Immunology 1990 **71** 218–223

Generation and characterization of a neutralizing rat anti-rmTNF-α monoclonal antibody

R. LUCAS, K. HEIRWEGH,* A. NEIRYNCK, L. REMELS, H. VAN HEUVERSWYN* & P. DE BAETSELIER
*Unit of Cellular Immunology, Free University of Brussels and * Innogenetics, Belgium, division Gent*

Accepted for publication 6 June 1990

SUMMARY

A rat anti-recombinant mouse tumour necrosis factor-alpha (rmTNF-α) monoclonal IgM antibody (1F3F3) with high specific binding activity for rmTNF-α was generated. The 1F3F3 monoclonal antibody (mAb) neutralizes the cytotoxic activity *in vitro* of rmTNF-α on L929 cells and inhibits the binding of radiolabelled rmTNF-α to its putative receptor on L929 cells. The 1F3F3 mAb binds to monomeric, dimeric and trimeric rmTNF-α and does not bind to reduced rmTNF-α, indicating that the recognized epitope is sensitive to denaturation. Using the 1F3F3 mAb as a capturing antibody and a biotinylated anti-rTNF-α as a detecting antibody, we have developed a sandwich ELISA that can specifically detect biologically active mTNF-α with a detection limit of 10 pg mTNF-α/well. This assay correlates well with the classical L929 cristal violet assay for the detection of bioactive rmTNF-α in biological fluids. The 1F3F3 mAb inhibits various *in vitro* biological activities of the rmTNF-α, such as the TNF-α-mediated tumouricidal activity of activated macrophages, the rmTNF-α-dependent stimulation of neutrophil degranulation and the growth-promoting effect of rmTNF-α. *In vivo* the 1F3F3 mAb inhibits lipopolysaccharide (LPS)-induced endotoxic shock. In conclusion, the 1F3F3 mAb is a useful tool to probe rmTNF-α activity both *in vitro* and *in vivo*.

INTRODUCTION

Although initially described as an inducer of haemorrhagic necrosis of certain transplantable tumours in mice primed with Bacillus Calmette–Guérin and subsequently challenged with endotoxin (Carswell *et al.*, 1975), the polypeptide hormone tumour necrosis factor-alpha (TNF-α) has recently been shown to be implicated in various immune reactions. Indeed TNF-α belongs, together with the interferons (IFN) and the interleukins (IL), to the family of cytokines: hormones that participate in a complex network of interactive signals between cells of immunological and non-immunological origin (Old, 1987).

Studies on different cell lines have identified diverse functional activities for this cytokine, including inhibition of lipoprotein lipase in adipocytes (Torti *et al.*, 1985), bone resorption in osteoclasts (Bertolini *et al.*, 1986), induction of class I major histocompatibility antigens in endothelial cells and fibroblasts (Collins *et al.*, 1986) and activation of the c-myc oncogene in osteosarcoma (Kirstein & Baglioni, 1988). In addition, TNF-α is also a potent angiogenic factor (Leibovitch *et al.*, 1987), a growth-promoting agent for fibroblasts (Sugarman *et al.*, 1985) and a degranulation inducing agent on neutrophils (Scuff-Werner *et al.*, 1987). Furthermore, TNF-α induces synthesis of plasminogen activator inhibitors (Medcalf, Kruithof & Schleuning, 1988) and production of other cytokines, such as macrophage colony-stimulating factor (CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-1 (Kauhansky *et al.*, 1988). All these studies indicate that TNF-α is an important mediator of inflammation (Tracey *et al.*, 1986).

Considering these diverse activities of TNF-α, the question is raised as to whether different distinct functional epitopes of mTNF-α are implicated in each activity. To tackle this important question we have generated mAb against mouse TNF-α. One of these mAb, i.e. 1F3F3, was found to neutralize various activities of mTNF-α *in vivo* and *in vitro*. Furthermore, we have used these antibodies to develop an ELISA assay that specifically detects biologically active mTNF-α.

Abbreviations: CFA, complete Freund's adjuvant; *E.coli*, *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; IFA, incomplete Freund's adjuvant; i.f.p., intrafootpad; IgG, immunoglobulin G; IgM, immunoglobulin M; i.p., intraperitoneal; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PMN, polymorphonuclear cells; rmTNF-α, recombinant mouse tumour necrosis factor-alpha; s.c., subcutaneous; SDS-PAGE, sodium dodecylsulphate–polyacrylamide gel; TBS, Tris-buffered saline.

