Two Discrete Types of Tumor Necrosis Factor-resistant Cells Derived from the Same Cell Line

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ABSTRACT

From the murine fibrosarcoma cell line L929s, which is sensitive to tumor necrosis factor (TNF)-mediated cell lysis, two discrete types of TNF-resistant variants were derived by TNF selection. Cells of the first type (named L929r1) were not sensitized to TNF cytotoxicity by cotreatment with either inhibitors of protein or RNA synthesis, or γ-interferon, despite the presence of a functional γ-interferon response. L929r1 constitutively produced TNF in the supernatant and expressed membrane-bound TNF, which was not bound to the TNF receptor. In fact, TNF receptors could not be demonstrated on L929r1 cells, not even after low pH treatment and/or incubation with antiserum to TNF. L929r1 exhibited a stable TNF-resistant phenotype in the absence of further TNF selection. No evidence could be obtained that TNF acted as an autocrine growth factor for these cells. L929r2, the second type of TNF-resistant L929 cells, became sensitive to TNF lysis in the presence of RNA or protein synthesis inhibitors, or in the presence of γ-interferon. TNF induced the secretion of interleukin 6 in these cells, additionally showing that functional TNF signaling in these cells indeed takes place, but does not lead to cell lysis under normal conditions. L929r2 did not produce TNF, also not upon stimulation with exogenous TNF. The number and binding affinity of TNF receptors were not consistently different between L929s and L929r2 cells. In the absence of further TNF selection, L929r2 gradually reverted to TNF sensitivity. This sensitivity was not reversible to TNF resistance by the gene-regulatory agents 5-azacytidine or sodium butyrate. Treatment with these agents also did not affect the TNF degradation of TNF to an extent similar to that of TNF-sensitive cells. 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. 'Research was supported by the Belgian ASLK, FGWO, and IUAP. 4 To whom requests for reprints should be addressed, at the Laboratory of Molecular Biology, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium. 5 Supported by the IWONL. 6 Supported by the BWTK. 7 The abbreviations used are: TNF, tumor necrosis factor; A2387, actinomycin D; azaC, 5-azacytidine; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; IFN, interferon; IL, interleukin; LT, lymphotoxin; r, resistant to TNF cytotoxicity; rh, recombinant human; rm, recombinant murine; s, sensitive to TNF cytotoxicity; VSV, vesicular stomatitis virus.
10^4 and 1 x 10^5 IU/mg of protein, respectively. rmIFN-γ had a specific activity of 10^5 IU/mg protein. Purified rhLT, expressed in Escherichia coli (28), was provided by Dr. A. Porter, National University of Singapore, Singapore. The LT used had a specific activity of 2.2 x 10^4 IU/mg. Rabbit polyclonal antiserum against murine or human TNF were provided by J. Van der Heyden (Roche Research Gent, Gent, Belgium).

Determination of Cellular Sensitivity to Viral Infection and Assay for Antiviral Activity of TNF and IFN-γ. Serial dilutions of rmIFN-γ or rmTNF (at highest final concentrations of 1000 IU/ml and 6 x 10^4 IU/ml, respectively) were transferred to 96-well microtiter plates containing cells seeded at 2 x 10^4/well 24 h earlier. After 24 h of incubation, culture medium was replaced and aliquots of VSV or encephalomyocarditis virus were added to each well. Twenty-four or 48 h later, viable cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma; Poole, United Kingdom), and colorimetrically assayed (29).

Isolation of TNF-resistant Variants by TNF Selection and Culture Conditions for TNF-resistant Cells. rmTNF at 4000 IU/ml was used throughout the whole selection procedure. L929 cells were seeded at 5000 cells/well in 96-well plates. Twenty-four h later, TNF was added. Every 5 days culture medium was replaced by fresh medium containing fresh TNF. On day 19 of culture, the culture wells were microscopically investigated and those containing a single colony of surviving cells were switched to TNF-free conditions. Next, two different selection protocols were followed. The first consisted of a readdition of TNF on day 30 of culture for another 30-day period, with weekly refreshings. Cells derived in this way were found to be stably TNF resistant. They were subcloned by limiting dilution. Cells derived in this selection procedure, further designated as L929r2, were found to have an unstable TNF-resistant phenotype and were therefore further cultured every second week in the presence of 1500 IU rmTNF/ml. Before use in assays, L929r2 cells were incubated for 3 to 7 days in TNF-free medium.

