Induction of Unresponsiveness to Tumor Necrosis Factor (TNF) after Autocrine TNF Expression Requires TNF Membrane Retention*

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Tumor necrosis factor (TNF) has a specific gene-inducing activity on many cell types and exerts a cytotoxic effect on a number of tumor cell lines. However, several tumor cell types are resistant to TNF-induced effects, and some of these produce TNF. We previously demonstrated that introduction of an exogenous TNF gene in the TNF-sensitive cell line L929A induced autocrine TNF production and unresponsiveness to the cytotoxic activity of TNF. This resistance required biologically active TNF and was correlated with complete down-modulation of the TNF receptors on the cell surface. We have now characterized this process in more detail. The role of expression of the membrane-bound TNF preform and its subsequent proteolytic processing in the induction of TNF unresponsiveness was investigated. Exchange of the TNF presequence for the signal sequence of interleukin-6 resulted in production of secreted TNF, but not in induction of TNF resistance. On the other hand, expression of non-secretable, membrane-bound TNF generated complete TNF unresponsiveness. To explore whether the requirement for anchoring reflects a specific functional role of the TNF presequence, the latter was replaced by the membrane anchor of trimeric chicken hepatic lectin. Expression of this construct induced complete TNF unresponsiveness. Hence, the role of the TNF presequence in the induction of TNF unresponsiveness only involves its function as a membrane anchor, which permits oligomerization of the TNF molecule into a biologically active homotrimer.

Tumor necrosis factor (TNF) is a pleiotropic cytokine that is primarily produced by activated macrophages and some T-lymphocyte subsets. TNF exerts a wide range of biological activities related to inflammation, mitogenesis, differentiation, immune modulation, and antitumor immunity (1–3). These activities are induced through interaction with specific cell surface receptors expressed on almost every cell type, except unstimulated T-lymphocytes and erythrocytes. In man and mouse, two types of TNF receptor have been characterized, namely TNF-R55 and TNF-R75, with molecular masses of 55 and 75 kDa, respectively. TNF effects are mainly mediated by TNF-R55, whereas the role of TNF-R75 as a signal transducer is mostly confined to T-lymphocytes (4).

TNF is synthesized as a 26-kDa, type II transmembrane proform (5), which is biologically active (6). This precursor is processed by a metalloprotease (7–9), which results in release of mature, trimeric TNF consisting of 17-kDa subunits. Membrane-bound TNF mediates TNF effects at the local, paracrine level via cell-cell contact (10), whereas diffusible TNF acts at longer distances, generating systemic responses to this cytokine. Both TNF forms induce killing of TNF-sensitive target cells (6, 11). TNF-producing cells are completely resistant to TNF-induced cytoxicity (12–15). Remarkably, transfection with an exogenous TNF gene under a constitutive promoter converts even very sensitive cell lines, like L929, to TNF production and to complete resistance to TNF-induced cytotoxicity; this resistance correlates with and can be explained by the absence of TNF receptors on these cells (16).

We have now further analyzed this system of complete unresponsiveness. Expression of a TNF gene leads to disappearance from the plasma membrane of TNF-R55 and TNF-R75, despite unaltered levels of corresponding mRNAs. The TNF presequence fulfills a crucial role in this process by its function as a membrane anchor.

MATERIALS AND METHODS

Cell Lines and Cell Culture—The fibrosarcoma cell lines L929aA (16), L929c2 (17), and WEHI 164 cl 13 (18) were cultured as described previously. These cell lines were repeatedly screened for Mycoplasma by a DNA-fluorochrome assay and were found to be negative.