Correspondence: R. Lucas, Dept. of General Biology, Unit of Cellular Immunology (IMOL2), Free University of Brussels, Paardenstraat 65, 1640 Sint-Genesius-Rode, Belgium.

MATERIALS AND METHODS

Cell lines

The murine TNF-α-producing macrophage cell line PU and the L929 fibrosarcoma cell line were generously provided by Dr L.

Fransen of Innogenetics, Gent, Belgium. The 3LL-S cell line is an extremely TNF-sensitive clone of the 3LL Lewis lung carcinoma (Remels & De Baetselier, 1987).

Animals

BALB/c mice and LOU rats, of 6–12 weeks of age, were purchased from the SCK Mol, Mol, Belgium. Swiss Nu/Nu mice, up to 6 weeks of age, were purchased from Iffa Credo, France.

TNF

Purified recombinant mouse TNF-α, produced in *E. coli*, was generously provided by Innogenetics. Preparations displayed a specific activity of 10^8 U/mg.

^{51}Cr-release assay

3LL-S cells and L929 cells were labelled during 3 hr at 37° with 20 μC Na_2CrO_4 (2×10^6 cells/ml). After three washings 10^4 cells/well were plated out in a 96-well plate, containing 10^5 peritoneal macrophages that had been activated during 24 hr with lipopolysaccharide (LPS) (100 ng/ml)/lymphokine (= 10% v/v of supernatants of concanavalin A-stimulated rat splenocytes). After 18 hr the supernatants of the wells were measured in a γ-counter. The spontaneous lysis of the 3LL-S cells was detected by measuring the radioactivity contained in the supernatant of those wells only containing the radiolabelled 3LL-S cells, and the maximal lysis corresponds with the radioactivity found in the supernatants of the wells in which 1% SDS was added 1 hr before the measurement.

The percentage specific lysis =

$$\frac{\text{experimental lysis} - \text{spontaneous lysis}}{\text{maximum lysis} - \text{spontaneous lysis}} \times 100.$$

TNF bioassay

Cytolytic activity of TNF-α was quantified using the ^{51}Cr-release assay, described previously, with labelled L-929 cells. One-hundred microlitres of the cell suspension were mixed with 50 μl of a TNF standard and 50 μl medium in 96-well flat-bottomed culture plates and 18 hr later cell lysis was measured in a γ-counter.

Alternatively, cytolytic activity of TNF-containing supernatants was quantified using the L929 cell-killing assay (Ruff & Gifford, 1981). L929 cells in RPMI-1640 + 10% foetal calf serum (FCS) were treated with actinomycin D (1 μg/ml) at a cell concentration of 3×10^5 cells/ml. One-hundred microlitres of the suspension were mixed with 50 μl of serially diluted sample (with purified TNF-α as a standard) and 50 μl medium in 96-well flat-bottomed culture plates. After 18 hr of culture at 37°, viability of the cell cultures was assessed by dye uptake analysis. The latter was accomplished by decanting the medium from the plates and staining the cells for 10 min with a 0·5% solution of cristal violet dissolved in a 1:5 mixture of methanol:water. Plates were rinsed extensively in distilled water and dye uptake was assessed at 450 nm with a Titertek Multiscan MCC 340 ELISA Reader (Flow Laboratories, McLean, VA).

TNF neutralization

Neutralization of TNF-α-dependent cytolytic activity by antibodies was quantified by preincubating 1000 U rTNF-α with a dilution range of the neutralizing anti-TNF antibodies for 1 hr before adding the rTNF to the cells in the ^{51}Cr-release assay or in the L929 cell-killing assay.

Production of anti-rTNF-α mAb

The neutralizing 1F3F3 mAb and the non-neutralizing 15B6H2 mAb were produced using the following immunization protocol: LOU rats of 8 weeks of age were immunized i.p. at Day 0 with 25 μg of purified rTNF-α emulsified in CFA and boosted 22 days later i.p. with the same quantity of rTNF-α in IFA. At Day 45 a booster injection i.p. of 50 μg rTNF-α was given in phosphate-buffered saline (PBS). Three days later the animals were bled, killed, and the spleen used to produce mAb. Rat–mouse hybridomas were prepared by fusion of immune rat splenocytes or lymph node cells to the HAT-sensitive murine myeloma cell line NSO with polyethylene glycol 1500 at a splenocyte/lymph node cell:NSO ratio of 10:1. Anti-rTNF-α mAb-producing hybrids were cloned twice by limiting dilution (0·6 cell/well) in HAT-medium. The mAb-producing hybridomas were then injected in nude mice in order to generate ascites. The rat anti-rTNF-α mAb were purified from ascites using a CNBr-activated Sepharose column containing coupled mouse anti-κ-chain rat antibodies. Purified antibody was diluted to 1 mg/ml, dialysed into PBS and stored at −20°.