Determination of Cellular Sensitivity to TNF or LT. Cells were tested for TNF sensitivity as described (19, 30). In short, cells were seeded in 96-well plates in 0.1 ml medium at 2 x 10^4 or 2500 cells/well (for 20- and 72-h assays, respectively). Twelve to 18 h later, serial dilutions of TNF or LT alone or in combination with either ActD (Sigma; final concentration, 1 μg/ml), CHX (Sigma; final concentration, 50 μg/ml), or rmIFN-γ (final concentration, 100 IU/ml) were added in 0.1 ml medium. After a 20 h- (for ActD and CHX assays) or 72 h-incubation period, surviving cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and colorimetrically assayed (29). Percentage of survival is expressed versus 100% = cells incubated without TNF in the presence of either ActD, CHX, or IFN-γ alone. Percentage of survival of cells incubated in the presence of these agents alone was never lower than 75% (versus 100% = cells incubated in culture medium alone). In some experiments, cells were treated for 72 h with 5, 10, or 20 μM azaC (Sigma) prior to seeding for TNF assay. These concentrations of azaC were found to be growth inhibitory for the cells. At higher doses, the percentage of survival was reduced to <25%. To test the effect of sodium butyrate (Sigma), cells were treated with 1 to 20 mM concentrations of this agent, either for a 24-h period prior to seeding for TNF assay, or additionally incubated in the presence of sodium butyrate during the whole TNF assay period. Proliferation of cells was assayed by measurement of incorporation of [6-'H]thymidine (Amersham International, Amersham, United Kingdom; 23 Ci/mmol), added to the cells at 3 μCi/ml for an 8- or 24-h period.

Determination of TNF Production. The amount of cytokin in conditioned medium of cells was determined on WEHI164C13 cells in a 24-h assay in the presence of 1 μg ActD/ml (25). The limit of detection of this assay was 0.1-1 pg rmTNF/ml. Neutralization was performed with polyclonal rabbit anti-rmTNF serum. For immunoblotting, conditioned medium of cells grown in DMEM supplemented with 5 μg insulin (from bovine pancreas; Sigma)/ml, 5 μg human transferrin (Sigma)/ml, and 5 μg sodium selenite (Sigma)/ml, was concentrated by ultrafiltration using Centricron and Centriprep microseparation devices (Amicon, Danvers, MA). For preparation of cell lysates, monolayers of cells were washed with ice-cold phosphate-buffered saline, followed by addition of ice-cold lysis buffer [120 mM NaCl, 50 mM Tris-HCl, 0.5% (w/v) Nonidet P-40, 0.05% (w/v) Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, 500 KIU aprotinin/ml; pH 7.6]. After 10 min of incubation on ice, lysates were collected and cleared. Samples of 50 μg protein were electrophoresed in sodium dodecyl sulfate-containing 12.5% polyacrylamide gels under reducing conditions. Electrophoresed proteins were electroblotted to nitrocellulose membranes in a Multiphor II transfer apparatus (Pharmacia LKB Biotechnology, Uppsa1a, Sweden) according to the manufacturer’s instructions. Blocking was overnight at room temperature in 50 mM Tris-HCl, 2 mM CaCl2, 80 mM NaCl, 0.2% (w/v) Nonidet P-40, 0.02% (w/v) Na3VO4, and 2.5% (w/v) instant dry milk; pH 8.0. Detection was by successive incubations with polyclonal rabbit anti-rmTNF serum and goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma), followed by quantitative enzymatic generation of formazan as described (31).

Determination of IL-6 Production. The presence of IL-6 in unconditioned culture supernatant was determined by its capacity to induce proliferation of the 7TD1 cell line (32). Neutralization by a specific antiserum (ascites fluid of the D690 6B4 hybridoma cell line; kindly provided by Dr. J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium) was used to confirm IL-6 identity.