Cytokines and Antisera—Purified E. coli-derived murine (m) TNF was produced in our laboratory and had a specific biological activity of 2 × 106 IU/mg (international standard according to the National Institute for Biological Standards and Control, Potters Bar, UK). Recombinant murine interferon (IFN)-β was also produced in our laboratory and had a specific activity of 3 × 106 units/ml, as determined on murine cells in an L929/vesicular stomatitis virus assay. Polyclonal rabbit antisera against mTNF was provided by Dr. J. Van der Heyden (Roche Research, Ghent, Belgium). Polyclonal rabbit antisera directed against amino acid sequence 99–115 (tip region of mTNF, Fig. 2) was a generous gift of Dr. R. Lucas (University Medical Center, Geneva, Switzerland). Polyclonal rabbit antisera against mTNF-R55 and mTNF-R75 was a kind gift of Dr. W. Buurman (State University of Limburg, Maastricht, The Netherlands).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out by PCR using mutagenic primers (6). The primers were designed on the basis of published data (19). The mutants were cloned into the retroviral expression vector pHR-SIN (20) and were stably transduced into L929 cell lines using the method of transduction (21). The mutants were then selected for their resistance to G418 (Roche). The cells were then cultured and assayed for TNF production as described previously (6).

* This work was supported by the Interuniversitaire Attractiepolen, the Fonds voor Geneeskdijf Wetenschappelijk Onderzoek, and the Vlaams Interuniversitair Instituut voor Biotechnologie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TNF, tumor necrosis factor; ActD, actinomycin D; CHX, cycloheximide; IFN, interferon; IL, interleukin; mTNF, murine tumor necrosis factor; neo', neomycin-resistant; NF, nuclear factor; TNF-R55, 55-kDa TNF receptor; TNF-R75, 75-kDa TNF receptor; wt, wild-type.

(Received for publication, September 24, 1997)
out with the pMa phasmid, which contains a chloramphenicol-sensitive gene (20). Using two oligonucleotides containing the mutations of interest, a plasmid conferring chloramphenicol resistance was obtained. Mutagenesis was performed with a kit from CLONTech. All mutations were verified by DNA sequencing.

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**Plasmid Construction and Transfection**—The interleukin (IL)-6/TNF fusion product containing the signal sequence of murine IL-6 (amino acids 24 to 1) at positions 9–9‘–5. The mutated IL-6 gene was then inserted as a blunt EcoRI-SalI fragment into pSV23s, a eukaryotic expression vector under control of the SV40 early promoter (21), which was cleaved with StuI-SalI. A unique BglII restriction site was introduced by site-directed mutagenesis at amino acid positions 2 and 3 of mature mTNF; this mutated TNF gene was inserted as an SalI fragment into SalI-cleave pSV23s. The pSV23s vector containing the IL-6-TNF chimeric gene was constructed as follows. The BglII/SalI fragment (containing mature mTNF) was ligated to the StuI-SalI fragment of pSV23s in order to insert the synthetic linker coding for amino acids 7 to 1 of the IL-6 signal sequence. The resulting construct was used for transfection of the TNF-sensitive L929 cell line with an exogenous TNF gene. After transfection, the cells were cultured in the presence of a cytokine activator, either IFN-α or IFN-β (22). The pSV2neo plasmid encoding the neo-resistant (neo”) gene under control of the SV40 early promoter was used as a selection marker in L929 transfections (23). The plasmid used for transfection was purified with columns from Qiagen (Chatsworth, CA). The plasmid was transformed into E. coli by electroporation using a Bio-Rad Gene Pulser (Hercules, CA).

**Determination of Biological Activity in Culture Supernatants**—Assessed in the supernatant of L929sA transfectants after 24 h of culture either untreated or treated with 40,000 IU/ml TNF.

**Flow Fluorocytometry**—Membrane-bound TNF was analyzed using polyclonal rabbit antisera directed against the tip region as described previously (17). The presence of TNF-R55 and TNF-R75 was determined by staining for 1 h at 4 °C with polyclonal rabbit antiserum coupled to a biotinylated DNA probe. Analyses were performed by flow fluorocytometry using a Coulter Epics 753 flow cytometer equipped with an argon-ion laser (Coulter, Hialeah, FL).