Iodination of the rTNF-α

The rTNF-α was radioactively labelled with ^{125}I using the Iodogen method (Markwell & Fox, 1978). Samples with more than 95% of the activity present in the TCA precipitate were selected for the experiments. The specific activity was 3000 c.p.m. U rTNF-α.

L929 binding assay

The binding of ^{125}I-labelled rTNF-α to L929 cells was tested as follows: 10^5 L929 cells/culture plate (diameter 3·5 cm) were incubated for 24-hr in the presence of 6 U ^{125}I-labelled rTNF-α, diluted in PBS, at 4°, in the presence or the absence of 1F3F3 mAb or 15B6H2 mAb. After 24 hr the cells were washed three times in PBS and were then lysed with 1% SDS in PBS. A specific binding was measured by incubating the 6 U of iodinated rTNF with 10,000 U of non-labelled rTNF. Equal volumes of the supernates were then counted in a γ-counter.

Biotinylation of antibodies

Purified rat anti-rmTNF-α mAb or rabbit anti-rmTNF-α polyclonal antibody (Innogenetics, Belgium) were biotinylated using the Amersham biotinylation kit (Amersham, Bucks, U.K.).

ELISA

Monoclonal anti-rTNF-α were detected using a direct ELISA system as described previously (Schreiber *et al.*, 1985). Four-hundred nanograms of partially purified rTNF-α were bound to 96-well plates (Nunc, Roskilde, Denmark) for 24 hr at 4° in PBS. Plates were incubated with alkaline phosphatase-labelled goat anti-rat IgG (Sigma) and, after a final wash, alkaline phosphatase-conjugated antibody was detected by the addition of 1 mM 4-nitrophenyl phosphate disodium salthexahydrate (= substrate) dissolved in the ELISA substrate buffer. Colour development was assessed at 405 nm with a Titertek Multiscan MC ELISA Reader.

An indirect ELISA was developed to quantify TNF-α in

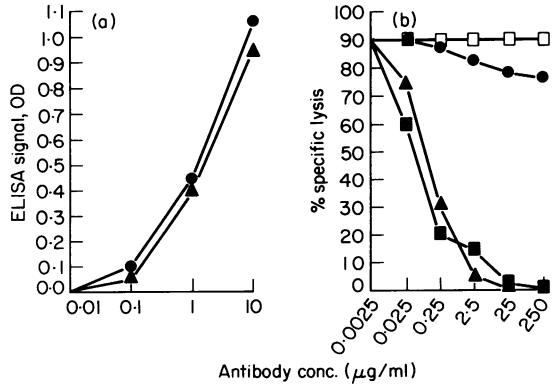

Figure 1. (a) Binding activity of the 1F3F3 (▲) and the 15B6H2 (●) mAb on solid-phase-coated rmTNF-α. (b) Neutralizing capacity of the anti-rTNF-α 1F3F3 mAb. The specific lysis of ^{51}Cr-labelled L929 cells was measured in the presence of 10,000 pg rTNF-α with or without (□) preincubation of the rmTNF-α with different concentrations of the 1F3F3 mAb (▲), the 15B6H2 mAb (●) or the neutralizing polyclonal anti-rmTNF-α Ab (■).

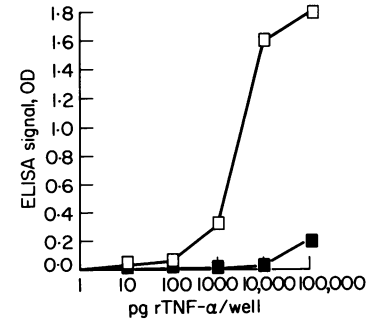

Figure 2. Sensitivity of the mTNF-α-specific capture ELISA. (□) rmTNF-α; (■) denatured rmTNF-α.

culture supernatants. Wells were precoated with 10 ng of the TNF-specific mAb 1F3F3 during 24 hr at 4°. After washing the wells were incubated with 100 μl of a TNF source for 2 hr at 37° and with 100 μl of a rTNF-α assay standard. Plates were washed again and exposed for 1 hr at 37° to 500 ng/well of biotinylated polyclonal rabbit anti-rTNF-α antibody. After washing, streptavidin-coupled alkaline phosphatase was added to the wells during 1 hr at 37°. Plates were then developed as described in the previous protocol.

