Ligand Passing: The 75-kDa Tumor Necrosis Factor (TNF) Receptor Recruits TNF for Signaling by the 55-kDa TNF Receptor*

(Received for publication, March 29, 1993, and in revised form, May 27, 1993)

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To understand the role of the 75-kDa tumor necrosis factor (TNF) receptor in non-lymphoid cells, the cytotoxic signaling and ligand binding activities of the 55-kDa (TNF-R1) and 75-kDa (TNF-R2) TNF receptors were investigated using agonist and antagonist antibodies specific for the two receptor types. This study indicates that although TNF-R2 can significantly reduce the TNF concentration required for cell killing, the mechanism by which this is accomplished is not through the generation of an intracellular signal by TNF-R2. Instead, TNF-R2 regulates the rate of TNF association with TNF-R1, possibly by increasing the local concentration of TNF at the cell surface through rapid ligand association and dissociation. We propose that other cell-surface receptors, such as the low affinity p75 nerve growth factor receptor, may utilize an analogous "ligand passing" mechanism.

Tumor necrosis factor (TNF), a potent cytokine produced primarily by activated macrophages, elicits a large number of biological effects including hemorrhagic necrosis of transplanted tumors, cytotoxicity, and inflammatory, immunoregulatory, proliferative, and antiviral responses (Beutler and Cerami, 1988; Fiers, 1991; Goeddel et al., 1986; Old, 1988). The first step in the induction of the various cellular responses mediated by TNF is its binding to specific cell-surface receptors. Two distinct TNF receptors of ~55 kDa (TNF-R1) and ~75 kDa (TNF-R2) have now been identified (Brockhaus et al., 1990; Hohmann et al., 1990; Old, 1988). The rabbit anti-murine TNF-R1 antibodies have been described previously (Tartaglia et al., 1991). Anti-TNF-R1 mAbs have also been described that effectively antagonize the TNF induction of many of these responses (Shalaby et al., 1991; Thoma et al., 1990). In addition, polyclonal and monoclonal antibodies (mAbs) directed against human TNF-R1 have been shown to behave as receptor agonists and to elicit several TNF activities such as cytotoxicity, fibroblast proliferation, resistance to chlamydiae, and synthesis of prostaglandin E2 (Engelmann et al., 1990; Espevik et al., 1990; Shalaby et al., 1990). Anti-TNF-R1 mAbs have also been described that effectively antagonize the TNF induction of many of these responses (Shalaby et al., 1990; Thoma et al., 1990). In addition, polyclonal antibodies to both murine TNF-R1 and TNF-R2 have been developed, and each has been shown to behave as a receptor-specific agonist and to induce a subset of mTNF activities (Tartaglia et al., 1991). Studies with the murine receptor agonist antibodies have demonstrated that the two receptors signal distinct TNF activities. TNF-R1 is responsible for signaling cytotoxicity and the induction of several genes, whereas TNF-R2 is capable of signaling proliferation of primary thymocytes and a cytotoxic T cell line (Tartaglia et al., 1991).

Paradoxically, several reports have described mAbs directed against human TNF-R2 that can partially antagonize the same TNF responses (e.g. cytotoxicity and NF-κB activation) that can be signaled through TNF-R1 (Hohmann et al., 1990; Naume et al., 1991; Shalaby et al., 1990). Furthermore, mTNF is much more effective than hTNF at killing some murine cell lines (Heller et al., 1992). This is also suggestive of TNF-R2 function since hTNF can bind murine TNF-R1, but not murine TNF-R2 (Lewis et al., 1991). The authors of several of these studies therefore concluded that there is redundancy in the function of the two TNF receptors. This model in which TNF-R2 signals many of the same activities as TNF-R1 is in marked contrast to the nonredundant signaling model used to explain data with receptor-specific agonists. To help reconcile these seemingly conflicting data, we have analyzed in detail the role TNF-R2 plays in influencing TNF's cytotoxic activity. Our findings indicate that TNF-R2 does not generate a signal that potentiates the cytotoxicity of TNF-R1, but instead passes TNF to TNF-R1, where signaling occurs.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant hTNF and recombinant mTNF (specific activity of >106 units/mg) were provided by the Genentech Manufacturing Group. The rabbit anti-murine TNF-R2 antibodies have been described previously (Tartaglia et al., 1991). Hamster mAbs against murine TNF-R1 (mAbs 170 and 176) and TNF-R2 (mAbs 32.4 and 54.7) were generated against the corresponding soluble receptor extracellular domains and supplied by K. Sheehan and R. Schreiber. Anti-TNF receptor mAbs 984 and 1040 inhibit the binding of TNF to human TNF-R1 and TNF-R2, respectively, and have been described previously (Pennica et al., 1992a, 1992b; Tartaglia and Goeddel, 1992). mTNF was purchased from Amersham Corp.

