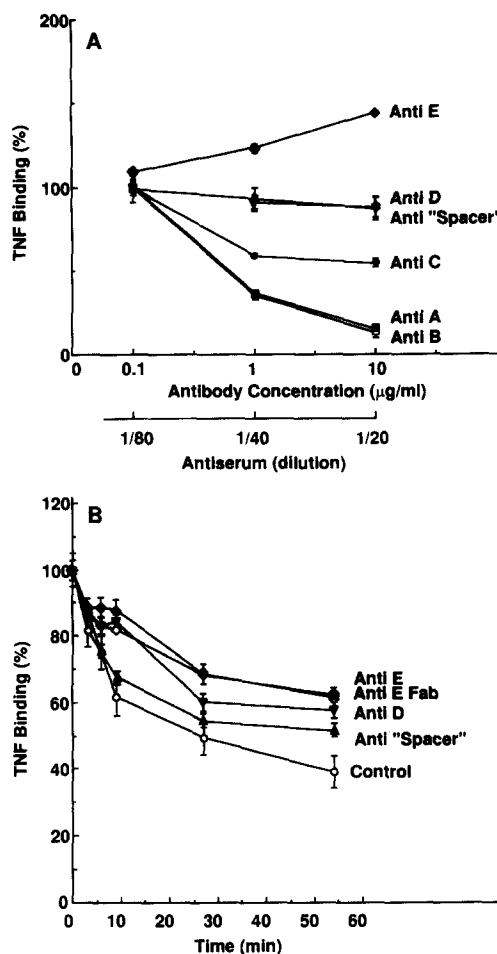


Abs observed with HeLa cells also pertain to other cells, we examined their effect on mouse A9 cells transfected with cDNA for the wild-type human p75-R (A9 p75WT) or for a cytoplasmically truncated mutant of it (A9 p75CT). All transfected clones exhibited a significantly reduced cytotoxic response to TNF, in correlation with the amounts of the expressed receptor, but responded normally to the cytotoxic effect of Abs against their murine p55-R. Unlike the HeLa p75WT cells, A9 p75WT and A9 p75CT cells did not exhibit a cytotoxic effect when treated with Abs against human p75-R, nor did these Abs enhance the cytotoxic effect induced in the cells by Abs against murine p55-R (Fig. 5 A and data not shown).

Although unable to initiate signaling for a cytotoxic effect in A9 p75WT or A9 p75CT cells, anti-p75-R Abs strongly affected the killing of these cells by TNF. These effects resembled those observed in HeLa p75CT cells. Abs or Fab monovalent fragments that bind to epitopes D or E, or to the spacer region, reduced the cytotoxic response of the cells to TNF. Abs or Fab fragments that bind to epitopes A and B and (though to a lesser extent) Abs or Fab fragments that bind to epitopes C enhanced the cytotoxic effect of TNF in these cells (Fig. 5, B and C and data not shown).

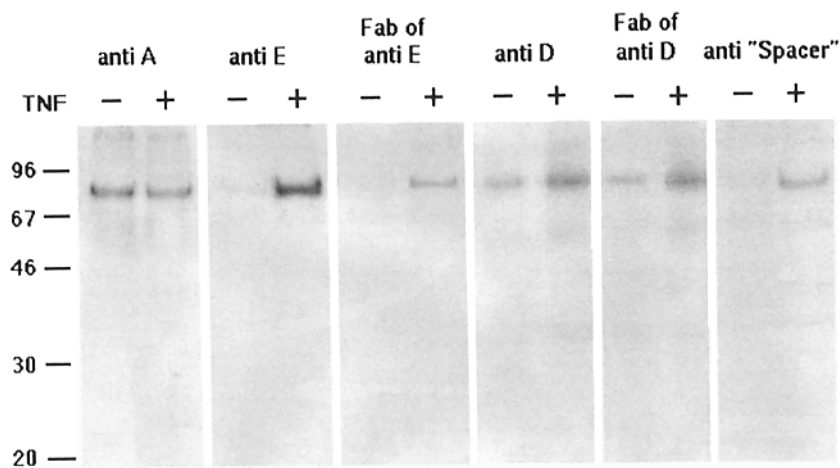
**Effects of Anti-p75-R Abs on TNF Binding to p75-R.** Abs binding to different epitopes in p75-R affected TNF binding in quite different ways. The pattern of these effects was almost opposite to that of their effects on TNF function (Fig. 6). Abs that bind to epitopes A, B, or C, which enhanced TNF function in HeLa p75WT cells, were found to inhibit TNF binding. This inhibitory effect was greater with the Abs that bind to epitopes A or B than to C. However, Abs against epitopes D or E or the spacer region, all of which inhibited TNF function, did not inhibit its binding. In fact, cells exposed to Abs against epitope E exhibited some increase in TNF binding (Fig. 6 A). Moreover, measurement of the dissociation of TNF from p75-R, which occurs rather rapidly (38), revealed that Abs against epitopes D or E and, to a lesser extent, Abs against the spacer region inhibit the dissociation of TNF from the receptor. Fab monovalent fragments of the Abs against epitope E had a similar inhibitory effect (Fig. 6 B).