Western blot analysis

A non-reducing parallel 15% SDS–PAGE gel was loaded with 1 μg active rTNF-α on the left side and with 1 μg reduced rTNF-α (reduced by heating at 56° during 1 hr) on the right side. Molecular weight markers were loaded in the middle. After the electrophoresis, the proteins were blotted on nitrocellulose by means of a 36 hr diffusion blotting (Bowen *et al.*, 1980). After cutting the nitrocellulose into 6-mm strips, the free sites on the strips were blocked with Tris-buffered saline (TBS) + 1% bovine serum albumin (BSA) during 60 min at room temperature. The strips were incubated during 1 hr at room temperature with the anti-rTNF-α mAb, which were biotinylated in order to increase the sensitivity, or with polyclonal rabbit anti-rTNF-α antibody, which was used as a positive control. After washing the strips, they were incubated during 1 hr at room temperature with streptavidin-coupled alkaline phosphatase in the case of the biotinylated mAb or with alkaline phosphatase-coupled goat anti-rabbit IgG in the case of the polyclonal antibodies. After washing the strips extensively the substrate was added. When a good signal/noise ratio of the colour reaction was reached, the reaction was stopped by adding the stopping buffer. The nitrocellulose filters were dried after the immunostaining and collected between Whatmann filters, protected from light.

Chemiluminescence

For chemiluminescence, human neutrophils were isolated according to the method developed by Boogaerts *et al.* (1983). Chemiluminescence was measured with a Beckman LS-7500 scintillation counter. In scintillation counter experiments, glass vials with a working volume of 5 ml were used. First, 4·7 ml of filtered PBS were added to these vials. Next, 100 μl of polymorphonuclear cells (PMN) suspension (10^6 PMN) were added. Prior to activation, these assay mixtures were supplemented with luminol, of which the final concentration was dependent of the concentration of the used rTNF-α stimulus. Immediately after the addition of the luminol, samples were counted for 'background' counts. Samples with background chemiluminescence (CL) exceeding 20,000 c.p.m. after stabilization were eliminated. Chemiluminescence responses are initiated by adding 100 μl of the rTNF-α stimulus.

$10T_{1/2}$ proliferation assay

10^4 $10T_{1/2}$ fibroblast cells/well were grown during 48 hr in serum-limited medium (DMEM + 0·2% FCS) for 72 hr in the presence of 50 U rTNF-α/well, supplemented with or without different concentrations of the 1F3F3 mAb. Wells treated only with the different concentrations of the 1F3F3 mAb were taken as a negative control. After 48 hr, cells were labelled with [^{3}H]thymidine and after the 72 hr incubation time, cells were lysed and the supernatants were counted in a β-scintillation counter.

Endotoxic shock survival curves

Groups of 10 male BALB/c mice of 14 weeks of age were injected i.p. with 500 μg/mouse of LPS (Difco, Michigan, U.S.A.) derived from *E. coli* 055:B5. The particular lot of endotoxin used in these experiments (Lot no. 778038) displayed an LD_{50} of 23·43 mg/kg (ipr-mus). Survival curves were generated from groups of BALB/c mice pretreated either with 50 μg 1F3F3 mAb, control rat IgM (developed in our laboratory) or saline and then injected 6 hr later with 500 μg LPS/mouse i.p. Animals were observed daily over a period of 2 weeks. This experiment was repeated four times in the same conditions.

RESULTS

Generation and characterization of a neutralizing anti-rmTNF-α mAb

Using the immunization protocol described in the Materials and Methods, we generated two rat anti-rmTNF-α mAb, both belonging to the IgM class, yet only one (i.e. 1F3F3) could neutralize the lytic action of rmTNF-α on L929 cells. The hybridoma secreting the neutralizing 1F3F3 mAb and a hybridoma secreting the non-neutralizing 15B6H2 mAb were cloned by limiting dilution, and positive, stable clones were injected in

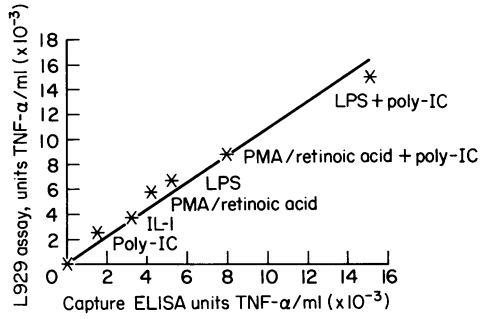

Figure 3. Detection of TNF-α in supernatants of stimulated PU cells. The mTNF-α concentration in supernatants of PU cells treated for 24 hr with different stimuli was detected with the mTNF-specific capture ELISA (*x*-axis). The results were correlated with the concentrations detected with the classical L929 assay (*y*-axis).

