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Tumour necrosis factor-like activity on paraformaldehyde-fixed monocyte monolayers

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SUMMARY

A tumour necrosis factor (TNF)-related protein appears to be associated in part with the monocyte surface membrane, since paraformaldehyde-fixed monocyte monolayers induced cytolysis of WEHI 164 clone 13 cells. Cytolysis was inhibited by neutralizing antisera raised against recombinant TNF (rTNF) and against the monocyte-derived cytotoxic factor, CF, which presumably is identical to natural TNF. Moreover, crude monocyte membrane preparations also contained cytotoxic activity, which was inhibited by antisera raised against rTNF and CF, and this activity was associated with particulate structures as it did not pass through filters with a pore size of 0·2 μm. Gamma-interferon (γ-IFN)-activated monocytes fixed with paraformaldehyde had more TNF-like activity than fixed unactivated monocytes, and the crude membrane preparations from γ-IFN-activated monocytes contained about five times more TNF-like activity than preparations from unactivated monocytes.

INTRODUCTION

Monocytes produce and release tumour necrosis factor (TNF), a protein that induces necrosis of tumours in animals and that is cytotoxic to various types of tumour cells *in vitro* (Old, 1985). TNF was initially found in sera from BCG-primed, endotoxin-treated mice and rabbits (Old, 1985; Carswell *et al.*, 1975). More recently, human TNF has been extensively characterized (Aggarwal *et al.*, 1985), and the gene coding for this protein has been cloned and expressed in *Escherichia coli* (Marmenout *et al.*, 1985; Pennica *et al.*, 1984; Shirai *et al.*, 1985; Wang *et al.*, 1985).

Lately it has become evident that TNF is a pleiotropic mediator that induces many diverse biological effects. In addition to its *in vitro* cytotoxic and *in vivo* necrosis activity, TNF has the ability to enhance fibroblast growth (Sugarman *et al.*, 1985; Vilček *et al.*, 1986), stimulate the acute-phase response in hepatoma cells (Darlington, Wilson & Lachman, 1986), stimulate collagenase and prostaglandin E_2 production by synovial cells and dermal fibroblasts (Dayer, Beutler & Cerami, 1985), produce fever (Dinarello *et al.*, 1986), decrease lipoprotein lipase activity in adipocytes (Beutler *et al.*, 1985), and activate neutrophils (Klebanoff *et al.*, 1986; Shalaby *et al.*, 1985) and endothelial cells (Pober *et al.*, 1986). Another monocyte-produced protein that also has the ability to induce these biological effects is interleukin-1 (IL-1) (Darlington *et al.*, 1986; Dinarello *et al.*, 1986; Pober *et al.*, 1986; Onozaki *et al.*, 1985; Schmidt *et al.*, 1982; Dayer *et al.*, 1986; Beutler & Cerami, 1985; Klempner, Dinarello & Gallin, 1978), which was initially detected as a factor inducing thymocyte proliferation (IL-1 activity) (Oppenheim *et al.*, 1986). In addition to being released from monocytes, it appears that IL-1 is associated with the monocyte/macrophage plasma membrane, as IL-1 activity has been detected on fixed macrophages (Kurt-Jones *et al.*, 1985) and in monocyte/macrophage membrane-containing preparations (Kurt-Jones *et al.*, 1985; Matsushima *et al.*, 1986). Moreover, IL-1 activity may be released from the monocyte cell surface upon exposure to proteolytic enzymes (Matsushima *et al.*, 1986).

Considering the many similarities between TNF and IL-1, it would seem likely that TNF is also in part a monocyte membrane-associated protein. This would be in agreement with results showing that neutralizing antiserum raised against a monocyte-derived cytotoxic factor (CF) that is very similar to TNF (Nissen-Meyer, Austgulen & Espevik, 1987) bound to the plasma membrane of monocyte, gamma-interferon-activated monocytes binding about three times more than unactivated monocytes (Kildahl-Andersen, Espevik & Nissen-Meyer, 1985). Although these results suggest that a TNF-related protein may be associated with the monocyte membrane, there is the uncertainty that the antiserum is not specific, and hence may detect other membrane-associated proteins. Analogous to what has been done for IL-1, in this study we have attempted to find support for the hypothesis that a TNF-related protein may be membrane associated, particularly of gamma-interferon-activated monocytes, by investigating whether or not TNF-like activity may be detected in monocyte membrane preparations and on the surface of fixed monocytes.