**Effects of TNF on Binding of Anti-p75-R Abs to Epitopes D and E and to the Spacer Region.** As shown above, Abs against epitopes D or E or the spacer region affected TNF binding and function even when in their monovalent form. This suggests that their effects are related to the conformation of the receptor molecules rather than to their extent of aggregation. The fact that these Abs affect TNF binding and function even though the site at which they bind to p75-R seems remote from the one at which TNF binding takes place suggested that the conformation of the receptor at the Ab binding site is affected by TNF. To test these notions, we compared the ability of different Abs against the receptor to bind to it before and after TNF binding. Three kinds of tests were employed (see Materials and Methods). In the first, the actual binding of the Abs to HeLa p75WT cells was quantified by flow cytometry. No difference between TNF-treated and



**Figure 6.** Inhibition or enhancement of TNF binding to p75-R by the various anti-p75-R Abs. (A) Effects of the various anti-p75-R Abs on TNF binding. The effects of Abs against epitope A (■, number 14), B (□, -41), C (●, -36) D (▼, -67) and E (◆, -32) and of Abs against the spacer region (▲) on the binding of radiolabeled TNF to HeLa p75WT cells were assessed as described in Materials and Methods. All Abs binding to the same epitope affected TNF binding similarly (data not shown). (B) Kinetics of the dissociation of radiolabeled TNF from HeLa p75WT cells in the absence of Abs (○) and in the presence of an Ab against epitope D (▼, number 67) or E (◆, -70) or its Fab monovalent fragments (◇, all applied at a concentration of 10 µg/ml) or of Abs against the spacer region (▲, at a dilution of 1:20).

untreated cells was observed (data not shown). The other two tests probed the efficacy of Ab binding to p75-R by subjecting the Ab-receptor complexes to detergent treatment. In one, binding efficacy was evaluated by assessing the recovery of the receptor immunoprecipitated from detergent extracts of HeLa p75WT cells. In the other, the amounts of Abs bound to the receptor were quantified by a cell-ELISA procedure. In the latter two tests, TNF was found to enhance the binding to the receptor of Abs against epitopes E or D or the spacer region, but not that of Abs against the other epitopes. TNF treatment increased the efficacy with which these Abs and their Fab monovalent fragments could immunoprecipitate p75-R from detergent extracts of the cells. No such



**Figure 7.** TNF enhances the binding of Abs to epitopes D and E and to the spacer region of p75-R: assessment by immunoprecipitation. Subconfluent cultures of HeLa p75WT cells were treated with TNF (10,000 U/ml) for 10 min. They were then incubated for 90 min at 4°C with the following Abs: an Ab against epitope A (number 13), an Ab against epitope E (number 32) or its Fab monovalent fragments, an Ab against epitope D (number 67) or its Fab monovalent fragments, each at a concentration of 10 µg/ml, or Abs against the spacer region at a dilution of 1:100. Unbound Abs were washed off and the cells were lysed with RIPA buffer, followed by precipitation of the receptor–Ab complexes, SDS-PAGE (10%) and Western blotting, as described in Materials and Methods.

effect was observed with Abs against epitopes A, B, or C (Fig. 7 and data not shown). TNF treatment also significantly increased the efficacy with which Abs against epitopes E or D or against the spacer region, but not Abs against A, B, or C, bound to (glutaraldehyde-fixed) cells in a cell-ELISA test where cells were rinsed with a detergent solution (Fig. 8 and data not shown). These effects were specific for TNF. They were observed with both TNF- $\alpha$  and TNF- $\beta$  (lymphotoxin), but not after treatment with IL-1 $\alpha$ , which in many respects functions similarly to TNF, or with IFN- $\gamma$ , shown to enhance TNF-R expression in HeLa cells (52). In a kinetic study, they could be discerned as early as 1 min after TNF was applied to the cells and reached a maximum within 5 min (data not shown).