Cytotoxicity Assays—Murine L929 cells (2 × 10⁴ cells/well) were seeded into 96-well microtiter plates in 100 μl of medium (low glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) and incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. The medium was then brought to 10 μg/ml cycloheximide (CHX), and the anti-TNF receptor antibodies or TNF was added to the wells and serially diluted. The plates were incubated for an additional 16 h (or for the indicated time period), and the viable cells were stained with 20% methanol containing 0.5% crystal violet. The dye was eluted with 0.1 N sodium citrate, 0.1 N citric acid in 50% ethanol, and absorbance was measured at 540 nm. HeLa,R2-1 cells (2 × 10⁴ cells/well) were seeded into 96-well microtiter plates in 100 μl of medium (50:50 low glucose Dulbecco's modified Eagle's medium/Hams's F-12 medium supplemented with 10% fetal calf serum, 1% l-glutamine,

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1 The abbreviations used are: TNF, tumor necrosis factor; TNF-R1, 55-kDa TNF receptor; TNF-R2, 75-kDa TNF receptor; mAbs, monoclonal antibodies; mTNF, murine TNF; hTNF, human TNF; CHX, cycloheximide; IL-2, interleukin-2; NGF, nerve growth factor.

2 R. F. Weber and D. V. Goeddel, unpublished data.
100 units/ml penicillin, 100 µg/ml streptomycin, and 400 µg/ml G418) and incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. The medium was then brought to 10 µg/ml CHX, and the anti-TNF receptor antibodies or TNF was added to the wells and serially diluted. The plates were incubated for an additional 24 h, and the viable cells were assayed as described above. Reported values are the means of triplicate determinations.

**TNF Association and Dissociation Experiment**—For association kinetics, 10⁶ U937 cells in phosphate-buffered saline, 0.1% bovine serum albumin, 0.02% sodium azide (PBSA) were incubated with 80 pg [³²P] TNF in a final volume of 200 µl on ice. After predetermined times, samples were centrifuged and then washed twice with PBSA and re-centrifuged. Nonspecific binding was determined in the presence of 0.5 µM unlabeled TNF. All values are the means of duplicate determinations. Additional details of the binding assays are as described elsewhere (Pennica et al., 1992b).

For dissociation kinetics, 2.5 × 10⁶ U937 cells in 5 ml of PBSA were incubated with 80 pg [³²P]TNF on ice. After 3 h, 100 ns unlabeled TNF was added, and 200-µl aliquots were removed at various times, centrifuged, washed, and re-centrifuged. The time at which unlabeled TNF was added was taken as time 0. Nonspecific binding was determined through an identical assay in which 100 ns unlabeled TNF was added at the beginning of the 3-h incubation. All values are the means of duplicate determinations. Additional details of the dissociation assay are as described elsewhere (Pennica et al., 1992b).

**RESULTS**

**Inhibition of TNF-mediated Cytotoxicity by TNF Receptor-specific Antibodies**—To help clarify the functional roles of the two TNF receptors, we examined the ability of anti-TNF receptor-specific mAbs to inhibit the killing of murine L929 cells by mTNF. Antibodies specific for murine TNF-R1 were found to completely inhibit cytotoxicity at all TNF concentrations tested. This result was seen both in a 9-h assay in the presence of CHX (Fig. 1A) and in a 24-h assay in the absence of CHX (Fig. 1B) and is consistent with an essential role of TNF-R1 in signaling cytotoxicity. Interestingly, saturating concentrations of anti-murine TNF-R2 antibodies partially inhibited cytotoxicity (Fig. 1, A and B). Significant inhibition by the anti-TNF-R2 antibodies was not observed at high TNF concentrations, but nevertheless, these antibodies caused a significant shift in the TNF dose response. This ability of TNF-R2 to reduce the TNF concentrations required for killing can also be seen in a comparison of the cytotoxicity dose-response curves of murine and human TNFs (Fig. 2). Human TNF binds only murine TNF-R1 and does not recognize murine TNF-R2 (Lewis et al., 1991). Therefore, the hTNF killing dose-response curve is shifted rightward relative to the mTNF killing curve. Similar effects of the receptor-specific antibodies and ligand species specificity were also observed in the NIH 3T3 cell line (data not shown).

The partial inhibition of cytotoxicity by anti-TNF-R2 antibodies and the complete inhibition by anti-TNF-R1 antibodies are consistent with either of two possible models. In the first model, both receptors are capable of activating signal-transducing pathways that ultimately lead to cell death. At high receptor occupancy, signals from TNF-R1 alone can result in cell killing, whereas signals from TNF-R2 alone are insufficient. However, at lower receptor occupancy, signals from TNF-R2 could synergize with signals from TNF-R1 and result in increased TNF sensitivity. In the second model, only signals generated by TNF-R1 result in cell killing, and signals from TNF-R2 play no role. However, in this model, TNF-R2 facilitates the binding of TNF to TNF-R1, resulting in the triggering of TNF-R1 at lower TNF concentrations.