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MATERIALS AND METHODS

Monocytes

Human monocytes were separated from defibrinated venous blood as previously described (Hammerstrøm, 1979) and cultured in RPMI-1640 medium (Gibco, Paisley, Renfrewshire, U.K.) containing 25% pooled human serum (The Blood Bank, Trondheim, Norway), 0·1 mM L-glutamine and 30 μg/ml gentamicin (human serum-containing medium, HS-M).

In experiments where monocytes were cultured for the preparation of membrane-containing fractions, monocytes separated from defibrinated blood were washed twice in Hanks' balanced salt solution (HBSS), suspended to a concentration of 4×10^6 cells/ml in HS-M, and 1·5 ml of the cell suspension was added to 35-mm tissue culture wells (Costar 3506). Non-adherent cells were then removed after 90 min incubation at 37°, and the adherent cells were washed three times with HBSS. After 1 day of culture at 37°, the medium was changed, adding 1·5 ml fresh HS-M per culture well. After culturing for 3 days at 37°, the medium was removed and fresh HS-M (1·5 ml/culture well) with or without 1000 units/ml recombinant gamma-interferon (γ-IFN) (produced by Genentech Inc., South San Francisco, CA, and kindly provided by Boehringer Ingelheim, Vienna, Austria) was added, and the monocytes were cultured further for 24 hr. On the fourth day of culture the medium was removed and the monocytes were ready for preparation of the membrane-containing fractions as described below.

In experiments where monocytes were cultured for fixation, monocytes separated from defibrinated blood were washed twice in HBSS, suspended to a concentration of 4×10^6 cells/ml in HS-M, and 200 μl of the cell suspension were added to 6-mm culture wells (Costar 3596). Cells were then treated in a manner identical to that described above, except that the volumes added per 6-mm culture well was 200 μl instead of 1·5 ml per 35-mm culture well as described above.

Target cells

WEHI 164 murine fibrosarcoma cells (Röllinghoff & Warner, 1973) were obtained from Dr H. W. Löms Ziegler-Heitbrock (Institute of Immunology, University of Munich, FRG). WEHI 164 clone 13 cells were obtained by cloning WEHI 164 cells by limiting dilution in microplates, and they were selected on the basis of their extreme sensitivity to TNF-induced cytolysis (Espevik & Nissen-Meyer, 1986). The cells were maintained as stationary cultures in RPMI-1640 containing 10% fetal calf serum (Gibco), 0·1 mM L-glutamine and 30 μg/ml gentamicin (fetal calf serum-containing medium, FCS-M).

Preparation of monocyte membrane-containing fraction

Monocytes activated with γ-IFN (1000 U/ml) in nine 35-mm culture wells and unactivated monocytes in a corresponding number of wells were washed three times in RPMI-1640, after which 0·5 ml of RPMI was added per well and the monocytes were detached using a rubber policeman. The monocytes in the resulting cell suspension were then lysed by nitrogen cavitation in a nitrogen bomb (Parr Instrument Co., Moline, IL) after equilibration at 600 p.s.i. of nitrogen for 10 min, similar to the method described by Lemonnier *et al.* (1978). The nuclei were pelleted by centrifugation at about 800 ***g*** for 5 min, after which the resulting supernatant was centrifuged at 37,000 ***g*** for 20 min to obtain a pellet containing monocyte membranes. This pellet was resuspended in 1 ml RPMI-1640 and assayed for TNF-like activity using the MTT assay.

Fixation of monocytes

The monocytes were fixed essentially as described by Kurt-Jones *et al.* (1985). Unactivated and γ-IFN-activated monocytes in 6-mm culture wells were washed three times with RPMI-1640 and then fixed with 1% paraformaldehyde in RPMI-1640 (100 μl per well) for 15 min at room temperature. The cells were then washed three times with FCS-M and incubated in FCS-M (200 μl per well) at 37° for 24 hr, after which the cells were washed once in FCS-M and then assayed for TNF activity using the ^{51}Cr-release assay.