**Mapping of Epitope E.** The three Abs against epitope E (but none of the 20 other mAbs against p75-R) recognized p75-R in Western blot analysis, even when the receptor molecules were analyzed after their complete denaturation and reduction. This enabled us to map epitope E by Western blot analysis of the binding of the Abs to various bacterially expressed deletion mutants of the extracellular domain of p75-R (Fig. 9 B). We found (Fig. 9 A) that NH<sub>2</sub>-terminal truncation of the receptor to Cys 163 and COOH-terminal truncation to Thr 179 did not affect recognition by the three Abs. However, all further truncations prevented recognition, suggesting that epitope E corresponds to the part of the protein that extends between the residues Cys 163 and Thr 179. Interaction of the Abs with a synthetic peptide consisting of amino acids 163-179 confirmed this identification (Fig. 9 C).

As with reduction and denaturation, alkylation with iodoacetamide did not affect recognition of the receptor by Abs against epitope E in Western blot analysis. However, recognition was completely prevented when the receptor was first reduced and then immediately alkylated (see Materials and Methods; data not shown). Recognition was also prevented by mutational replacement of one of the cysteine residues in the epitope region by alanine (3-181 C → A in Fig. 9 B). These findings indicated that the structure to which the Abs bind is not the reduced form of the protein, but the cysteine

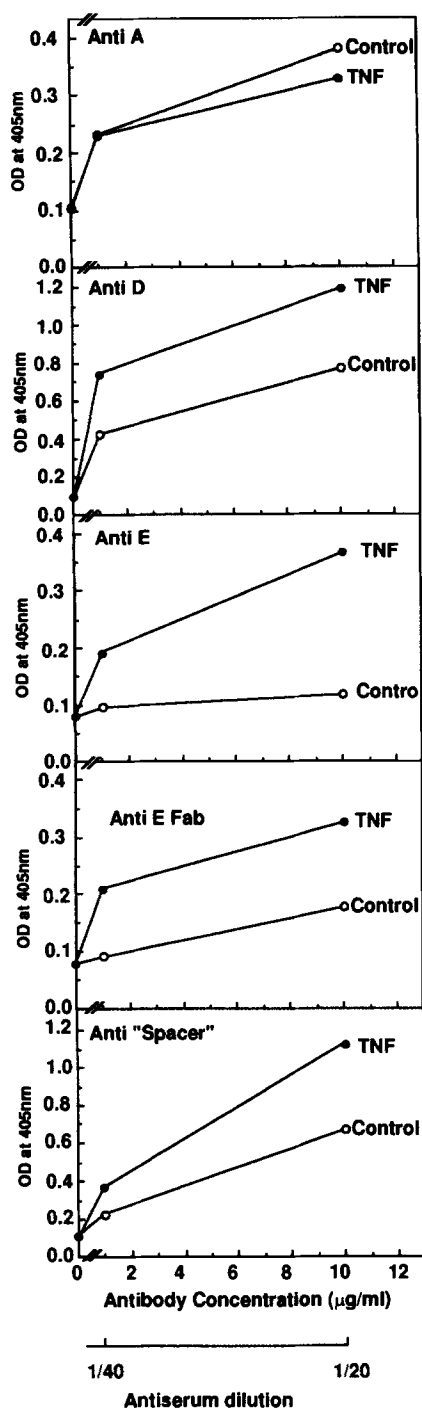
loop formed between the cysteine residues at positions 163 and 178. The fact that this protein can be recognized in Western blotting after its complete reduction and denaturation suggests that the primary structure of the protein in this region dictates correct refolding of the denatured receptor, as well as reformation of the cysteine link.