**Agonist Antibodies to TNF-R2 Behave as Antagonists in Cytotoxicity Assay**—To begin to distinguish between a signaling or a binding accessory role of TNF-R2, we first tested whether antibodies capable of specifically activating TNF-R2 would act as TNF antagonists or agonists in the L929 cytotoxicity assay (Fig. 3). We have previously shown that these antibodies are capable of potently activating TNF-R2 and inducing the proliferation of murine thymocytes and a T cell line (Tartaglia et al., 1991). However, in a cytotoxicity assay, these TNF-R2-activating antibodies do not act as agonists and do not shift the dose-response curve to the left. Instead, they act as antagonists and push the dose-response curve to the right. The effect of the anti-TNF-R2 polyclonal antibodies in this assay again points to a role of TNF-R2 in affecting cytotoxicity. However, the fact that antibodies capable of activating TNF-R2 are acting as antagonists argues against a signaling role.

**Occupancy of TNF-R2 by TNF Does Not Enhance the Killing Signal Generated by a Weak TNF-R1 Agonist**—To test whether occupancy of TNF-R2 by TNF can generate a signal that synergizes with a weak TNF-R1 signal, we examined the effect of increasing concentrations of murine TNF on L929 cells in which access to TNF-R1 was blocked by a saturating concentration of a weak TNF-R1 agonist antibody (Fig. 4). If TNF-R2 can generate a cytotoxicity signal, then mTNF would be ex-

**Fig. 1.** Inhibition of TNF-induced cytotoxicity in L929 cells by anti-TNF receptor antibodies. A, L929 cells were pretreated for 1 h with control hybridomas supernatant (1:10 dilution) (△), anti-TNF-R1 mAb 170 (1:10 dilution of hybridomas supernatant) (○), or 22 µg/ml anti-TNF-R2 mAb 32.4 (■), followed by treatment with the indicated concentrations of murine TNF and 10 µg/ml CHX for 9 h. B, L929 cells were pretreated for 1 h with control hybridomas supernatant (1:10 dilution) (△), anti-TNF-R1 mAb 170 (1:10 dilution of hybridomas supernatant) (○), anti-TNF-R1 mAb 176 (1:10 dilution of hybridomas supernatant) (□), 22 µg/ml anti-TNF-R2 mAb 32.4 (■), or 22 µg/ml anti-TNF-R2 mAb 54.7 (△), followed by treatment with the indicated concentrations of murine TNF for 34 h. Cell viability was determined as described previously (Tartaglia and Goeddel, 1992).

**Fig. 2.** Comparison of L929 cytotoxicity in response to murine and human TNFs. Cells were treated with the indicated concentrations of murine or human TNF and 10 µg/ml CHX for 10 h. Cell viability was determined as described previously (Tartaglia and Goeddel, 1992).
TNF-R2 recruits TNF for signaling by TNF-R1

**Fig. 3. Inhibition of TNF-induced cytotoxicity in L929 cells by anti-TNF-R2 agonist antibodies.** L929 cells were pretreated for 1 h with either preimmune serum (1:100 dilution) or rabbit anti-TNF-R2 polyclonal serum (1:100 dilution), followed by treatment with the indicated concentrations of murine TNF and 10 μg/ml CHX for 19 h. Cell viability was determined as described previously (Tartaglia and Goeddle, 1992).

**Fig. 4. Effect of TNF-R2 occupancy on cytotoxicity induced by weak TNF-R1 agonist.** L929 cells were pretreated for 1 h with either preimmune serum (1:100 dilution) or rabbit anti-TNF-R2 mAb 176 (1:10 dilution of hybridoma supernatant), followed by treatment with the indicated concentrations of murine TNF and 10 μg/ml CHX for 18 h (the weak agonist activity of mAb 176 was detected in cytotoxicity assays that were performed in the presence of CHX and that were >12 h in duration). Cell viability was determined as described previously (Tartaglia and Goeddle, 1992).

**Fig. 5. TNF-induced cytotoxicity of L929 cells in response to independent occupation of two TNF receptors.** Two 36-well plates of L929 cells were treated with the indicated concentrations of hTNF for 1 h at 4°C. The cells were then further treated for 1 h at 4°C with an excess of an antagonist antibody to TNF-R1 (mAb 170, 1:10 dilution of hybridoma supernatant). 1 μg/ml mTNF (a dose sufficient to achieve 100% killing in the absence of blocking antibody) was then added to one plate (C), whereas the other was not further treated (O). 10 μg/ml CHX was added to all cells, and the temperature of the experiment was shifted to 37°C for 10 h. Cell viability was determined as described previously (Tartaglia and Goeddle, 1992).