Colorimetric MTT (tetrazolium) assay for measuring TNF-like activity

The MTT tetrazolium cytotoxicity assay (Mosmann, 1983) was used to measure the percentage of dead cells. Clone 13 WEHI 164 target cells (2×10^4 cells in 100 μl FCS-M) were added to 6-mm microculture wells (Costar 3596) together with various amounts of recombinant TNF (rTNF) (kindly provided by Biogen S. A., Geneva, Switzerland and KNOLL/BASF, Ludwigshafen, FRG) or monocyte membrane-containing fractions. The final volume in each well was adjusted to 150 μl FCS-M. After 20 hr of incubation at 37°, 10 μl MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Chemical Co., Poole, Dorset, U.K.) at a concentration of 5 mg/ml in phosphate-buffered saline was added. The cultures were then incubated for 4 hr at 37°, after which 50 μl supernatant were removed from each well and replaced with 100 μl 0·04 N HCl in isopropanol. After dissolving the dark blue formazan, the optical density of each well was measured with a Dynatech MR 600 Microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of dead target cells was calculated as:

$$\% \text{ dead cells} = 100 - \frac{\text{OD in wells with TNF}}{\text{OD in control wells without TNF}} \times 100.$$

Under these assay conditions, OD corresponding to no target cell death (OD in control wells) was generally between 0·9 and 1·1 and OD corresponding to 100% cell death (same as reference wells without target cells) was 0·0.

^{51}Cr-release assay for measuring TNF-like activity on the plasma membrane of fixed monocytes

WEHI 164 clone 13 cells that had been labelled with ^{51}Cr as described previously (Austgulen *et al.*, 1986) were added to 6-mm microculture wells (5×10^3 cells in 200 μl FCS-M per well) containing the fixed monocytes. After culturing for 8 hr at 37°, 50 μl HBSS were added to each well. To some wells 50 μl of 2·5% SDS were added instead of 50 μl HBSS in order to determine the total amount of releasable ^{51}Cr. The microculture plates were then centrifuged (400 ***g***, 10 min) and the radioactivity was determined in 100-μl samples of cell-free supernatants from wells containing fixed monocytes (experimental release, *E*), wells without fixed monocytes (spontaneous release, *S*), and wells to which SDS had been added (total release, *T*). The percentage specific lysis was calculated as:

$$\frac{E-S}{T-S} \times 100.$$

Under our labelling conditions the total release, T, was generally between 2000 and 3000 c.p.m., and the spontaneous release, S, after 8 hr was 20–25% of the total release.

Antiserum against rTNF and the monocyte-derived cytotoxic factor (CF)

Antiserum against rTNF was made by injecting a chinchilla rabbit three times intradermally at 14-day intervals with 12 μg, 80 μg and 70 μg rTNF (99% pure, kindly provided by Biogen, S. A., and KNOLL/BASF), respectively. The two first injections were given in the presence of 50% Freund's complete adjuvant, and the third injection with 50% Freund's incomplete adjuvant.

Antiserum against CF (presumably natural TNF; Nissen-Meyer *et al.*, 1987) was made by multiple intracutaneous injections into a rabbit of CF, after purification on a Mono S cation exchanger, Mono P chromatofocusing column and a Sephacryl S-200 gel filtration column as described elsewhere (Kildahl-Andersen *et al.*, 1985). In order to prepare CF-containing monocyte supernatants used for purifying CF, human monocyte monolayers cultured for 3 days were activated for 24 hr with lymphokines prepared from BCG-exposed human lymphocytes, after which the lymphokine-containing medium was removed and RPMI-1640 containing 0·1 μg/ml LPS was added, all as described by Kildahl-Andersen *et al.*, (1985). After incubating the monocyte cultures for 7 hr, the CF-containing monocyte supernatants were rended cell-free by low-speed centrifugation, and were stored at −20°. The monocyte supernatant had a protein concentration of 1 mg/ml, of which somewhere between 0·1 and 0·01 μg/ml appears to be TNF-related proteins (J. Nissen-Meyer, manuscript in preparation), and the purification procedure resulted in about a 10^3–10^4-fold reduction in the content of contaminating proteins (Nissen-Meyer & Kildahl-Andersen, 1984).

Statistics

Results are given as the mean ± standard deviation of quadruplicate determinations in single experiments.

RESULTS

TNF-like activity in monocyte membrane-containing fractions

Membrane-containing preparations from both unactivated and γ-IFN-activated monocytes contained cytotoxic activity (Fig. 1). Judging from the dose–response curves, the preparation from γ-IFN-activated monocytes contained about five times more of the cytotoxic agent than the preparation from unactivated monocytes (Fig. 1).