The mapping of epitope E to the COOH-terminal end of the cysteine-rich module points to a correlation between the effects of the various Abs against the extracellular domain of p75-R on TNF function and the location of the epitope to which they bind (Table 1). Abs that bind to the membrane-proximal part of the extracellular domain—to its COOH-terminal cysteine loop and the spacer region—have different effects on TNF function and binding from those of most of the Abs that bind to the membrane-distal part. Only the two Abs that bind to epitope D showed a mixed type of effect, raising the possibility that this epitope is located at the boundary between the two regions.

## Discussion

Involvement of p75-R in the cytotoxic effect of TNF was explored in this study by probing the function of this receptor with Abs that bind to different regions in its extracellular domain. The results (summarized in Table 1) indicated that p75-R contributes to the cytotoxic effect by at least two kinds of activities: its own signaling activity and control of the access of TNF to p55-R.

Examination of the effects of Abs on the viability of HeLa cells manipulated to express large amounts of wild-type p75-R indicated that p75-R plays a role in signaling for the cytotoxic effect of TNF. Anti-p75-R Abs were cytotoxic to these cells even in the absence of TNF or anti-p55-R Abs, and enhanced the cytotoxic effect of anti-p55-R Abs. These effects of the anti-p75-R Abs were correlated with their ability to cross-link the receptor molecules, suggesting that the signaling occurs as a consequence of receptor aggregation. To some extent, these effects were also dependent on the site to which the Abs bind within the receptor. Abs against epi-



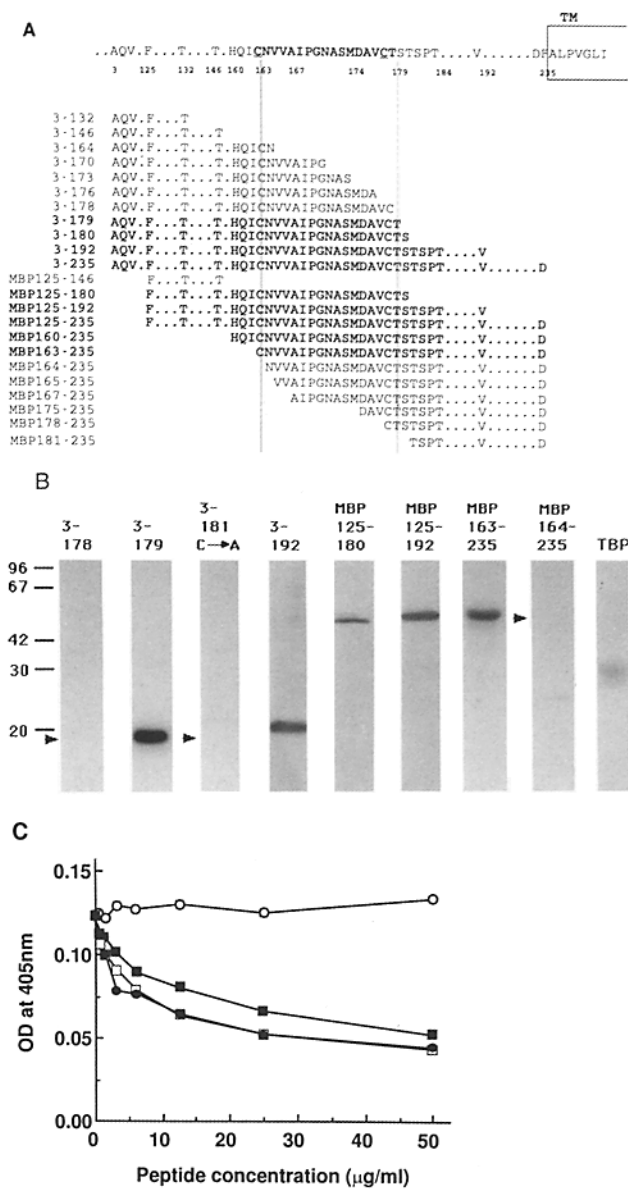
**Figure 8.** TNF enhances the binding of Abs to epitopes D or E and to the spacer region of p75-R: assessment by cell-ELISA. Effects of TNF on the binding of mAbs against epitopes A (number 13), D (number 67), and E (number 32), of Fab monovalent fragments of the Ab against epitope E, and of Abs against the spacer region. TNF (10,000 U/ml) was applied to HeLa p75WT cells for 10 min. The cells were then fixed with glutaraldehyde. Binding of the Abs applied at the indicated concentrations, after washing of unbound Abs with RIPA buffer, was assessed as described in Materials and Methods. Similar results were obtained when the binding of Ab number 32 or of its Fab fragments was quantified by applying radio-labeled preparations of these Abs to the cells.