Possible Mechanism of TNF-R2 Accessory Role—The mechanism by which TNF-R2 can affect the association rate of TNF binding to TNF-R1 is accessible to a given TNF molecule. However, if the role of TNF-R2 is to facilitate binding to TNF-R1, then it should not enhance cytotoxicity when TNF-R1 cannot take place, did not potentiate the killing that is obtained by occupancy of TNF-R1 alone (Fig. 5). This result indicates that the binding of TNF to TNF-R2 does not enhance cytotoxicity unless the same TNF molecules that bind to TNF-R2 also have access to TNF-R1 and thus argues against a direct signaling role of TNF-R2.

TNF-R2 Increases Association Rate of TNF Binding to TNF-R1—The biological effects described above are consistent with TNF-R2 facilitating the binding of TNF to TNF-R1. In an attempt to provide direct biochemical data for this phenomenon, the association rates of TNF binding to the two TNF receptors were examined. The association of 125I-TNF with TNF-R1 alone, with TNF-R2 alone, and with the two receptors simultaneously was determined using U937 cells treated with blocking antibodies to TNF-R2, with blocking antibodies to TNF-R1, and in the absence of blocking antibodies, respectively (Fig. 6A). The experimentally observed rate constants (kassoc) were derived from the slope of semilogarithmic transformed plots of the initial association data and were calculated to be 0.002 and 0.037 min⁻¹ for TNF-R1 and TNF-R2, respectively (Fig. 6B). Thus, when the two TNF receptors are isolated, TNF associates nearly 20 times as rapidly with TNF-R2 as compared to TNF-R1. The association rate of TNF binding to TNF-R1 in the presence of TNF-R2 was derived from the binding that is inhibited by the anti-TNF-R1 antibody (Fig. 6A) (differential between TNF binding to both receptors simultaneously versus TNF-R2 only) and is also shown in Fig. 6B. A comparison of the slope of this plot (0.023 min⁻¹) with the slope of the association plot for TNF-R1 alone (0.002 min⁻¹) shows that accessible TNF-R2 increases the rate of association of TNF with TNF-R1 ~10-fold. This result is consistent with a previously unexplained observation by Hohmann et al. (1990), who noted that the Kd for all TNF-binding sites on HL-60 cells was ~7-fold lower than that measured for isolated TNF-R1 sites. It is likely that this ability of TNF-R2 to enhance the binding of TNF to TNF-R1 explains the enhanced cytotoxicity observed at low TNF concentrations since TNF-R2 is not capable of generating a synergistic signal.

Possible Mechanism of TNF-R2 Accessory Role—The mechanism by which TNF-R2 can affect the association rate of TNF binding to TNF-R1 is also of interest. To address this, we have attempted to distinguish between two different models. In the first model, TNF can form heterocomplexes between the two different TNF receptors. This model is attractive because it is easy to conceptualize, and also there is a well characterized precedent for it in the case of interleukin-2 (IL-2) binding to its receptors (Lowenthal and Greene, 1987; Wang and Smith, 1988). IL-2 binding to the p55 IL-2 receptor is rapid, whereas IL-2 binding to the isolated p75 IL-2 signaling receptor is slow. When both receptors are present on the same cell, IL-2 binds...
judged by the binding inhibited by a TNF-R1 antagonist anti-
formation model, in which heterocomplex formation is likely to
TNF-R1. This would not be a prediction of the heterocomplex
body). Another prediction of this model is that the dissociation
min time point, yet when TNF-R2 was accessible, binding to
TNF-R1 was almost saturated at the 30-min time point (as
rate of TNF from TNF-R2 is independent of the presence of
with TNF-R1 when TNF-R2 is accessible (derived from the difference
TNF from TNF-R2 is rapid enough to account for the observed
facilitation of binding to TNF-R1.

certain predictions. One prediction is that the dissociation of
TNF-R2 (either two or three receptor) (Loetscher et al.,
1991; Pennica et al., 1992a, 1992b) and were used as a model for TNF binding to TNF-R2. Dissociation of TNF from 293/R2 cells (TNF-R2) was found to be surprisingly rapid (t_{1/2} = 10 min) for a high affinity receptor (Fig. 7A). Therefore, the rapid rates of both association with and dissociation from TNF-R2 could account for its observed TNF-R1 binding facilitation. In contrast, dissociation of TNF from HeLa cells (TNF-R1) was found to be quite slow, with a half-life of >3 h (Fig. 7A).

The dissociation of TNF from U937 cells for the two TNF receptors simultaneously and for both receptors individually was also examined (Fig. 7B). Again, dissociation from TNF-R2 was found to be rapid, whereas dissociation from TNF-R1 was slow. It is also important to note the shape of the dissociation curves of TNF bound to both receptors. The parallel nature of these dissociation curves (whose absolute values are shifted as a result of TNF-R1 binding) indicates that the dissociation of TNF from TNF-R2 is unaffected by TNF-R1 and therefore argues against heterocomplex formation.