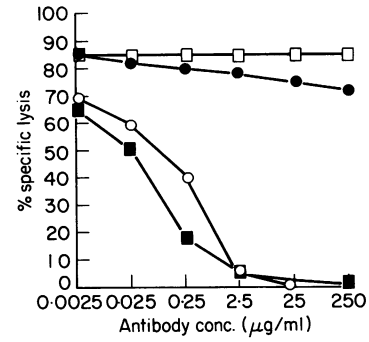

Figure 4. Inhibition of macrophage-mediated tumor cell cytotoxicity by the 1F3F3 mAb. The specific lysis of TNF-sensitive 3LL-S cells in the presence of LPS/lymphokine-activated peritoneal macrophages (E:T = 10) was measured with or without (□) preincubation of the macrophages with the 1F3F3 mAb (■), the 15B6H2 mAb (●) or a rabbit anti-TNF polyclonal antibody (○).

nude mice to generate ascites. The IgM mAb were purified from the ascitic fluid by affinity chromatography on a monoclonal anti-rat κ-chain–Sepharose column.

The two purified mAb were analysed for their anti-rmTNF-α binding activity by direct ELISA and Western blotting. As shown in Fig. 1a, the two anti-rmTNF-α mAb gave a comparable binding profile on rmTNF-α bound to plastic plates, yet only the 1F3F3 mAb could detect monomeric, dimeric and trimeric rmTNF-α bound to nitrocellulose Western blots (data not shown). This Western blot signal by the 1F3F3 mAb was nearly completely abolished when probing reduced rmTNF-α, indicating that the epitope recognized by the IF3F3 mAb is sensitive to denaturation. As expected, the purified 1F3F3 mAb and not the 15B6H2 mAb could potently neutralize the lytic activity of rmTNF-α on L929 cells to the same extent as a polyclonal rabbit anti-rTNF-α (0·5 ng/U TNF-α) (Fig. 1b). The neutralizing activity of 1F3F3 was found to be species specific, since no neutralizing activity against human rTNF-α or rat rTNF-α could be detected with 1F3F3 (data not shown).

According to its neutralizing activity, the 1F3F3 mAb could be expected to interfere with the interaction of rmTNF-α and the putative TNF-α receptor on L929 cells. Hence we tested the capacity of 1F3F3 to block the binding of radiolabelled rmTNF-α on L929 cells. The 1F3F3 mAb inhibited the binding of 60 pg rTNF-α on 10^5 L929 cells to an extent of 98%, whereas the non-neutralizing 15B6H2 did not interfere with the binding of rmTNF-α on TNF-α-sensitive L929 cells.

Utilization of the 1F3F3 mAb to detect biologically active rmTNF-α

The experiments described above indicate that the 1F3F3 mAb binds on a biologically important epitope of rmTNF-α and that such a mAb might be useful to detect biologically active rmTNF-α in various biological fluids. Hence a double antibody ELISA was developed using the 1F3F3 mAb and biotinylated rabbit anti-rmTNF-α to capture and detect mTNF-α. As shown in Fig. 2, the ELISA assay composed of the 1F3F3 mAb as a capturing antibody and polyclonal anti-rmTNF-α as a detecting antibody allowed the detection of rmTNF-α below 0·2 ng/ml (i.e. 20 pg/well) with a linear response up to 10,000 pg rmTNF-α per well (correlation coefficient = 0·97). Furthermore, this detection system enabled only the detection of biologically active mTNF-α since rmTNF-α denatured by heating (30 min at 100°) or by reducing conditions (treatment with 2-mercaptoethanol) could not be detected by this double antibody ELISA up to concentrations below 10,000 pg/well. This double antibody ELISA assay was subsequently used to detect mTNF-α in biological fluids such as supernates from activated macrophages. To this end a macrophage cell line (PU cells) was stimulated by different macrophage activating agents and the supernates were analysed for the presence of active mTNF-α by the antibody ELISA assay and the classical L929 cristal violet staining assay (see the Materials and Methods). As demonstrated in Fig. 3, a nearly perfect correlation was found between the two assays for the detection of mTNF-α in supernatants of activated macrophages, thus confirming the validity of the 1F3F3 antibody assay as an alternative to probe for biologically active mTNF-α.