In order to determine whether or not the cytotoxic activity was due to a TNF-related protein, we studied the effect on the cytotoxic activity of antiserum raised against rTNF, and antiserum raised against the monocyte-derived cytotoxic factor CF. This cytotoxic factor is similar, if not identical, to rTNF (Nissen-Meyer *et al.*, 1987), and hence it presumably is natural TNF. Both antisera neutralized the activity of rTNF (Fig. 2), whereas pre-immune sera had no effect (results not shown; Nissen-Meyer *et al.*, 1987; Kildahl-Andersen *et al.*, 1985). Per volume of undiluted sera, the rTNF antiserum was 30–50 times more potent than the CF antiserum in neutralizing the cytotoxic activity of rTNF (Fig. 2). The same was true for neutralization of the cytotoxic activity in CF-containing monocyte supernatants (data not shown). The cytotoxic activity in the membrane-containing fractions was neutralized by both the rTNF and the CF antisera (Fig. 3), indicating that the cytotoxic activity in the membrane-containing fraction was due to a TNF-related protein. Somewhat surprisingly, however, the CF antiserum was, on a per volume basis, more potent in neutralizing the cytotoxic activity in these membrane-containing fractions than the rTNF antiserum.

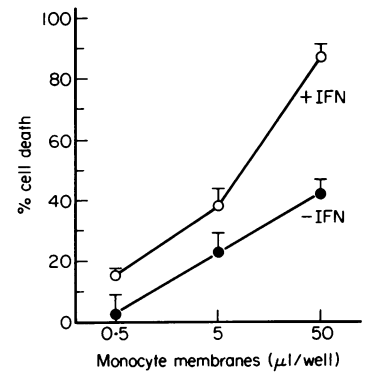

Figure 1. Cytotoxic activity in monocyte membrane fractions. Monocyte membrane fractions were prepared from γ-IFN (1000 U/ml) exposed (○——○) and unexposed (●——●) monocytes as described in the Materials and Methods, and the fractions were tested for cytotoxicity activity using the MTT assay. An equal number of monocytes were used to isolate membrane fractions from γ-IFN-exposed and unexposed monocytes. Four similar experiments were performed.

The amount of the TNF-related protein in the membrane-containing fractions was reduced by approximately 90% when the fractions were passed through low protein-binding filters (Gelman Acrodisc filters) with a pore size of 0·2 μm, whereas no detectable cytotoxic activity was lost when rTNF- and CF/TNF-containing monocyte supernatants were passed through these filters. This indicates that the TNF-like activity in the membrane-containing fractions was in fact associated with membrane fragments or other particulate structures, and was thus not due to free TNF contaminating the membrane-containing fractions.

TNF-like activity associated with fixed monocytes

The above results suggest that the TNF-like activity may be associated with particulate structures, perhaps monocyte surface membrane fragments. In order to obtain evidence to suggest that the activity was in fact associated with the monocyte surface membrane, the TNF-like activity on fixed monocytes was determined in an analogous manner as has been done for IL-1 (Kurt-Jones *et al.*, 1985). Both unactivated and γ-IFN-activated fixed monocytes induced cytolysis of target cells; however, γ-IFN-activated monocytes were much more cytotoxic than unactivated monocytes (Fig. 4). The cytotoxic activity was inhibited by both the rTNF and the CF antisera (Fig. 4), indicating that the cytotoxic activity was due to a TNF-related protein located on the surface of the fixed monocytes. As was the case for the membrane-containing fractions, the CF

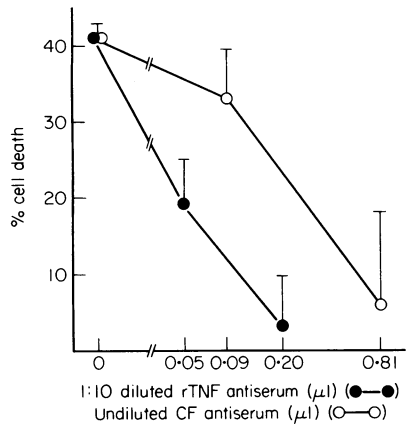

Figure 2. Effect of increasing amounts of rTNF antiserum and CF antiserum on cell death induced by rTNF (1 pg/culture well). Cell death was measured using the MTT assay, and the numbers given along the abscissa indicate the amount (in microlitres) of rTNF antiserum at a 1:10 dilution and undiluted CF antiserum added per culture well. Three similar experiments were performed.