TNF-binding Assay. TNF was radioiodinated by using Iodo-Gen (Pierce Chemical Co., Rockford, IL) to a final specific activity of 6 x 10^4 cpm/μg. Binding studies were performed on L929 cells grown in suspension, as binding assays on confluent monolayers always resulted in a high level of aspecific binding (up to 80%). Cells were trypsinized and seeded 24 h before the binding assay at 2 x 10^6 cells/ml in 90-mm bacterial Petri dishes (Steri1in, Hounslow, United Kingdom). Under these conditions, L929 cells continue to proliferate for 4-5 days and retain their TNF-resistant/sensitive phenotype (data not shown). Cells were harvested and incubated for 4 h at 4°C with varying concentrations of 125I-TNF in 200 μl binding medium (DMEM supplemented with 10% FCS and 0.01% Na3VO4). After binding, the cells were washed twice with ice-cold binding medium, pelleted through a mixture of silicon oil and paraffin (84/16; v/v), and their radioactivity was determined in a gamma counter. Specific binding of 125I-TNF was determined by subtracting the amount of radioactivity bound to cells in the presence of a 500-fold excess of unlabeled TNF from the radioactivity bound in the presence of 125I-TNF alone. Nonspecific binding of 125I-rmTNF was about 10% of total binding. The number of TNF receptors per cell and the dissociation constant (Kd) were determined by Scatchard plot analysis (33). To remove putative receptor-bound, endogenously produced TNF, cells were pretreated for 12 h at 37°C with polyclonal rabbit anti-rmTNF serum at 2000 neutralization units/ml and/or incubated for 5 min at 4°C with glycine-HCl buffer (50 mM, containing 150 mM NaCl; pH 3.0).

Immunofluorescence. L929 cells were cultured as described for TNF-binding assays. All further treatments were performed in phosphate-buffered saline supplemented with 0.5% (w/v) bovine serum albumin (Sigma) and 0.1% (w/v) Na3VO4. Cells were first incubated for 1 h at 4°C with 2.5 μg/ml primary antibody. Primary antibodies were either the murine anti-mouse H-2k monoclonal IgG antibody H142-23 (in the form of ascites, used at a 1/1000 dilution; Serotec, Oxford, United Kingdom) or the neutralizing rat anti-rmTNF monoclonal IgM antibody 1F3F3D4 (34). As an irrelevant primary antibody, we used the rat IgM antibody, produced by the RA3-3A1/6.1 hybridoma (American Type Culture Collection), which recognizes the mouse B-cell marker B220. Rat primary antibodies were used at 10 μg/ml. Binding of the anti-H-2k antibody was revealed by biotinylated sheep anti-mouse immunoglobulin (affinity purified; Amersham), using Texas Red-conjugated streptavidin (Amersham) as third reagent. For the rat IgM antibodies, fluorescein-conjugated goat anti-rat IgG (cross-reactive with rat IgM; Tago, Burlingame, CA) was used as the secondary reagent. Before use, the primary and secondary antibody solutions were 2470.
DISCRETE TYPES OF TNF-RESISTANT L929 CELL LINES

**RESULTS**

Isolation of Two Types of TNF-resistant L929 Variants by TNF Selection. Upon incubation of L929s cells in the presence of 4,000 IU rmTNF/ml, the frequency of surviving cells was found to be about 1 in 5,000 to 10,000 cells. Two different TNF selection procedures (details described in “Materials and Methods”) yielded two discrete types of TNF-resistant variants. From cells surviving the first selection procedure (a total of 48 days of TNF selection, with an intermittence of an 11-day culture period in TNF-free medium from day 19 on), four independently derived clones were analyzed. All were found to have the same phenotype of TNF resistance and are further referred to as L929r1-type cells (L929r1.1 to L929r1.4). After a different TNF selection procedure (a total of 88 days of TNF selection, with an intermittence of a 50-day culture period in the absence of TNF, starting on day 19), cells from one culture well only (out of 10) survived. These cells were found to have a TNF-resistant phenotype distinct from that of L929r1-type cells, and are further designated as L929r2-type cells. All L929 cell lines were found to express similar levels of murine H-2k major histocompatibility complex antigens as parental L929s cells (assessed by immunofluorescence analysis; not shown). Also the total protein profiles, as revealed by 2-dimensional gel electrophoresis, were analogous for L929s and derivatives, additionally indicating that the TNF-resistant cells are authentic L929 derivatives.

Sensitivity to TNF, or to TNF Combined with ActD, CHX, or IFN-γ. A representative result of determination of TNF sensitivity of L929s and derivatives is shown in Fig. 1 and Table 1. When applying TNF alone, both L929r1 and L929r2-type cells were found to be resistant to TNF cytotoxicity over the broad concentration range of TNF tested. They were also resistant to LT (highest dose tested, 4 × 10^5 IU/ml; results not shown). L929r2, but not L929r1-type cells, could be rendered sensitive to TNF-mediated cell lysis by cotreatment with either ActD, CHX, or IFN-γ (data for CHX are not shown). Under these conditions, L929r2 cells were still 8 to 100 times less TNF sensitive than parental L929s cells, depending on the type of assay used.