Effect of TNF-R2 Expression Level on Binding Facilitation

rapidly to the p55 chain, thereby restricting IL-2 to a two-
dimensional search for the p75 chain that ends in rapid IL-
2-p55-p75 heterocomplex formation. In the alternative model,
TNF cannot form heterocomplexes between the two different
receptors. In this case, TNF-R2 would function to increase the
local concentration of TNF at the cell surface by rapid ligand
association and dissociation.

We and others have demonstrated previously that TNF can
induce the formation of homocomplexes of both TNF-R1 and
TNF-R2 (either two or three receptors/TNF trimer) (Loetscher
et al., 1991; Pennica et al., 1992a, 1992b). However, cross-link-
ing and immunoprecipitation experiments have so far been
unable to demonstrate the formation of TNF receptor hetero-
complexes on the cell surface even though homocomplex forma-
tion is readily detectable under these same conditions. These
negative data have forced us to consider the model of TNF-R2
association function in the absence of heterocomplex formation.
Although this model is difficult to test directly, it does have
certain predictions. One prediction is that the dissociation of
TNF from TNF-R2 is rapid enough to account for the observed
facilitation of binding to TNF-R1. An example of the rapidity of
this binding facilitation is shown in Fig. 6A. Very little TNF
associated with TNF-R1 in the absence of TNF-R2 at the 30-
min time point, yet when TNF-R2 was accessible, binding to
TNF-R1 was almost saturated at the 30-min time point (as
judged by the binding inhibited by a TNF-R1 antagonist anti-
body). Another prediction of this model is that the dissociation
rate of TNF from TNF-R2 is independent of the presence of
TNF-R1. This would not be a prediction of the heterocomplex
formation model, in which heterocomplex formation is likely to
alter the dissociation rate of TNF from TNF-R2, as has been
observed for the dissociation of IL-2 from the p55 chain of the
IL-2 receptor system (Lowenthal and Greene, 1987; Wang and
Smith, 1987).

To test these predictions, we examined the dissociation of
TNF from the TNF receptors on HeLa, 293/R2, and U937 cells.
HeLa cells have been shown to express exclusively or predomi-
antly TNF-R1 (Brookhaus et al., 1990), and we have therefore
used TNF binding to HeLa cells as a model of TNF-R1 binding.
293/R2 cells have been engineered to express TNF-R2 to 100-
fold higher levels than endogenous receptors (Pennica et al.,
1992b) and were used as a model for TNF binding to TNF-R2.

FIG. 6. Association kinetics of 125I-TNF binding to two TNF
receptors on U937 cells. A, cells were preincubated with 27 pg/ml
anti-TNF-R1 mAb 984 (○) or with 38 pg/ml anti-TNF-R2 mAb 1040 (□)
or in the absence of blocking antibody (●) for 1 h at 0°C. 0.08 nm
125I-TNF (specific activity of 46 Ci/pg) was then added, and the cells
were further incubated. At the indicated times, aliquots were removed,
washed as described under "Experimental Procedures," and counted for
radioactivity. The dashed curve (●) shows the deduced association of
TNF with TNF-R1 when TNF-R2 is accessible (binding in the absence
of blocking antibody minus binding in the presence of anti-TNF-R1 m Ab
984). B, shown are semilogarithmic plots of initial association data.
The data are plotted as ln(B/B_0) versus time, where B_0 is specific
binding at steady state and B is specific binding for the association
time shown. □, specific association in the presence of anti-TNF-R1; ○,
specific association in the presence of anti-TNF-R2; ●, specific association
with TNF-R1 when TNF-R2 is accessible (derived from the difference
between association in the absence of blocking antibody and association
in the presence of anti-TNF-R1).

FIG. 7. Dissociation kinetics of 125I-TNF binding to two TNF
receptors. A, HeLa (○) and 293/R2 (□) cells were incubated with 0.08
nm 125I-TNF at 0°C for 3 h. A 1000-fold excess of unlabeled TNF was
then added, and aliquots were removed at the indicated time points.
Aliquots were washed as described under "Experimental Procedures,"
and radioactivity was determined. Total binding and nonspecific bind-
ing at time 0 for 293/R2 cells were 19,280 and 1472, respectively, and for
HeLa cells were 3129 and 1536, respectively. B, U937 cells were
preincubated either with 27 pg/ml anti-TNF-R1 mAb 984 (○) or with 38
pg/ml anti-TNF-R2 mAb 1040 (□) or in the absence of blocking antibody
(●) for 1 h at 0°C. 0.08 nm 125I-TNF was then added at 0°C for 3 h. A
1000-fold excess of unlabeled TNF was then added, and aliquots were
removed at the indicated time points. Aliquots were washed as de-
scribed under "Experimental Procedures," and radioactivity was deter-
mained.