**Determination of TNF Sensitivity, IL-6 Production, and Activation of the Nuclear Factor (NF)-κB**—TNF sensitivity was determined as described previously (17). The presence of IL-6 in unconcentrated supernatants was determined by its capacity to induce proliferation of 7TD1 cells (24). Activation of NF-κB was measured by an electrophoretic mobility shift assay. Nuclear extracts and binding reactions were carried out as described previously (25). The double-stranded oligonucleotide containing the NF-κB site from the IL-6 promoter was 32P-labeled; after purification, 50,000 cpm was used for the binding assay.

**Northern Blotting**—Poly(A)+ mRNA was isolated using a FastTrack mRNA Isolation Kit (In Vitrogen, San Diego, CA). 5 μg of poly(A)+ mRNA was separated by electrophoresis through a 1.4% agarose-formaldehyde gel and transferred to a Hybond-N+ membrane (Amersham Life Science). The RNA was UV-fixed, and hybridization of the membrane was achieved at 42 °C in the presence of formamide. A 65-base pair BstEII-BamHI fragment of pBLUmtNF55 and a 1300-base pair BamHI-BglII fragment of pCmTNFR75 were used as probes. As control for the quality of RNA loaded, a probe for glyceraldehyde-3-phosphate dehydrogenase was used. Probes were 32P-labeled with a Random Primed labeling kit (Boehringer, Mannheim, Germany).

**RESULTS**

**TNF Secretion Is Not Required for Induction of Unresponsiveness to TNF after Autocrine TNF Production**—Transfection of the TNF-sensitive L929 cell line with an exogenous TNF gene induces TNF production and TNF unresponsiveness (16). To determine whether proteolytic processing of the membrane TNF proform is necessary for the induction of this resistance, we assayed two membrane-bound, non-secretable TNF mutants, which were described previously (11). TNFΔ1–9K11E, in which, in addition to deletion of the first nine amino acids of mature TNF, Lys at position 11 was replaced by Glu, is a biologically active mutein. The TNF mutant TNFΔ1–12, with deletion of the first 12 amino acids of mature TNF, is considerably less biologically active (11). As shown in Table I, L929sA cells transfected with TNFΔ1–12 were partially TNF-resistant, whereas TNFΔ1–9K11E-producing L929sA cells became fully resistant to TNF cytotoxicity. This resistance is similar to that observed in L929sA cells transfected with a wild-type (wt) TNF, which produce both membrane-bound and secreted TNF. L929sA cells transfected only with a neo“ selection marker retained their sensitivity to the cytoktic activity of TNF. Since TNF also mediates a gene-inducing activity in L929 cells, the latter activity was analyzed by assaying IL-6 production and NF-κB activation. TNF stimulation could not induce IL-6 (Table I) or activate NF-κB (Fig. 1) in wtTNF-producing or TNFΔ1–9K11E-producing L929sA cells, whereas addition of

| Transfected gene | Colony | TNF production<sup>a</sup> | TNF sensitivity<sup>b</sup> | IL-6 production<sup>c</sup> |
|------------------|--------|--------------------------|--------------------------|--------------------------|
|                  |        | Supernatant Membrane None | + | None + NF |
| neo”             | B1     | <0.1 ND 2 | <10 6000 | |
| neo” + wtTNF     | E2     | 0.1 ND 2 | <10 7000 | |
| neo” + TNFΔ1–12  | C2     | 25 + >40,000 | <10 100 | |
| neo” + TNFΔ1–9K11E | C3   | 7 + >40,000 | <10 100 | |
| neo” + TNFΔ1–12  | F3     | <0.1 + 4444 | <10 1226 | |
| neo” + TNFΔ1–12  | F6     | <0.1 + 187 | <10 2200 | |
| neo” + TNFΔ1–12  | G1     | <0.1 + >40,000 | <10 100 | |
| neo” + TNFΔ1–12  | G7     | <0.1 + >40,000 | <10 100 | |

<sup>a</sup> Assessed in unconcentrated culture supernatant from the cytoktic activity on the highly sensitive cell line WEHI 164 cl 13. Membrane-bound TNF was detected by flow cytometry; ND, not detectable.