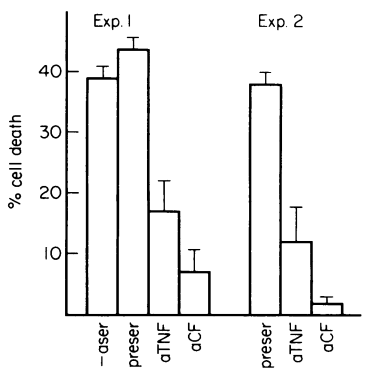

Figure 3. Effect of rTNF antiserum and CF antiserum on cell death induced by membrane fractions prepared from γ-IFN (1000 U/ml)-activated monocytes. Fifteen microlitres of monocyte membrane fractions were added per culture well plus 1 μl per culture well of either preimmune serum (from rabbits subsequently immunized with rTNF) (preser), rTNF antiserum (aTNF) or CF antiserum (aCF). To some wells neither preimmune serum nor antiserum was added (−aser). Data from two different experiments are shown; the membrane fractions used in Experiments 1 and 2 were different, but the membrane fraction used in Experiment 1 was the same as that used in Fig. 1. The cytotoxic activity was measured using the MTT assay. Four similar experiments were performed.

antiserum was more potent in neutralizing the cytotoxic activity associated with the fixed monocytes than the rTNF antiserum, despite the fact that the rTNF antiserum was 30–50 times more potent than the CF antiserum in neutralizing rTNF- and CF/TNF-containing monocyte supernatants.

DISCUSSION

The results demonstrate that a TNF-related protein is associated in part with the monocyte surface membrane, as paraformaldehyde-fixed monocyte monolayers showed cytotoxic activity that was inhibited by neutralizing antisera raised against rTNF and against the monocyte-derived cytotoxic factor, CF, which presumably is identical to natural TNF (Nissen-Meyer *et al.*, 1987). Moreover, preparations of monocyte membranes contained TNF-like activity associated with particulate structures that did not pass through filters with a pore size of 0·2 μm. Fixed monocytes that had been activated by exposure to γ-IFN had more TNF-like activity than fixed unactivated monocytes, and the membrane-containing fraction prepared from γ-IFN-activated monocytes contained about five times more TNF-like activity than the corresponding fraction prepared from unactivated monocytes.

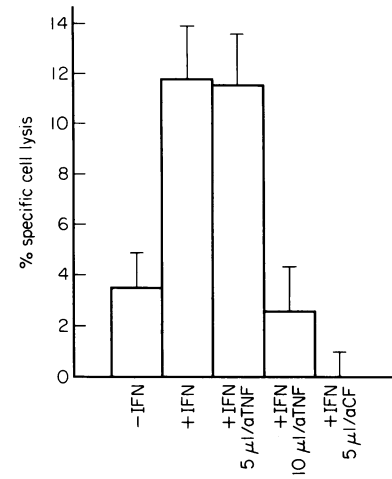

Figure 4. Target cell lysis induced by paraformaldehyde-fixed monocytes and the effect of rTNF antiserum and CF antiserum on lysis. Monocytes were either exposed to γ-IFN (1000 U/ml) (+IFN) or unexposed (−IFN) before fixation, and the ability of the fixed monocytes to induce cell lysis using the ^{51}Cr-release assay was measured in the absence and presence of the indicated amount (μl per culture well) of rTNF antiserum (aTNF) and CF antiserum (aCF). Four similar experiments were performed.

When using the WEHI 164 clone 13 cells for measuring cytotoxicity, all the cytotoxic activity in the CF-containing monocyte supernatants is neutralized by rTNF antiserum, similar dose–response curves are obtained for rTNF and supernatants, and the cytotoxic activity in the supernatants elutes in the same manner as rTNF upon chromatofocusing, gel filtration and SDS–polyacrylamide gel electrophoresis (Nissen-Meyer *et al.*, 1987). Moreover, the CF antiserum inhibits all the cytotoxic activity of rTNF (Nissen-Meyer *et al.*, 1987; Fig. 2). Thus, when using the clone 13 cells for measuring cytotoxicity, as has been done in this study, the cytotoxic activity released by the monocytes appears to be entirely due to a protein (presumably natural TNF) that is similar or related to rTNF.