3 D. Pennica and D. V. Goeddel, unpublished data.
4 H. Loetscher and W. Leslauer, personal communication.
Most cell lines in which we have detected enhanced triggering of TNF-R1 via TNF-R2 express TNF-R2 on the order of a few thousand receptors/cell. To determine whether high level expression of TNF-R2 can further accentuate its accessory effects, we examined a HeLa cell line (HeLa.R2-1) (Tartaglia et al., 1993) that expresses high levels of cell-surface TNF-R2 (52,000 TNF-R2 molecules/cell) from a transfected cDNA. HeLa cells were chosen for this transplantation analysis because parental HeLa cells do not express significant levels of TNF-R2 (Brockhaus et al., 1990), and their sensitivity to TNF is not affected by TNF-R2 antagonist antibodies (data not shown). Surprisingly, high level expression of TNF-R2 actually reduced sensitivity at low TNF concentrations, as determined by a comparison of TNF-induced cytotoxicity in HeLa.R2-1 cells in the presence and absence of a TNF-R2 antagonist antibody (Fig. 8A). The inhibitory effects of TNF-R2 at low (and not high) TNF concentrations would be consistent with TNF-R2 titrating the TNF from the assay medium, thus leaving less available to reach TNF-R1. We therefore examined how narrow the window of TNF-R2 expression levels must be to effectively concentrate TNF at the cell surface and yet not reduce the available TNF concentration by titration.

HeLa.R2-1 cells were treated with a low TNF concentration (0.01 ng/ml), and the effective concentration of TNF-R2 was incrementally increased by addition of decreasing concentrations of a TNF-R2 antagonist antibody (Fig. 8B). In agreement with Fig. 8A, cells with little or no accessible TNF-R2 (high antibody concentrations) are more sensitive to TNF than are cells with high levels of accessible TNF-R2 (little or no added antibody). However, cells treated with intermediate concentrations of anti-TNF-R2 antibody show even higher sensitivity to TNF compared with cells treated with high antibody concentrations. Together, these results indicate that low level expression of TNF-R2 can facilitate triggering of TNF-R1, but high level expression of TNF-R2 is detrimental to TNF-R1 triggering at low TNF concentrations. Thus, cells must carefully regulate the level of TNF-R2 expression to optimize enhanced triggering of TNF-R1.

**Discussion**

Although not yet systematically investigated, the majority of cell types and tissues appear to express both TNF receptors (Lewis et al., 1991; Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). Therefore, it is important to understand the individual roles of these two receptors in cell signaling, both to assess whether greater clinical specificity of TNF actions can be realized at the level of receptor activation and to better understand the biology of this important cytokine. To help address this question, several groups have generated antibodies that are specific for either TNF-R1 or TNF-R2. However, despite the availability of these powerful immunological tools, the individual signaling roles of the two TNF receptors are still under considerable debate.

A significant breakthrough in our understanding of TNF receptor function came from the observation that antibodies against TNF-R1 can act as specific agonists for this receptor and can signal several diverse TNF activities such as cytotoxicity, fibroblast proliferation, resistance to chlamydiae, and synthesis of prostaglandin E2 (Engelmann et al., 1990; Espevik et al., 1990; Shalaby et al., 1990). These studies demonstrated that activation of TNF-R1 alone is sufficient to mimic many TNF activities in diverse cell types. Agonist antibodies specific for TNF-R2 can also mimic TNF and induce a small subset of TNF activities including the enhancement of T cell proliferation (Tartaglia et al., 1991). However, TNF-R2 agonists have so far been demonstrated to directly signal only in some lymphoid cell types and do not induce the same set of activities induced by TNF-R1 agonists even in cells expressing similar levels of both receptors (Tartaglia et al., 1991; Wong et al., 1992a, 1992b).

The role of the relatively high levels of TNF-R2 in non-lymphoid cells is therefore still an important and unresolved issue. Several reports have described mAbs directed against TNF-R2 that can partially antagonize the same TNF responses that are induced by TNF-R1 agonists (Hohmann et al., 1990; Shalaby et al., 1990). These reports suggested that there is redundancy in the function of the two receptors. However, a redundant signaling model is clearly in conflict with the demonstration, using receptor-specific agonist antibodies, that the two receptors signal distinct and largely nonoverlapping sets of activities. Central to this controversy is the role of the two TNF receptors in mediating cytotoxicity. In cell lines examined so far, only anti-TNF-R1 (and not anti-TNF-R2) antibodies are able to mimic TNF's cytotoxic activity, even in cell lines expressing both receptors (Tartaglia et al., 1991, 1993; Wong et al., 1992a). Nevertheless, there are clear data showing that antibodies that block binding of TNF to TNF-R2 partially inhibit TNF killing (Shalaby et al., 1990). In addition, several groups have identified murine cell lines that are killed much more readily by murine TNF than by human TNF (Fraena et al., 1986; Heller et al., 1992; Rice et al., 1990), which might also be indicative of some role of TNF-R2 in cell killing.