<sup>b</sup> Units required to obtain 50% cell death within 18 h in the presence of ActD; > means that 50% cell death was not observed with the indicated TNF concentration.

<sup>c</sup> Assessed in the supernatant of L929sA transfectants after 24 h of culture either untreated or treated with 40,000 IU/ml TNF.
exogenous TNF could still induce IL-6 production in TNFΔ1–12 transfectants (Table I). Given these results, we conclude that TNF does not need to be processed into a soluble, secreted form to induce unresponsiveness to TNF-mediated effects.

Membrane Retention of TNF Is Necessary for the Generation of TNF Unresponsiveness after Autocrine TNF Production—To express TNF exclusively as a secreted protein, we exchanged the TNF presse in the classical signal sequence of IL-6 (Fig. 2). Cotransfection of this chimeric IL-6-TNF gene with the neo' gene in L929sA cells and G418 selection yielded only marginal numbers of G418-resistant colonies, none of which produced TNF (data not shown). This failure to isolate IL-6.TNF transfectants suggested that the transfectants were counter-selected, which means that expression of the IL-6-TNF gene resulted in cell death. To verify this hypothesis, the IL-6.TNF gene was transfected in L929sA, a TNF-resistant and non-TNF-producing cell line derived from TNF-sensitive L929-r2 cells (17). After transfection with the IL-6.TNF gene and G418 selection, a normal number of G418-resistant L929sA colonies were obtained. These transfectants released between 1 and 40 IU/ml TNF. Unlike wtTNF-transfected L929sA cells, no membrane-bound TNF form could be detected in the IL-6.TNF transfectants by flow fluorocytometric analysis (Fig. 3). These results demonstrate that expression of the IL-6.TNF chimeric gene gives rise to biologically active TNF, which is produced exclusively as a secreted protein. Furthermore, IL-6.TNF-producing transfectants were isolated only after transfection of the TNF-resistant L929sA variant, and not with the TNF-sensitive L929sA cell line, the TNF-mediated counter-selection was presumably the direct cause of the negligible transfection efficiency obtained with L929sA cells.

Although L929sA cells are resistant to the cytotoxic activity of TNF, the cells become sensitive in the presence of ActD or cycloheximide (CHX), inhibitors of RNA or protein synthesis, respectively (17). This feature allowed us to study whether the secreted IL-6-TNF chimera and wtTNF expressed in L929sA differ in the induction of resistance to the cytotoxic combination of TNF and ActD, CHX, or staurosporine. The latter is a non-specific protein kinase C inhibitor, which also sensitizes L929sA cells to TNF effects (26). As shown in Table II, IL-6.TNF transfectants exhibited extensive cell death after treatment with ActD, CHX, or staurosporine alone. Apparently, these cells were killed as a result of the combined activity of autocrine-produced TNF and sensitizing agents. In contrast to this, wtTNF transfectants, producing both membrane-bound and secreted TNF, exhibited full resistance to the sensitizing drugs alone or supplemented with exogenous TNF (Table II). A similar differential responsiveness was observed by following the induction of IL-6. In agreement with the observation that L929sA cells still respond to TNF by producing IL-6, introduction of the constitutively expressed IL-6.TNF chimeric gene induced high levels of IL-6 production in the transfectants (an autocrine stimulation), whereas introduction of the wtTNF gene eliminated the inducible IL-6 response observed in the control transfectants (Table II). Clearly, omission of the intermediary step of the membrane-bound form in the course of TNF biosynthesis abolished the capacity of endogenously produced TNF to generate unresponsiveness.