Stability of TNF Resistance. In the absence of further TNF selection, L929r1-type cells were found to be TNF resistant in a stable way over a period of at least 1 year (with weekly passaging). This was not the case for L929r2 cells which, over a culture period of 4 to 8 weeks in the absence of TNF selection, gradually reverted to a TNF-sensitive phenotype similar to that of L929s cells. This rapid loss of TNF resistance suggests that this feature was not due to a cellular mutation, but was rather related, for example, to an unstable epigenetic change. L929r2 cells, either in the TNF-resistant state or after having become TNF-sensitive, were treated with azaC or sodium butyrate, agents known to affect the expression of many cellular genes most probably without involvement of mutagenesis (35, 36). Treatment with the DNA-hypomethylating agent azaC has been reported to induce TNF sensitivity in a TNF-selected TNF-resistant cell line (37). However, TNF sensitivity of L929r2 was not influenced by azaC or butyrate, which also did not alter the TNF-sensitive/resistant phenotype of L929s and L929r1-type cells (results not shown).

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* R. Beyaert, unpublished results.
L929r1-type Cells Produce TNF. Conditioned media from L929r1-type cells were found to possess cytotoxic activity toward L929s and WEHI164c113 cells which could be neutralized completely by antiserum to murine TNF (not shown). L929r1-type clones varied in the amount of TNF produced, with maximum production levels detected in the supernatant varying between 100 IU (for L929r1.3) and 15,000 IU (for L929r1.1) (produced in 10 ml culture medium over a period of 4 days by a culture of 2.5 × 10⁴ cells inoculated in a 10-cm² culture flask).

Immunoblotting revealed M, 17,000 TNF in the supernatant, and a M, 26,000 TNF form in lysates of L929r1-type cells (Fig. 2). Activated macrophages also express these molecular forms of TNF (Fig. 3; Ref. 38), suggesting no aberrant TNF processing by the L929 fibrosarcoma cells.

Membrane-associated TNF could be visualized on L929r1-type cells by flow cytometry (Fig. 4). Treatment with glycine-HCl buffer, found to remove ¹²⁵I-TNF bound on L929s cells, did not or only marginally altered the immunofluorescence signal observed with anti-murine TNF antibodies on L929r1-type cells (Fig. 4E). Obviously, the majority of the TNF detected on these cells does not behave as receptor-bound M, 17,000 TNF.

In the experiments shown in Fig. 1 and Table 1, L929r1-type cells did not show enhanced sensitivity (as compared to L929s cells) to treatment with ActD, CHX, or IFN-γ alone, which could be expected if L929r1-type cells were sensitive to the combined action of these agents with the endogenously produced TNF (results not shown).

L929r2 Cells Do Not Produce TNF. Conditioned media from L929r2 cells did not contain cytotoxic activity toward L929s and WEHI164c113 cells. Also immunoblotting and flow cytometry analysis with anti-murine TNF did not reveal TNF (Figs. 2 and 4). To investigate whether L929r2 transiently produced TNF upon treatment with exogenous TNF, these cells were incubated with 20,000 IU human TNF/ml for different time periods, and analyzed for production of murine TNF by immunoblotting detection with an anti-murine TNF serum which was not cross-reactive with human TNF. No detectable endogenous TNF production was observed under these conditions either (Fig. 3). Also no cytotoxic activity was found in the supernatant of L929r2 cells, pretreated for 48 or 72 h with 1000 IU human TNF/ml, upon testing on WEHI164c113 cells in the presence of an antiserum to human TNF which did not neutralize murine TNF (data not shown; detection limit, 0.1 pg murine TNF/ml).