Inhibition of mTNF-α-mediated biological activities by the 1F3F3 mAb

TNF-α has been reported to act as the main effector molecule in the tumouricidal activity of activated macrophages on certain tumour cell lines (Urban *et al.*, 1983). Hence the neutralizing activity of the 1F3F3 mAb was tested on the tumouricidal activity of LPS and lymphokine-activated peritoneal macrophages against a macrophage-sensitive 3LL Lewis lung carcinoma cell line, developed in our laboratory (Remels & De Baetselier, 1987). The results shown in Fig. 4 indicate that the 1F3F3 mAb strongly inhibited the lytic activity of tumouricidal macrophages against 3LL-S target cells.

Besides its lytic activity on various cell lines, TNF-α has also been reported to be a potent activator of the degranulation of granulocytes (Scuff-Werner *et al.*, 1987). Hence we have tested the neutralizing activity of the 1F3F3 mAb on the rmTNF-α-induced degranulation of human PMN, as measured via luminol-aided chemiluminescence. As shown in Fig. 5, the 1F3F3 mAb exerted a strong inhibitory and dose-dependent effect on the rmTNF-α-mediated activation of human neutrophils, whereas the non-neutralizing 15B6H2 mAb had only a low inhibitory effect and a control rat IgM antibody showed no inhibition at all.

Another biological activity of TNF-α is the capacity to act as

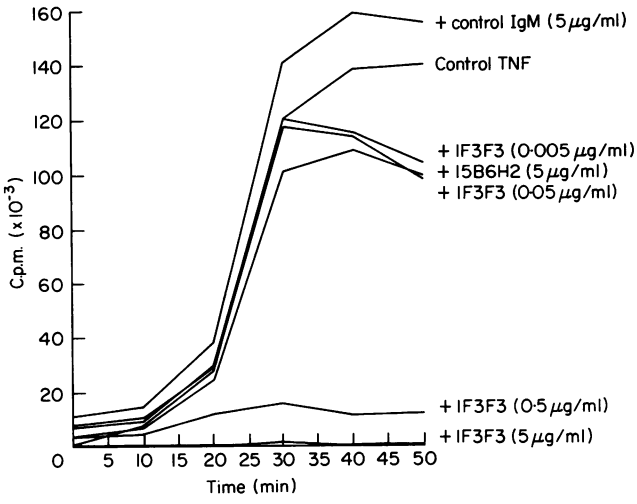

Figure 5. Inhibition of rmTNF-α-mediated degranulation of human neutrophils by the 1F3F3 mAb. The rmTNF-α-mediated degranulation of human neutrophils was measured via luminol-aided chemiluminescence (10^{-7} M luminol) with or without preincubation of the cells with the 1F3F3 mAb, the 15B6H2 mAb and a control rat IgM.

a growth promoting agent for certain fibroblastic cell lines (Sugarman *et al.*, 1985). Accordingly, rmTNF-α (5000 pg/ml) could promote the growth of the fibroblastic cell line $10T_{1/2}$ in serum-limiting conditions up to 280% of the control growth value. This growth-promoting effect of rmTNF-α on $10T_{1/2}$ cells could also be completely blocked by the 1F3F3 mAb in a dose-dependent way (data not shown).

Inhibition of lethal LPS-induced endotoxic shock by the 1F3F3 mAb

BALB/c mice were injected i.p. with 10^6 neutralizing units of the 1F3F3 mAb, or with an equal amount of a control IgM 6 hr before an i.p. injection of 500 μg/mouse of LPS (*E. coli*), in order to test the ability of the 1F3F3 mAb to protect mice from the lethal effects of endotoxic shock. Figure 6 shows that whereas 80% of the non-pretreated mice or 87% of the control mice that were pretreated with irrelevant rat IgM died, only 30% of the animals pretreated with the 1F3F3 mAb succumbed to the lethal effects of endotoxin after 12 days. This protective effect of the 1F3F3 mAb gradually decreased when the mice were pretreated with, respectively, 10^5 (55% survival) and 10^4 neutralizing units of the 1F3F3 mAb (40% survival) (data not shown). This experiment clearly shows the usefulness of the 1F3F3 mAb to probe TNF-α activity *in vivo*.