By indirect immunofluorescence we have shown earlier that CF antiserum binds to the surface of monocytes, and that activating the monocytes with γ-IFN results in about a three-fold increase in the amount of antiserum that binds, an increase that correlates with an increase in the ability of the monocytes to kill tumour cells (Kildahl-Andersen *et al.*, 1985). Other cell types tested did not bind the CF antiserum. There is some uncertainty, however, when using indirect immunofluorescence to localize cellular proteins, since the reliability in the interpretation of the

results depends on the specificity of the antibodies used. Although the CF antiserum was raised against a CF preparation purified 10^3–10^4-fold judging from the reduction in protein content, the antiserum appears to bind to some serum proteins (albumin, immunoglobulins) and may possibly also bind to unknown monocyte proteins other than TNF. We have recently performed immunofluorescence studies with seven different monoclonal antibodies raised against rTNF and natural TNF, and found that several of the monoclonal antibodies among themselves stain monocytes very differently, presumably due to the antibodies also recognizing cellular proteins other than TNF, proteins which are probably very dominant in the monocytes (T. Espevik, unpublished observations). We were, however, unable to detect significant membrane staining with any of the monoclonal antibodies (T. Espevik, unpublished observations). This also appears to be the case for antibodies against IL-1, which only stain intracellular structures in monocytes after LPS and PHA stimulation (Bayne *et al.*, 1986). It is therefore possible that immunofluorescence microscopy is generally not sensitive enough to detect membrane-bound TNF-related proteins nor IL-1, perhaps because of steric hinderance or low amounts of these membrane-associated proteins. The method used in this study where one detects both TNF-like activity and the inhibition of this activity by antiserum against TNF, is more specific for probing for the presence of TNF-related proteins. This method is also more sensitive, since it is based on a bioassay system using the extremely TNF-sensitive WEHI 164 clone 13 cells (Espevik & Nissen-Meyer, 1986) for assaying TNF-like activity.

IL-1, a monocyte produced protein that has many biological activities in common with TNF (see the Introduction), is associated with the surface membrane of monocytes and macrophages, as has been shown using paraformaldehyde-fixed cells (Kurt-Jones *et al.*, 1985). It has been suggested that IL-1 is intracellularly synthesized as a 31,000 molecular weight precursor protein that undergoes sequential cleavage into an intermediate 23,000 MW membrane-associated form, and finally to a 17,000 MW extracellular protein (Oppenheim *et al.*, 1986). From the DNA sequence coding for TNF it also seems that TNF is synthesized initially as part of a precursor protein containing a long hydrophobic region (Pennica *et al.*, 1984), and one might speculate that this hydrophobic region functions to embed TNF in the monocyte membrane.

The biological significance of the monocyte cell surface-associated TNF-like activity is unclear. However, one might speculate that the accumulation of a TNF-like protein on the cell surface of γ-IFN-activated monocytes may in part explain how γ-IFN enhances monocyte-mediated killing, since TNF is an effector molecule involved in monocyte-mediated killing of at least some types of tumour cells (Urban *et al.*, 1986; Ortaldo *et al.*, 1986; Philip & Epstein, 1986; Espevik & Nissen-Meyer, 1986; Nissen-Meyer *et al.*, 1987). TNF is thus probably similar or identical to the monocyte-derived cytotoxin(s) that has also been shown to be involved in monocyte-mediated killing (Austgulen *et al.*, 1986; Espevik, Kildahl-Andersen & Nissen-Meyer, 1986; Matthews, 1983; Männel, Falk & Meltzer, 1981). Both our rTNF antiserum and the CF antiserum inhibited monocyte-mediated killing of certain tumour cells (Nissen-Meyer *et al.*, 1987; Austgulen *et al.*, 1986; Espevik *et al.*, 1986). However, although the rTNF antiserum was on a per volume basis 30–50 times more potent that the CF antiserum in neutralizing the cytotoxic activity of rTNF- and TNF-containing monocyte supernatants, the CF antiserum is more potent than the rTNF antiserum in inhibiting monocyte-mediated killing of some types of tumour cells, especially K562 cells, which are relatively resistant to killing by rTNF (J. Nissen-Meyer and T. Espevik, manuscript in preparation). Moreover, as shown in the study, the CF antiserum was also more potent in inhibiting monocyte-membrane associated TNF-like activity. One might speculate from this that the CF antiserum recognizes a variant form of TNF, structurally somewhat different from both rTNF and most of the TNF released from the monocytes, and that this variant form of TNF is present on the monocyte surface membrane, especially on γ-IFN-activated monocytes. Thus, this membrane-associated TNF may conceivably be the form of TNF of major importance in monocyte-mediated killing of tumour cells, which requires effector to target cell contact for killing, such as K562 cells, and which otherwise appears to be relatively resistant to cytolysis induced by rTNF and free TNF released from monocytes. Free extracellular TNF would, on the other hand, largely contribute to monocyte-mediated killing of cell types that are extremely sensitive to rTNF, such as WEHI 164 clone 13 cells, and which do not appear to require effector to target cell contact for efficient killing.

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