TNF Receptor Number and Affinity. Using ¹²⁵I-rmTNF, specific TNF binding could not be demonstrated on L929r1-type cells, also not after treatment with polyclonal antiserum to murine TNF and/or low pH medium to remove possibly receptor-bound endogenous TNF (data not shown). For L929s and L929r2 cells, differences in the number and affinity of TNF receptors were sometimes observed. However, these differences were not reproducible over several experiments and could mostly not be related to the TNF resistance observed. For example, depending on the experiment, the number of TNF receptors on L929r2 cells was found to be either higher (Fig. 5A), lower (Fig. 5B), or similar to (not shown) the number of TNF receptors on L929s cells. Therefore, we believe that it is unlikely that such differences can account for the dramatic difference in TNF sensitivity between these cell lines. This is also in agreement with the finding by others regarding the absence of correlation between TNF receptor expression and TNF resistance (4, 39). Thus, TNF resistance of L929r2 as such seems not to be related unequivocally to altered binding capacity of TNF.

TNF Induces IL-6 Secretion in L929s and L929r2 Cells but Not in L929r1-type Cells. TNF is known to induce production of IL-6 in fibroblasts (40). L929 cells constitutively secreted low amounts of IL-6 (Table 2). Upon treatment with TNF, this basal level was found to be enhanced by at least a factor of 10 in L929s and L929r2 cells, but not in L929r1-type cells. These data additionally indicate that TNF signaling occurs in L929r2, but not in L929r1-type cells.

Involvement of TNF in Growth of L929r Cells. Production of cytokines by tumor cells frequently involves an autocrine growth-stimulatory signal (41). A growth-promoting activity of TNF on some primary cell cultures and on tumor cells has been reported (42-45). A dose titration of rmTNF (40,000 to 0.05 IU/ml) was added to low-density cultures (200 cells in 200 µl culture medium/microwell) or to starved, high-density cell cultures of L929r1.1 or L929r2 cells, and proliferation was assayed 1, 2, 4, or 6 days later. In no case was TNF-induced growth stimulation observed (data not shown). Proliferation of L929r1.1 could also not be inhibited by addition of polyclonal antiserum to murine TNF to low-cell-density cultures (data not shown). Taken together, these data suggest no role for TNF as an autocrine growth factor for L929r1-type cells.

Colony Morphology. Recently, a relationship between cell colony morphology and TNF resistance has been reported (12, 46): colonies from TNF-sensitive cells (L929, human histiocytoma U937 and established rabbit kidney cells RK13) were found to be widely spread, whereas they were closely packed in the corresponding TNF-resistant cells derived by TNF selection. Although we found a dense colony morphology for the L929r1.1 clone (Fig. 6), neither other r1-type cells nor the r2-type TNF-resistant L929 cells grew as dense colonies, but instead formed colonies with a loose appearance similar to parental L929s cells (Fig. 6). Thus, there is no clear relationship
Fig. 3. Immunoblot analysis with anti-rmTNF of: lysate of L929r1.1 cells, either untreated (Lane 1) or pretreated with 200 ng/ml rhTNF for 7 h (Lane 2); lysate of L929r2 cells, either untreated (Lane 3) or pretreated with 200 ng/ml rhTNF for 2 h (Lane 4), 4 h (Lane 5), 7 h (Lane 6), or 24 h (Lane 7); lysate of PUS-1.8 cells, either untreated (Lane 8) or treated for 12 h with 50 ng lipopolysaccharide/ml (E. coli 0111:B4; Difco Laboratories, Detroit, MI) and 20 ng/ml phorbol-12-myristate 13-acetate (Sigma) (Lane 9). Arrows indicate M, 26,000 and M, 17,000 forms of TNF. Ordinate, molecular weight in thousands.

Fig. 4. Flow cytometry analysis of mTNF expression on L929s (A); L929r1.1 (B); L929r2 (C); L929r1.3 (D); and L929r1.1 cells treated for 3 min at 4°C with glycine-HCl buffer (50 mM, containing 150 mM NaCl; pH 3.0) (E). Cells were treated either with secondary antibody alone (Curve 1) or with anti-murine TNF and secondary antibody (Curve 2). No other signal than that of staining with the secondary antibody alone was found with an irrelevant rat IgM (not shown).

between cell colony morphology and TNF resistance in our L929 cell lines.

Sensitivity of L929s and Derivatives to Viral Infection and Antiviral Action of TNF and IFN-γ. As TNF is known to have antiviral effects in vitro (47), we investigated the sensitivity of TNF-resistant L929 cells, whether or not pretreated with TNF, to infection with VSV. Both types of TNF-resistant L929 cells were found to have a sensitivity to VSV infection similar to that of parental L929s cells and were found to be unprotected against infection by TNF pretreatment (Fig. 7). Similar results were observed for encephalomyocarditis virus (data not shown).