To help resolve these conflicting viewpoints, we have analyzed in detail the role of TNF-R2 in influencing cytotoxicity. We observed that mAbs to TNF-R1 could completely inhibit cytotoxicity at all tested TNF concentrations, indicative of an absolute requirement of the binding of TNF to TNF-R1. How-

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**FIG. 8. Effect of TNF-R2 expression level on binding facilitation.** A. HeLa.R2-1 cells were preincubated for 1 h in either the absence □ or presence ○ of 38 μg/ml TNF-R2 antagonist mAb 1040. Cells were then further treated for 24 h with 10 μg/ml CHX and the indicated concentrations of TNF. Cell viability was determined as described previously (Tartaglia and Goeddel, 1992). Data shown are the means of triplicate determinations. B. HeLa.R2-1 cells were pretreated for 1 h with the indicated concentrations of TNF-R2 antagonist mAb 1040. Cells were then further treated with a combination of 10 μg/ml CHX and 0.01 ng/ml TNF. Cell viability was determined as described previously (Tartaglia and Goeddel, 1992). Data shown are the means of triplicate determinations.

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5 R. Schreiber, personal communication.
ever, in agreement with previous reports using the human U937 cell line (Shalaby et al., 1990), we observed that monoclonal antibodies that block binding of TNF to TNF-R2 partially inhibit TNF toxicity at low TNF concentrations. Interestingly, we found that anti-TNF-R2 polyclonal antibodies capable of activating TNF-R2 also exhibited antagonistic activity with respect to cytotoxicity. This result suggests that while the binding of TNF to TNF-R2 may be important for facilitating cytotoxicity, the activation of TNF-R2 and the generation of a TNF-R2 signal are unlikely to be involved. It is therefore possible that the binding of TNF to TNF-R2 serves only to facilitate the binding of TNF to TNF-R1. To rule out the possibility that TNF initiates a signal through TNF-R2 that agonist antibodies cannot mimic, we tested whether the binding of TNF itself to TNF-R2 could potentiate the partial killing mediated by a weak TNF-R1 agonist antibody or a TNF-R1-specific ligand. Under these conditions, in which the TNF molecules binding TNF-R2 do not have further access to TNF-R1, no effects of TNF-R2 occupancy were observed. Taken together, these results argue against a signaling role of TNF-R2 in cytotoxicity and suggest that the role of TNF-R2 is to channel TNF to TNF-R1, which is then responsible for all signal generation.

To examine the biochemical mechanism by which TNF-R2 might facilitate the activation of TNF-R1 by TNF, we examined the binding of 125I-TNF to its two receptors. This study showed that TNF associates much more rapidly with TNF-R2 than with TNF-R1. However, when TNF has access to both receptors on the same cell, the presence of TNF-R2 greatly enhances the rate of association of TNF with TNF-R1. This enhancement is not likely to be a result of an intracellular signal by TNF-R2 since it occurs at 0°C and is not mimicked by TNF-R2 agonist antibodies. The ability of TNF-R2 to enhance association of TNF with TNF-R1 is likely to account for the many inhibitory effects observed with anti-TNF-R2 antibodies in non-lymphoid cell types. It has also been proposed, based on ligand species specificity arguments, that TNF-R2 contributes to the in vivo toxicity of TNF (van Ostade et al., 1993). The ability of TNF-R2 to facilitate TNF-R1 triggering may be the primary mechanism for this enhanced toxicity.

An important feature of this accessory role of TNF-R2 is that it does not require intracellular signaling by TNF-R2, despite the observations that antagonists to either receptor can inhibit the same cellular response. This model is therefore also consistent with data on the nonoverlapping activities of TNF-R1 and TNF-R2 agonists (Tartaglia et al., 1991), the completely neutralizing activities of TNF-R1 antagonists observed with cell lines expressing both receptors (Tartaglia et al., 1993; Thoma et al., 1990; this study), and the lack of homology between the two TNF receptor intracellular domains.