Role of the TNF Presequence in Induction of Unresponsiveness—The requirement for membrane retention in the induction of unresponsiveness to TNF implies an involvement of the TNF presequence either structurally, as a membrane anchor, or functionally, involving (signal-transducing) amino acid sequence information. To answer this question, we exchanged the TNF presequence for the membrane anchor of CHL (27). CHL is a trimeric, type II transmembrane liver glycoprotein receptor (28, 29), which contains an anchor allowing trimerization of the extracellular domains. Transfection of L929sA cells with the CHL.TNF chimeric construct (Fig. 2) yielded a normal number of colonies, all of which secreted high amounts of biologically active TNF, varying within the range of 30–300 IU/ml (Table III). These L929sA transfectants were completely unresponsive to the cytotoxic and gene-inducing activity of TNF, which means that they behaved in the same way as L929sA cells transfected with the wtTNF gene (Table III).

Immunoprecipitation and immunoblotting of cell lysates revealed a 27.8-kDa protein band corresponding to the expected CHL.TNF proform and several higher M, forms (Fig. 4A), which presumably correspond to heterogeneity in glycosylation. The presence of membrane-bound fusion protein was also confirmed by flow fluorocytometric analysis, where the levels of immunofluorescence remained unchanged after acidic treat-
Induction of Unresponsiveness to TNF

TABLE II
Responsiveness to TNF of L929r2 cells transfected with wtTNF or chimeric IL-6.TNF

| Transfected gene | Colony | Survivala | TNF sensitivityb | IL-6 productionc |
|------------------|--------|-----------|------------------|-----------------|
|                  |        | ActD      | CHX              | STS             | ActD      | CHX              | STS             | None + TNF |
| neo'             | B1     | 75        | 75               | 71              | 6          | 2                 | 1               | 100        |
|                  |        |           |                  |                 |            | 2                 |                 | 2000       |
|                  | B2     | 80        | 80               | 67.5            | 2          | 2                 | 1               | 95         |
|                  |        |           |                  |                 |            | 2                 |                 | 120        |
| neo' + wtTNF     | C3     | 80        | 70               | 61              | >40,000    | 20,000            | >40,000         | 150        |
|                  |        |           |                  |                 |            |                   |                 | 200        |
| neo' + IL-6.TNF  | C9     | 70        | 80               | 75              | >40,000    | 40,000            | >40,000         | 5300       |
|                  |        |           |                  |                 |            |                   |                 | 7000       |
|                  | E2     | 10        | 4.5              | 4.5             | NR         | NR                | NR              | 7000       |
|                  |        |           |                  |                 |            |                   |                 | 8000       |
|                  | E3     | 15        | 3.5              | 5.5             | NR         | NR                | NR              |            |

a Cells were treated with ActD (1 μg/ml), CHX (25 μg/ml), or staurosporine (STS, 1.5 μM) in the absence of exogenous TNF. Cell survival in control cultures was 100% for all colonies.
b Units required to obtain 50% cell death in the presence of the indicated inhibitor; > means that 50% cell death was not observed with the TNF concentration mentioned; NR, not relevant.
c Assessed in the supernatant of L929sA transfectants after 24 h of culture either untreated or treated with 40,000 IU/ml TNF.

TABLE III
Characterization of L929sA cells transfected with wtTNF or CHL.TNF

| Transfected gene | Colony | TNF productiona | TNF sensitivityb | IL-6 productionc |
|------------------|--------|-----------------|------------------|-----------------|
|                  |        | IU/ml           | IU/ml            | pg/ml           |
| neo'             | B1     | <0.1            | 2                | <10             |
|                  |        |                 |                  | 6000            |
|                  | B2     | <0.1            | 2                | <10             |
|                  |        |                 |                  | 7000            |
| neo' + wtTNF     | C2     | 25              | >40,000          | <10             |
|                  |        |                 |                  | 10              |
|                  | C3     | 7               | >40,000          | <10             |
|                  |        |                 |                  | 10              |
| neo' + CHL.TNF   | D2     | 250             | >40,000          | <10             |
|                  |        |                 |                  | 10              |
|                  | D3     | 100             | >40,000          | <10             |
|                  |        |                 |                  | 10              |