topes A, B, C, and D, at the membrane-distal part of the receptor's extracellular domain, had stronger effects than those that bind to epitope E or to the spacer region (Fig. 2). However, the epitope E and anti-spacer Abs showed increased cytotoxicity when their ability to cross-link the receptor molecules was enhanced by their cross-linking with anti-Ig Abs. The partial site dependence of their effects may reflect differences in the ability of the Abs to impose an orientation on the receptor molecules resembling that attained when TNF binds to them. Abs that bind to epitopes A, B, C, and D may be better able to bring about this orientation, possibly because their binding sites to the receptor are close to the region at which TNF binds (as reflected in the ability of the Abs that bind to epitopes A, B, and C to interfere with TNF binding, Fig. 6 A).

Similar to the signaling activity of p75-R, signaling for the cytotoxic effect by p55-R is triggered upon receptor aggregation (33). Yet, in spite of this similarity in mode of triggering of the receptors, and the apparent identity of their impact on the cell, it seems that the mechanisms of signaling for cell death by the two receptors differ, at least at their initial step. This difference was evident from the way in which stimulation of each receptor affected the activity of the other. HeLa cells prestimulated with Abs against p55-R became almost completely unresponsive to the cytotoxic effect of these Abs (33, and the present study), yet showed a response greater than the additive effect of the two receptors when subsequently stimulated with Abs against p75-R. It thus seems that the signals provided by p75-R are able to reverse the desensitization of p55-R or perhaps to uncover preformed p55-R-mediated signals in the desensitized cells and act in concert with them. The possible occurrence of receptor-specific desensitization is consistent with a prior study which indicated that desensitization of cells to TNF cytotoxicity (51, 55, 56) involves, in addition to activities that antagonize the cytotoxic effect itself (57-59), activities that inhibit signaling-related mechanisms (60).

Although capable of triggering a cytotoxic effect in HeLa cells, the anti-p75-R Abs were unable to do so in A9 cells, irrespective of whether the transfected human p75-R that they expressed was of full-length or cytoplasmically truncated. The nature of this difference between the A9 and the HeLa cells is not clear. It is unlikely to reflect a species specificity barrier, as human p75-R does have the ability to trigger TNF effects in mouse cells (37). Moreover, a recent study (38) demonstrated that Abs against endogenous mouse p75-R cannot trigger a cytotoxic effect in cells of the mouse L929 line, which are closely related to the A9 cells. Perhaps the difference is related to the way in which TNF cytotoxicity is regulated in the two cell lines. In L929 and A9 cells, unlike in HeLa cells, preexposure to TNF or to Abs against p55-R does not result in desensitization (55, and data not shown). It thus appears that the desensitization mechanisms which restrict the signaling for cell death in HeLa cells, and which seem to be reversed upon triggering of p75-R, do not operate in A9 cells.

Effects of the anti p75-R Abs on TNF cytotoxicity were correlated only in part with their own ability to trigger cyto-



**Figure 9.** Mapping of epitope E. (A) Schematic representation of the bacterial constructs used for mapping. Recombinant peptides corresponding to parts of the extracellular domain of p75-R were expressed in *Escherichia coli*. Constructs differing in their COOH termini were obtained by use of the pET8c vector, whereas products differing in their NH<sub>2</sub>-termini were obtained as fusion proteins with the maltose binding protein (MBP constructs) by use of the pMalcRI vector. Recognition of these constructs by Abs against epitope E was assessed by Western blot analysis, as described in Materials and Methods and exemplified in B below. Constructs that reacted with the Abs are denoted in bold letters. The three mAbs against epitope E exhibited identical reaction patterns with the constructs. The sequence of amino acid residues in the extracellular domain of p75-R is shown in part. Residues found to be within the region of epitope E are denoted in bold letters. The two cysteine residues in it are underlined. (TM) Transmembrane domain. (B) An example of the Western blot used to map epitope E. After IPTG induction, samples (0.1 ml) of bacterial cultures expressing the indicated products were lysed in SDS-PAGE sample buffer and analyzed by SDS-PAGE (12% acrylamide). Western blotting was performed with mAb number 32 and goat anti-mouse Igs linked to horseradish peroxidase in the first and second steps of probing, respectively, as described in Materials and Methods. The urine-derived soluble form of p75-R (TBP, 0.36 µg [2]) served as a positive control. (Arrow-