The mechanism by which TNF-R2 facilitates association of TNF with TNF-R1 may also be novel as it does not appear to involve the stable association of the two receptor types with one another to form heterocomplexes. Chemical cross-linking and immunoprecipitation experiments, which can readily demonstrate TNF-induced aggregation of multiple TNFs-R1 or TNFs-R2 in homocomplexes, have not detected heterocomplexes on the cell surface under these same conditions. In addition, the kinetics of dissociation of TNF from a cell expressing both TNF receptor types is a simple sum of the dissociation from the two receptors individually. This is in marked contrast to receptor chains that form heterocomplexes such as the IL-2 receptor system (Lowenthal and Greene, 1987; Wang and Smith, 1987). Why TNF receptors seem only to form homocomplexes and not heterocomplexes is also of interest. One possibility is the 57-amino acid spacer sequence between the transmembrane region and the presumed cysteine-rich ligand-binding domain of TNF-R2 (Smith et al., 1990). This spacer region is heavily O-glycosylated and forms an extended structure (Pennica et al., 1993), which may also be quite rigid (Jenoff, 1990). The ligand-binding domain of TNF-R2 may therefore be further from the membrane surface than the ligand-binding domain of TNF-R1, which contains no such spacer region. This would make it unlikely that the compact TNF trimer could simultaneously interact with both receptors. It is possible that preventing heterocomplex formation is critical to achieve proper TNF responses since a complex between TNF-R2 and TNF-R1 would likely result in a dominant negative effect on TNF-R1 signaling, as has been observed with complexes between truncated TNF-R1 and full-length TNF-R1 (Tartaglia and Goeddel, 1992).

The kinetic properties of TNF binding to TNF-R2 suggests a mechanism by which TNF-R2 might increase the apparent association rate of TNF binding to TNF-R1. In this model, the rapid association of TNF with and the dissociation of TNF from TNF-R2 would serve to increase the local concentration of TNF at the cell surface, resulting in more rapid association with TNF-R1. This "passing" model does not require heterocomplex formation. It is therefore consistent with data in several studies (Engelmann et al., 1990; Loetscher et al., 1991; Tartaglia and Goeddel, 1992) that suggest that TNF, which exists as a trimer, must cross-link three molecules of TNF-R1 to deliver an effective signal. However, the possibility that TNF-R2 facilitates association of TNF with TNF-R1 by formation of a transient heteroreceptor complex cannot yet be ruled out.

The physiological importance of the ability of TNF-R2 to regulate TNF-R1 triggering is also of interest. In vivo, TNF is often present at rather low concentrations and is also rapidly cleared. It is therefore possible that many cells are exposed to TNF under conditions in which the ligand concentrating effects of TNF-R2 are essential for proper TNF-R1 responses. Therefore, the expression of a receptor capable of rapid ligand association and dissociation provides a novel mechanism for increasing a cell's sensitivity to a transient flux of a regulatory molecule. Rapid shedding of the TNF-R2 extracellular domain (Pennica et al., 1992b) might then be a mechanism for a cell to reduce sensitivity. In some cases, dramatically reduced TNF sensitivity might be achieved by expression of TNF-R2 at higher than optimal levels (as suggested by studies on high level expression of TNF-R2 in HeLa cells (Fig. 8)). Such mechanisms may, in fact, be superior to regulating the level of TNF-R1, where very high expression would be required to obtain cellular responses at TNF levels ×100-fold below its K_i for TNF-R1, as is the case with many cellular responses to TNF.

Whether similar mechanisms of regulating ligand binding are used by other receptor systems remains to be established. However, it seems unlikely that the TNF system alone would utilize this mechanism to control signaling sensitivity. Several known membrane receptors, whose exact functions remain obscure, could potentially fulfill a ligand passing or concentrating role analogous to that of TNF-R2. The function of the low affinity p75 NGF receptor is currently unresolved since most of the direct signaling of the neurotrophic factors appears to be mediated by the trk family of receptors (Jing et al., 1992). Nevertheless, the p75 NGF receptor still plays an important role in neural development (as demonstrated with targeted mutations in mice) (Lee et al., 1992) and, under at least some conditions, can increase the binding of NGF to trk (Hempstead et al., 1991). Interestingly, the binding kinetics of NGF to the p75 NGF receptor is very fast relative to trk binding (Hartman et al., 1992). In addition, there is no evidence to indicate that the p75 NGF receptor forms a heterocomplex with the trk receptors (Jing et al., 1992). It is therefore possible that rapid
dissociation from the p75 NRG receptor concentrates neurotrophic factors near the cell surface. It has also been suggested that the transforming growth factor-β type III receptor serves a ligand presentation rather than a signaling role (Lopez-Casillas et al., 1991; Wang et al., 1991). Although information on binding kinetics and complex formation is not yet available for this receptor, ligand passing is a potential mechanism of action. In the case of the IL-1 receptor system, only the type I receptor has been shown to be active in signal transduction (Stylianou et al., 1992). The IL-1 type II receptor, which dissociates from IL-1 more rapidly (Horuk and McCubrey, 1989), may therefore serve to regulate the binding of IL-1 to the type I receptor. Further studies on the properties of these and other receptor systems are needed to establish the generality of regulating ligand binding in this manner.

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