a Assessed in unconcentrated culture supernatant.
b Units required to obtain 50% cell death within 18 h in the presence of ActD; > means that 50% cell death was not observed with the indicated TNF concentration.
c Assessed in the supernatant of L929sA transfectants after 24 h of culture either untreated or treated with 40,000 IU/ml TNF.

Unresponsiveness through Membrane-bound TNF-mediated Down-modulation of TNF-R55 and TNF-R75—TNF exerts its cytotoxic and gene-inducing activity through binding and subsequent clustering of its receptors TNF-R55 and/or TNF-R75. We previously described the down-modulation of TNF receptor molecules on the cell surface after autocrine production of TNF (16). Therefore, the expression level of TNF receptors on the plasma membrane of L929sA transfectants expressing wtTNF, membrane-bound TNFα1–9K11E, TNFα1–12, or CHL.TNF fusion gene was determined. As shown in Fig. 6, using 125I-TNF, no specific TNF binding could be demonstrated on L929sA cells expressing wtTNF, TNFα1–9K11E, or CHL.TNF, even not after treatment of the cells with acidic glycine-HCl buffer. Similar lack of binding was observed after treatment with polyclonal antiserum directed against mTNF to remove possibly receptor-bound endogenous TNF (data not shown). L929sA transfectants expressing membrane-bound TNFα1–12 showed considerably reduced TNF binding compared with control neo' transfectants (Fig. 6). This is in agreement with the lower efficacy in rendering the transfected cells TNF resistant (Table I). Pretreatment with acidic glycine-HCl buffer did also not enhance the level of TNF binding by the TNFα1–12 transfectants, indicating that the down-modulation of the cell-surface TNF receptors was not due to interference by receptor-bound TNF. It may be noted that soluble TNFα1–12 is almost biologically inactive (30). To investigate the time course of the down-regulation of the TNF receptors, we examined how soon after mTNF production in L929sA cells the down-modulation became evident. Therefore, we determined the cytotoxic effect of exogenous TNF and the expression of both TNF receptor types on L929sA cells transfected with pMx-mTNF gene, in which mTNF expression is under control of the IFN type I (IFN-α or IFN-β)-inducible Mx promoter (22). Under uninduced conditions, the L929sA pMx-mTNF transfectants were TNF-sensitive (Fig. 7), whereas partial TNF resistance occurred after 3-h IFN-β administration and complete TNF resistance was evident after 6 h IFN-β pretreatment (Fig. 7). This induction of TNF resistance was correlated with the down-modulation of cell-surface TNF-R55 and TNF-R75, as revealed by immunofluorescence on the L929sA pMx-mTNF transfectants (Fig. 8). We further investigated whether the absence of cell-surface TNF receptors was due to down-regulation of TNF receptor gene transcription. Northern blot analysis of mRNA levels did not reveal silencing of the respective genes in the unresponsive transfectants (Fig. 9). This result indicates that the lack of TNF receptors on the cell surface must be due to a post-transcrip-
DISCUSSION

TNF is synthesized as a 26-kDa, type II transmembrane proform, consisting of the TNF presequence and the mature 17-kDa TNF subunit, which is released from the membrane by proteolytic cleavage. Considering the fact that the membrane-bound proform is biologically active, trimerization must have already occurred at this stage (6, 11, 31). Following TNF production, for example by transfection with an exogenous TNF gene, TNF-sensitive cells, such as the L929sA murine fibrosarcoma, become resistant to TNF-mediated cytotoxicity (16). We previously reported that mere expression of the TNF presequence was not sufficient for induction of unresponsiveness, and that a biologically active TNF molecule is required, suggesting that an orthodox ligand/receptor interaction is involved. The resistance could be explained by the complete absence of TNF receptors on the cell surface (16).