Hence, TNF has no antiviral effect on TNF-resistant L929 cells. Apparently, also endogenously produced TNF does not confer antiviral protection to L929r1-type cells.

L929s as well as L929r cells could be protected from virus infection by pretreatment with IFN-γ (Fig. 7). This finding shows that the failure of IFN-γ to enhance or to induce TNF sensitivity in L929s and L929r1-type cells, respectively (Fig. 1; Table 2 Production of IL-6 by L929s and derivatives

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<tr>
<th>L929 cell line</th>
<th>Amount of IL-6 produced (pg/ml)</th>
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<tr>
<td>s</td>
<td>Untreated 135 ± 23</td>
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<tr>
<td>r1.1</td>
<td>TNF-treated 1934 ± 114</td>
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<td>r1.3</td>
<td>87 ± 12</td>
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<td>r2</td>
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<td>r2</td>
<td>208 ± 75</td>
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* Cells (10⁶) were seeded in 10-cm² wells in 1 ml culture medium. Twenty-four h later, fresh culture medium with or without 5000 IU rhTNF/ml was added for 24 h, after which the supernatant was harvested. IL-6 activity herein was assayed as described in “Materials and Methods.”

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Fig. 6. Colony morphology of L929s and its TNF-resistant derivatives. Cells were seeded at 200 cells/75-cm² flask, fixed 7–9 days later with paraformaldehyde, and stained with crystal violet. Bar, 200 μm (A) or 80 μm (B).
L929r1-type cells remained resistant to TNF, even after addition of ActD, CHX, or IFN-γ, agents known to sensitize several tumor cell lines to TNF cytotoxicity. The observation that treatment with IFN-γ protects L929r1-type cells to viral infection shows that the failure of IFN-γ to induce TNF sensitivity cannot be attributed to a defective IFN-γ signaling in these cells. Apparently, the mechanism of TNF resistance present in L929r1-type cells is quite stable, even upon blocking of de novo expression of the TNF gene by ActD or CHX. However, we cannot exclude that inhibition of RNA or protein synthesis during the TNF assay also blocks (re-)expression in L929r1-type cells of factors required for TNF-induced cytolysis.

TNF receptors on L929r1-type cells could not be revealed by treatments known to disrupt or prevent interaction of TNF with its receptor (incubation with acidic glycine-HCl buffer and/or with antiserum to murine TNF). These treatments also did not remove membrane-bound TNF (as revealed by flow cytometry), additionally indicating that the TNF present on L929r1-type cells is not receptor bound, and most probably is the membrane-bound M, 26,000 TNF proform (50). Our observations are in contrast with the findings of Spriggs et al. (23), who could detect specific TNF binding on a continuously TNF-producing breast carcinoma cell line after treatment with antibodies to TNF. The reason for this discrepancy is unclear for the moment. It is unlikely that this discrepancy relates to absence of TNF receptors in L929r1-type cells, as cells with a similar phenotype can be generated at high frequency by transfection of an expression vector for the murine TNF gene in a TNF-sensitive L929s subclone with an extremely low frequency of spontaneous TNF-resistant cells. A possible explanation might be that TNF synthesis modulates TNF receptor expression/function in order to prevent toxicity by autocrine TNF. This might, for example, occur by desensitization of TNF receptors by intracellular interaction with TNF before these receptors are coupled to signaling systems. A related mechanism might be internalization of TNF receptors before extracellular secretion of TNF, as has recently been reported to occur in lipopolysaccharide-stimulated macrophages (51).

The second type of TNF-resistant cells, named L929r2, did not produce detectable amounts of TNF, also not after treatment with exogenous TNF. No consistent differences in the number or binding affinity of TNF receptors were found for L929s and L929r2 cells. The latter were found to become sensitive to TNF-mediated cell lysis in the presence of ActD, CHX, or IFN-γ, showing that the pathway of TNF cytotoxicity is still present and potentially active in these cells. Apparently, a protein synthesis-dependent protection mechanism is active in this cell line. The nature of this salvage pathway is completely unknown for the moment, but its expression in L929r2-cells seems to be fairly unstable, as the cells gradually reverted to TNF sensitivity over a 1- to 2-month culture period without TNF selection. This irrevocable reversion to TNF sensitivity might be due to unstable gene-regulatory mechanisms. Treatment with the DNA-hypomethylating agent azaC, reported to induce TNF sensitivity in a TNF-resistant cell line (37), did not alter the TNF responsiveness of L929r2 cells in any stage of TNF resistance. Therefore, it is unlikely that gene inactivation by methylation is involved in modulation of the TNF-resistant/sensitive phenotype of L929r2-type cells. Reacquirement of TNF sensitivity could also be related to silencing of genes

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Table 1), is due to the absence of a functional IFN-γ response in these cells.