cidal activity, alone or in the presence of anti-p55-R Abs (see Table 1). The partial nature of these correlations suggests that p75-R contributes to the cytotoxic function of TNF not only by its signaling activity, but also in another manner. The differences between the signaling effects of the Abs alone and their overall effects on TNF cytotoxicity were particularly notable in the transfected A9 cells and in HeLa cells expressing cytoplasmically truncated p75-R. Even though in these cells the Abs themselves did not trigger signaling, they had a significant effect on the killing of the cells by TNF. This was true also when the Abs were applied as Fab monovalent fragments (Table 1), indicating that this Ab-induced modulation, unlike triggering of the signaling activity of p75-R, does not involve cross-linking of p75-R. Two ways of modulation, depending on the site of Ab binding to the receptor, were observed. Abs that bind to the membrane-proximal part of the extracellular domain of p75-R, i.e., to its COOH-terminal cysteine loop or to the spacer region, inhibited the cytotoxic effect of TNF. This inhibition was observed in all transfectants studied, including the HeLa p75WT cells. Abs that bind to epitopes A, B, or C at the membrane-distal part of the extracellular domain, and which in HeLa p75WT cells triggered signaling for cytotoxicity, enhanced the cytotoxic effect also in cells that could not respond to signaling by p75-R (A9 cells and HeLa p75CT). In the latter cells, all the above Ab effects on TNF cytotoxicity were also observed when the Abs were applied as Fab monovalent fragments (Table 1).

Several points of evidence indicate that these two ways of modulation reflect an ability of p75-R to control the access of TNF to p55-R. (a) Because modulation was also observed in cells in which p75-R could not signal for cytotoxicity, it must reflect the effect(s) of p75-R (specifically, of its extracellular domain) on the cytotoxicity signaled by p55-R. (b) The modulation affected only the triggering, not the actual mechanism of signaling. Cells whose killing was triggered by agonistic Abs against p55-R showed no protection from the killing when treated with Abs that bind to the membrane-proximal part of the extracellular domain of p75-R, nor enhancement of it when treated with Fab monovalent fragments of Abs that bind to the distal part of the extracellular domain. (c) Modulation of TNF function by the different Abs was inversely related to their effect on TNF binding to p75-R. Potentiation of TNF activity was observed with those Abs that displace TNF from p75-R, and inhibition of TNF function was seen with Abs that enhance its binding.

Two possible mechanisms by which the access of a ligand

(heads) Location of the recombinant peptides that did not react with the Ab. This location was defined both by Coomassie blue staining and by Western blot analysis of the proteins, using rabbit polyclonal Abs against the soluble form of p75-R (2). (Left) Molecular weight markers. (C) ELISA for the binding of synthetic peptides by an Ab against epitope E. Comparison for binding to Ab number 32 between peptides whose sequences correspond to residues 165-180 (○), 163-180 (■), 162-180 (□), or 160-180 (●) in p75-R and a recombinant protein that contains the sequence of epitope E (a fusion protein comprised of MBP linked to a recombinant protein corresponding to amino acids 125-192 in p75-R) was assessed as described in Materials and Methods. Similar results were obtained with the two other Abs against epitope E.

to its receptor can be controlled by other, coexpressed, receptors were recently proposed. One was presented in a study that, like the present one, concerned the interactions of the two TNF-Rs (38), and the other in a study of the interactions of the two receptors for IL-1 (61). The two proposed mechanisms have practically opposing consequences. According to the first, interactions between the two receptors can result in enhancement of signaling; it was suggested that the p75 TNF-R is capable of "ligand passing", i.e., of presenting TNF to p55-R in such a way that its binding to p55-R is enhanced. The second mechanism can account for inhibitory interactions between two receptors. It was suggested that the type II IL-1 receptor serves as a "decoy receptor" which inhibits the binding of IL-1 to the type I IL-1 receptor by its own ability to bind IL-1. Whether and to what extent either of these two ways of modulation contributes to the observations of the present study is not clear. Perhaps both are involved.