We have now elucidated in more detail the mechanism responsible for this induction of unresponsiveness. We first examined whether the synthesis of a membrane-bound proform, a feature characteristic of TNF biosynthesis, is required for this process. Elimination of membrane retention by exchanging the TNF presequence for the signal sequence of IL-6 resulted in complete loss of autocrine-induced desensitization, as indicated by a complete absence of TNF binding to the cell surface (16).

Fig. 7. Determination of TNF sensitivity of L929sA cells transfected with pMx-mTNF. The cells were either not pretreated (▲) or pretreated for 3 h (■), 6 h (●), or 12 h (◆) with 1000 units/ml IFN-β.

Fluorescence intensity (log)
by the occurrence of counter-selection following transfection of the chimeric IL-6.TNF gene into L929sA cells. However, the same construct could be transfected into TNF-resistant L929r2 cells, which yielded a normal number of transfecants producing secreted TNF. Using conditions in which L929r2 cells become TNF-sensitive, we confirmed that expression of IL-6.TNF could not induce TNF unresponsiveness. We conclude that membrane retention of TNF is crucial for desensitization to TNF-mediated effects such as cytotoxicity or gene induction. These results are supported by our finding that the biologically active, but uncleavable TNFΔ1–9K11E mutant (11) induced complete unresponsiveness. It should be mentioned that expression of the uncleavable, membrane-bound TNF mutant TNFΔ1–12 could only induce partial TNF unresponsiveness. In fact, it is known that recombinant soluble TNFΔ1–12 is virtually inactive (30). Considering that endogenous synthesis of biologically active TNF is required for the induction of TNF resistance (16), our observations suggest that TNFΔ1–12 is a membrane-bound TNF mutant with a considerably reduced biological activity. This is in agreement with our previous report, in which we demonstrated that membrane TNFΔ1–12 is less potent than TNFΔ1–9K11E for the induction of granulocyte-macrophage colony-stimulating factor production by PC60-R55/R75 and is, in contrast to TNFΔ1–9K11E, not able to induce TNF-R55-mediated apoptosis in U937 cells (11). Taken together, our data indicate that membrane-bound mTNFΔ1–12 mutant is not a good candidate to examine the physiological relevance of membrane-bound TNF \textit{in vivo}. For example, results obtained in transgenic mice using the membrane-bound mTNFΔ1–12 mutant to examine the role of membrane-bound TNF \textit{in vivo} should be interpreted with care. Our observations with the membrane-bound mutant TNFΔ1–9K11E clearly show that the presence of a biologically active TNF proform is not only crucial, but also sufficient for rendering TNF-sensitive cells unresponsive to TNF-mediated signaling. In view of these results, an involvement in this phenomenon of the TNF presequence must be considered. This effect could be restricted to a structural one, providing a membrane anchor appropriate for trimerization, or, in addition, may encompass specific sequence information leading to desensitization.

To answer this question, we exchanged the TNF presequence for the membrane anchor sequence of trimeric CHL. Normal expression levels, as shown by detection of both membrane-bound and secreted TNF, were obtained with this CHL.TNF fusion gene. This means that the CHL membrane anchor clearly supports trimerization. The transfecants obtained with the CHL.TNF gene exhibited complete unresponsiveness to TNF, as measured by lack of cytotoxicity, NF-κB activation, and IL-6 production. Hence, induction of unresponsiveness after autocrine production of TNF solely requires the retention of a biologically active molecule through a membrane anchor that is permissive for trimerization. Since there is no detectable homology between the TNF and CHL presequences, involvement of additional interacting components in the phenomenon of unresponsiveness is highly unlikely.