### DISCUSSION

In this report we describe the derivation and characterization of TNF-resistant sublines of the TNF-sensitive murine fibrosarcoma cell line L929s. Using different TNF selection procedures, two distinct types of TNF-resistant variants were obtained. We do not know, however, whether isolation of these two types is due to the differences in the selection protocols applied, as only a limited number of TNF-resistant cell clones have been characterized so far.

Cells of the first type, named L929r1-type cells by us, were similar to the TNF-resistant L929 cell line Lr (22), as they were also found to produce TNF constitutively and to lack demonstrable cell surface receptors for TNF. Others too have reported a correlation between TNF resistance rather exceptional, despite the observation that quite a number of tumor cell lines as well as primary cells contain TNF mRNA, either constitutively or upon TNF induction (24, 48, 49). However, the presence of TNF mRNA without concomitant synthesis of the TNF protein does not necessarily correlate with resistance to TNF cytotoxicity (24).

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coding for TNF resistance proteins. However, treatment of L929r2 cells with butyrate, known to induce de novo expression of many endogenous cellular genes (36), did not influence the TNF-sensitive/resistant phenotype of L929r2 cells. An unstable L929r2-like TNF-resistant phenotype was also observed for TNF-resistant variants selected from the TNF-sensitive WEHI164c113, MCF-7 (human breast carcinoma), and U937 (human histiocytic lymphoma) cell lines.10 Upon application of a strong TNF selection on the TNF-sensitive parental cell lines, we have always observed that a fraction of the cells did not succumb to TNF, not even after prolonged TNF selection. When the latter cells, surviving a 2-week culture period in the presence of TNF, were recultured in TNF-free medium for 1 week and then rechallenged with TNF, again only a fraction of them survived.10 At present, we can only speculate about possible mechanisms involved. Cells might initially withstand TNF-mediated cell lysis because inherent TNF resistance mechanisms are operative during a particular stage of the cell cycle, which is known to be correlated with TNF sensitivity (52). Due to continuous further TNF selection, such otherwise cell cycle-dependent resistance mechanisms might persist or allow the cells to build up additional protein synthesis-dependent TNF resistance mechanisms. As TNF cytotoxicity can be conditionally induced in such r2-type cells, this system may be a valuable tool in the search for parameters other than TNF production in the mechanism(s) of TNF resistance. Comparison of protein and phosphorylation patterns in sensitive and r2-type L929 cells as well as investigation of TNF signaling in these cells is presently under way. In this respect, we recently observed that L929r2 cells, in contrast to L929s, do not release arachidonic acid upon TNF treatment (53). In contrast, other actions of TNF, such as the induction of IL-6 secretion, still occur in r2-type cells, showing that cells can develop resistance to TNF cytotoxicity, yet retain their responsiveness to some other effects of TNF, such as specific gene induction. These data further indicate that signals responsible for distinct TNF actions (in this case cytotoxicity versus IL-6 induction) are via independent postreceptor pathways.

The observation of two discrete types of TNF-resistant derivatives of a single cell line suggests the existence of two discrete mechanisms by which cells can withstand the deleterious effects of TNF. It is unlikely that r1- and r2-types of TNF resistance are part of a single mechanism in which, for example, the r2-stage of TNF resistance precedes the r1-stage of continuous TNF synthesis. This conclusion is based on the observation that TNF production in L929r2 cells could not be induced by short-term treatment with exogenous TNF. Moreover, L929r2 cells did not become TNF producers upon a 2-month culture period either in the continuous presence of TNF or for alternating weeks with and without TNF.10 If tumor cells with discrete mechanisms of TNF resistance also occur in vivo, exploitation of the direct cytotoxic action of TNF in treatment of neoplasia might be seriously hampered. Hopefully, further research will provide more insight in the mechanism(s) involved in TNF resistance, and in this way contribute to the development of methods for circumvention of TNF resistance in vivo.

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