The enhancement of TNF cytotoxicity by its displacement from p75-R with Abs against epitopes A, B, and C (in those transfectants where p75-R cannot signal) indicates that this receptor can have an inhibitory effect on the function of p55-R. A "ligand passing" mechanism cannot account for this effect since it should result not in inhibition but in enhancement of signaling. The inhibitory effect can however be explained by assuming that p75-R functions as a decoy receptor. At the levels attained in the transfected cells of this study, this receptor, by binding TNF, may have decreased the concentration of TNF in the cell culture and thus reduced the activation of p55-R.

On the other hand, the inhibition of TNF cytotoxicity by Abs that bind to the membrane-proximal part of the extracellular domain of p75-R is unlikely to be due to its function as a decoy receptor. Although these Abs decrease the dissociation of TNF from p75-R, this decrease is rather mild and results in only a small enhancement of TNF binding to the cells (Fig. 6 A), without any significant decrease in the concentration of TNF in the cell growth media (our unpublished data). Perhaps the Abs, by inhibiting dissociation of TNF from p75-R, slow down ligand passing between the two receptors (38).

Even though the action of decoy receptors and the process of ligand passing result in opposing effects, they may well co-occur, since they relate to different ways of ligand binding. Decoy receptors affect the access of ligands in the cell's milieu. The way in which ligand passing occurs is not clear, but it seems plausible that it occurs by transient formation of a ternary complex of the ligand with the two receptors, without involvement of the ligand found in the fluid phase compartment.

The way in which binding of Abs to the membrane-proximal part of p75-R, quite remote from the ligand-binding site, decreases the rate of TNF dissociation from the receptor remains to be clarified. The data presented in this paper indicate that these Abs and TNF have mutually positive effects in their binding to the receptor. The amounts of the Abs

that bound to the membrane-proximal part of the extracellular domain were the same in cells treated or untreated with TNF. However, the increased ability of the Abs to remain associated with the TNF-bound receptor in the presence of detergent indicates an increased efficacy of Ab binding. A likely explanation for the mutual effects of the Abs and TNF in their interaction with the receptor is that the binding of Abs to the membrane-proximal part of the extracellular domain and the binding of TNF to the distal part cause similar conformational changes in the receptor, and thus enhance each other allosterically. Induced conformational changes have also been observed in the extracellular domains of a number of other receptors as a consequence of their binding to their respective agonists (62–64). Notably, both the structure of epitope E (cysteine loop) and its size (17 residues, significantly above the size characteristic of sequence epitopes) would allow it sensitively to reflect changes in receptor conformation.

The findings of this study, although obtained only by examining the cytotoxic activity of TNF, have general implications for our understanding of the function of the two TNF-Rs. The ability of p75-R to participate doubly in an effect signaled by p55-R—both by controlling the access of TNF to p55-R and by its own signaling activity—implies that, even if the two TNF-Rs can be triggered separately, they constitute part of one functional unit. The ways in which the expression and function of p75-R are regulated are therefore likely to affect not only those TNF activities that are triggered by this particular receptor, but also the intensity of the effects of TNF signaled by p55-R. Moreover, pharmaceutical agents affecting p75-R may have a general impact on TNF function, including those activities whose major signaling receptor is p55-R. Such pharmacological modulation is perhaps indicated by the mode of function of Abs that bind to the membrane-proximal part of the extracellular domain of p75-R, particularly those that bind to its COOH-terminal cysteine loop. Because they are able to inhibit the activation of p55-R by TNF, and are unable to trigger the signaling activity of p75-R (see Fig. 2), these Abs may be useful as inhibitors of TNF function. Particularly suitable targets for such inhibition are cells of the monocytic and lymphocytic lineages, where the predominant receptor is p75-R yet many of the TNF effects depend on the signaling activity of p55-R (65). Preliminary findings in our laboratory indicate that Abs against epitope E can inhibit the effects of TNF in such cells, just as in the transfected cells examined in the present study, even though TNF binding to them is enhanced. In view of the marked conservation of various structural features in the cysteine-rich module that characterizes the NGF/TNF receptor family, particularly the location of the cysteine residues, it seems plausible that these structural features have similar functions in the different members of this family. It would be of interest to determine whether the COOH-terminal cysteine loops in the extracellular domains of the various other receptors of this family display features similar to those observed in p75-R and are affected in the same way by Ab binding.

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