It has been proposed that myristoylation of the conserved Lys residue at position −57 of the TNF presequence might be important for membrane insertion of TNF (32). This assumption is not supported by our results, as we demonstrated that expression of a membrane-bound TNF form occurred after transfection with the CHL.TNF chimeric gene, and also with the deletion mutant TNFΔ62–55.2

The mechanism underlying autocrine, TNF-induced unresponsiveness involves down-modulation of cell surface-expressed TNF-R55 and TNF-R75. Neither treatment of the cells at pH 3.0 (to remove receptor-bound TNF) nor addition of TNF-neutralizing antibodies restored the expression of cell surface TNF receptors. This indicates that receptor down-modulation presumably occurs prior to the appearance of the TNF receptor on the plasma membrane. Since mRNA levels for both receptor types were unaltered in TNF-producing L929 transfecants, inhibition at the transcriptional level can be excluded. The observation that, soon after induction of autocrine TNF production, down-modulation of TNF receptors occurred and that the cells became completely TNF-resistant supports a mechanism whereby intracellular interaction between newly synthesized TNF and TNF receptor occurs, followed by degradation of the complexes. The rapid intracellular disappearance of TNF receptors explains the induction of TNF-non-responsiveness. We failed to demonstrate directly TNF-TNF receptor complex formation either by co-immunoprecipitation or by immunofluorescence co-localization studies (data not shown). This may be explained by the rapid degradation of TNF-TNF receptor complexes or to a very low expression level of TNF receptor. Examples of intracellular interaction between receptor and ligand are well known. On the one hand, retention of IL-3 or v-sis oncogene in the endoplasmic reticulum results in signaling within the cell (33, 34). On the other hand, it has been reported that retention of IL-6 in the endoplasmic reticulum leads to prevention of surface expression of the IL-6 receptor protein gp80, making the cells unresponsive to IL-6 (35). Considering that mRNA for gp80 is still present in these cells, it was proposed that IL-6 retained in the endoplasmic reticulum specifically interacts with de novo synthesized gp80, resulting in retention of the complexes. However, direct demonstration of retained gp80 also failed, possibly because of extremely low numbers of gp80 expressed per cell, or to rapid degradation of the IL-6-gp80 complexes formed.

Our proposed mechanism raises an important dilemma: if receptor clustering occurs already intracellularly, why does this not result in constitutive signaling such as suicide of sensitive cells or IL-6 production by L929r2 cells? Possibly, at the early stage of their synthesis, the intracellular domains of the receptors are not yet fully decorated with accessory proteins required for their function. It is attractive to assume that a cell, such as a macrophage or a T-lymphocyte, which produces a pleiotropic factor like TNF, has a mechanism to avoid being a target of its own product. Indeed, macrophages, which are stimulated to produce TNF, internalize their TNF receptor prior to TNF production (36). Additionally, activated lymphocytes, which produce membrane-bound lymphtoxin, no longer express lymphotoxin-β receptor at the cell surface (37). Furthermore, a phenomenon as described here has been observed also in the course of tumorigenesis. Melanoma tumor cells, which express membrane-bound Fas ligand, can induce apoptosis in tumor-infiltrating, immune effector cells bearing Fas.

\footnote{E. Decoster, unpublished data.}
but cannot receive a death signal themselves because they do no longer express Fas on their cell surfaces (38). In conclusion, synthesis of a membrane-bound cytokine and the resulting down-modulation of the corresponding receptors represent a more general mechanism, which allows a paracrine activity of the cytokine, while preventing it from acting in an autocrine manner.

Acknowledgments—We thank A. Raeymaekers for providing TNF preparations, as well as J. Van der Heyden, Dr. W. Buurman, and Dr. R. Lucas for donating antisera. We acknowledge Dr. K. Drickamer and preparations, as well as J. Van der Heyden, Dr. W. Buurman, and Dr. R. Lucas for donating antisera. We acknowledge Dr. K. Drickamer and

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