DISCUSSION

In order to identify the functional epitopes involved in the functional activity of TNF-α both *in vitro* and *in vivo*, a panel of mAb towards rmTNF-α was generated. One of these mAb, i.e. 1F3F3, was of particular interest since it could neutralize efficiently the TNF-α-dependent cytolytic activity of activated macrophages. This mAb could furthermore inhibit the rmTNF-α-induced degranulation of human neutrophils *in vitro* and the growth-promoting effect of rmTNF-α on the fibroblastic $10T_{1/2}$ cell line. Since Western blotting indicated that the 1F3F3 mAb recognizes an epitope of the rmTNF-α that is sensitive to denaturation, the 1F3F3 mAb was used to detect via ELISA picogram amounts of biologically active TNF-α in biological fluids. This ELISA detection system of TNF-α correlated fairly well with the classical L929 cytotoxicity assay. The herein described indirect ELISA assay has a major advantage over the L929 cytotoxicity assay since it specifically detects biologically active TNF, whereas the L929 cytotoxicity assay may measure a lytic activity that can be caused by other factors than TNF-α or by synergistic effects between TNF-α and other factors, such as IFN-γ. Recently Sheenan *et al.* (1989) described the generation of a hamster anti-TNF-α mAb TN3.19.12, which inhibits 100% of the lytic activity of either recombinant or natural TNF-α and protects mice from the lethal effects of endotoxic shock (measured on the 5th day after the LPS administration). Our experiments, with lower doses of LPS and longer observation times, clearly show a protective effect of the 1F3F3 mAb on the lethal effects of endotoxic shock, as measured on Day 12 after the LPS administration.

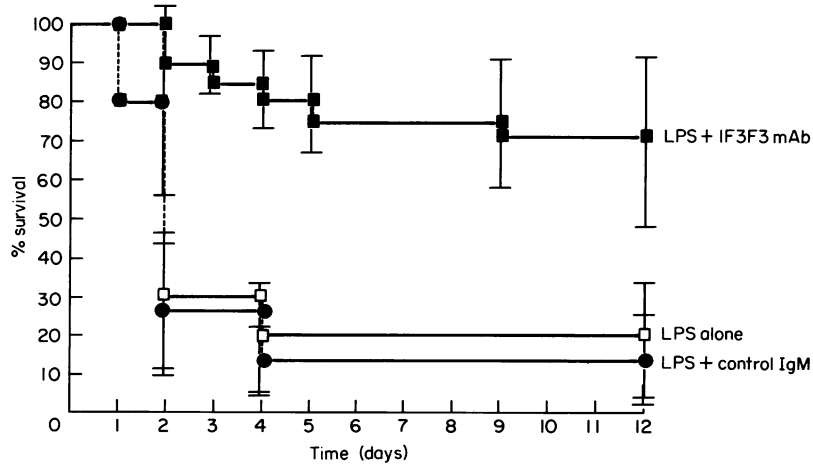

Figure 6. Survival curve of BALB/c mice injected with 500 μg LPS (*E. coli*)/mouse. BALB/c mice of 14 weeks of age were injected i.p. with 50 μg of the 1F3F3 mAb or with a control rat IgM, 6 hr prior to the injection of 500 μg of LPS i.p. The survival curve represents the mean values of four experiments (confidence limits: SD).

Since the 1F3F3 mAb can efficiently neutralize different biological activities of the rmTNF-α, such as degranulation induction of human PMN, cytotoxic activity against L929 cells and growth-promoting activity on $10T_{1/2}$ fibroblast cells, it is tempting to conclude that all these activities are induced by a single epitope or by closely related epitopes on the rmTNF-α molecule. However, binding competition experiments between the neutralizing 1F3F3 and the non-neutralizing 15B6H2 mAb indicated that the 1F3F3 mAb could efficiently inhibit the binding of the non-neutralizing 15B6H2 mAb to the polystyrene-coated rmTNF-α (data not shown). Such observations might indicate that binding of 1F3F3 on mTNF-α induces a conformational change in other mTNF-α epitopes, such as the epitope recognized by the 15B6H2 mAb. Hence one cannot exclude that the 1F3F3 interferes with the functional activities of mTNF-α in an indirect way by conformational modification.

Whatever the mechanism of action through which 1F3F3 mediates its neutralizing potential, it is clear that this mAb can be used as a useful tool to probe the activity of TNF-α *in vivo* and *in vitro*.

ACKNOWLEDGMENTS

This work was supported by a grant of the FGWO (number 3.0072.88). P. De Baetselier is a NFWO fellow and R. Lucas is an IWONL fellow.

Special thanks to Dr C. Roelant of the Laboratory of Hematology of the KUL, Leuven, for his inspiring help in the chemiluminescence experiments.

REFERENCES

Bertolini D.R., Nedwin G.E., Bringman T.S., Smith D.D. & Mundy G.R. (1986) Stimulation of bone resorption and inhibition of bone formation *in vitro* in human tumour necrosis factors. *Nature (Lond.)*, **319,** 516.

Boogaerts M.A., Vercelotti G., Roelant C.H., Verwilghen R.L., Jacob H.S. (1983) Importance of cell separation techniques for the study of granulocyte–platelet interactions. *Blood*, **61,** 46.

Bowen B., Steinberg J., Laemmli U.K. & Weintraub H. (1980) The detection of DNA-binding proteins by protein blotting. *Nucleic Acid Res.* **8,** 1.

Carswell E.A., Old L.J., Kassel R.L., Green S., Fiore N. & Williamson B. (1975) An endotoxin-induced serum factor that causes necrosis of tumours. *Proc. natl. Acad. Sci. U.S.A.* **72,** 3666.

Collins T., Lapierre L.A., Fiers W., Strominger J.L. & Pober J.S. (1986) Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A, B, antigens in vascular endothelial cells and dermatofibroblasts *in vitro*. *Proc natl. Acad. Sci. U.S.A.* **83,** 466.

Kauhansky K., Brudy V.C., Harlan J.M. & Adamson J.W. (1988) Tumor necrosis factor-α and tumor necrosis factor-β stimulate the production of granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and IL-1 *in vivo*. *J. Immunol.* **141,** 3410.

Kirstein M. & Baglioni C. (1988) Tumor necrosis factor stimulates proliferation of human osteosarcoma cells and accumulation of c-myc messenger RNA. *J. Cell. Physiol.* **134,** 479.

Leibovitch S.J., Polverini P.J., Sheppard H.M., Wiseman D.M., Shively V. & Nuseir N. (1987) Macrophage-induced angiogenesis is mediated by tumour necrosis factor-α. *Nature (Lond.)*, **329,** 630.

Markwell M.A.K. & Fox F.C. (1978) Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochemistry*, **17,** 4807.

Medcalf R.L., Kruithof E.K.O. & Schleuning W.D. (1988) Plasminogen activator inhibitor 1 and 2 are tumor necrosis factor/cachectin-responsive genes. *J. exp. Med.* **168,** 751.

Old L.J. (1987) Tumor necrosis factor: polypeptide mediator network. *Nature (Lond.)*, **168,** 330.

Remels L.M. & De Baetselier P.C. (1987) Characterization of 3LL-tumor variants generated by *in vitro* macrophage-mediate selection. *Int. J. Cancer*, **39,** 343.

Ruff M.R. & Gifford G.E. (1981) Tumor-necrosis factor. *Lymphokines* **2,** 236.

Schreiber R.D., Hicks L.J., Celada A., Buchmeier N.A. & Cray P.W. (1985) Monoclonal antibodies to murine γ-interferon which differentially modulate macrophage activation and antiviral activity. *J. Immunol.* **134,** 1609.

Scuff-Werner P.F., Scheurich P., Gottsmann K., Pfitzmaier K. & Nagel G.A. (1987) MCL-derived chemiluminescence enhancing activity on poly-morphonuclear neutrophils: TNF-α as an active principle. Bioluminescence and chemiluminescence. *Proc. of the IV Internat. Bioluminescence and Chemiluminescence Symposium, Freiburg, B.R.D.*, p. 129. John Wiley & Sons.

Sheenan K.C.F., Ruddle N.H. & Schreiber R.D. (1989) Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. *J. Immunol.* **142,** 3884.

Sugarman B.J., Aggarwal B.B., Hass P.E., Figari I.S., Palladino M.A. & Sheppard H.M. (1985) Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells *in vitro*. *Science*, **230,** 943.

Torti F.M., Dieckmann B., Cerami A. & Ringold G.M. (1985) A macrophage factor inhibits adipocyte gene expression. An *in vitro* model of cachexa. *Science*, **229,** 867.

Tracey K.J., Beutler B., Lowry S.F., Merryweather J., Wolpe S., Milsark I.W. *et al.* (1986) Shock and tissue injury induced by recombinant human cachectin. *Science*, **234,** 470.

Urban J.L., Sheppard H.M., Rothstein I.L., Sugarman B.J. & Schreiber H. (1986) Tumor necrosis factor: a potent effector molecule for tumor cell killing by natural macrophages. *Proc. natl. Acad. Sci. U.S.A.* **83,** 5233.