PREVENTION OF ENDOTOXIC SHOCK IN MICE USING ANTI-TUMOR NECROSIS FACTOR-ALPHA MONOCLONAL ANTIBODY

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Qasim Ayub, M.B.B.S.
Denton, Texas
December, 91

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Mouse tumor necrosis factor-alpha (TNF-α) was prepared by stimulating macrophage cell line RAW 264.7 with lipopolysaccharide (LPS) obtained from *Escherichia coli* strain 055:B5. The protein was purified from culture supernatant by affinity chromatography, using goat anti-human recombinant TNF-α polyclonal antibody coupled to a cyanogen bromide activated Sepharose 4B affinity gel. New Zealand white female rabbits were immunized with mouse TNF-α and a rabbit anti-murine TNF-α monoclonal antibody (mAb) was prepared, using modification of fusion techniques developed by Köhler and Milstein. Neutralizing antibody secreting hybridoma (MRH.3) was selected by L929 cytotoxicity assays and cloned by limiting dilution technique to obtain single cell clones. The selected clone was propagated in cell culture and used to produce ascites in mice primed with pristane. The mAb was purified on protein A columns and characterized as rabbit IgG that neutralized TNF-α but did not cross react with other cytokines or LPS. Intraperitoneal injection of mAb increased the percentage survival of BALB/c mice exposed to a lethal dose of LPS.

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CHAPTER 1

INTRODUCTION TO THE STUDY

Shock is defined as a state of inadequate perfusion of cells and tissues which at first leads to reversible hypoxia, but, if sufficiently protracted or grave, to irreversible cell and organ injury and death (1). Endotoxic, septic or bacterial shock results from infections with Gram-negative endotoxin producing bacteria, such as *Escherichia coli (E. coli), Klebsiella pneumoniae, Shigella, Serratia, Proteus* or *Pseudomonas* (2-4).

Endotoxin or lipopolysaccharide (LPS) is a 'toxin' that was first isolated from the culture filtrate of *Serratia marcescens* in 1943 by Shear *et al* (5). It was described as the active principle of endotoxin that is released on the death of Gram-negative bacteria and that induces hemorrhagic necrosis of tumors. The lipopoly-saccharide complex consists of polysaccharide with a covalently bound phospholipid component, termed lipid A, associated with the outer cell membrane of the Gram-negative bacteria (4, 6). These amphipathic molecules represent the O-antigens and the endotoxins of Gram-negative bacteria.

The polysaccharide consists of a core oligosaccharide and an O-specific chain region. The latter contains ~20-35 repeating oligosaccharide units and is considered important for protection of bacteria from host defenses. The core region has limited structural variability and consists of a branched hexose pentasaccharide outer core and a phosphorylated mannoheptose and 2-keto-3-deoxyoctonate inner core region in the *Salmonella* species (4).

The inner core oligosaccharide and the lipid A have been implicated in the physiological functions of the LPS (4). It has also been shown that LPS affects the host indirectly by eliciting production of host factors, the biological response modifiers or cytokines. The cytokines are polypeptide, protein or glycoprotein molecules produced by stimulated lymphocytes (lymphokines), or macrophages (monokines). Included among these host factors is tumor necrosis factor-alpha (TNF-α), cachectin, or necrosin (5, 7-21).

TNF-α is referred to as a monokine, as it is secreted principally by activated macrophages (5, 22-29). It is a 17 kilodalton (kDa) polypeptide that was discovered as a serum factor that caused necrosis of tumors and was isolated by Old, Carswell and their associates from serum of mice primed

with viable *Mycobacterium bovis* strain BCG (Bacillus Calmette-Guerin) and challenged with bacterial endotoxin (10, 20, 30). A historical background of TNF-α is presented in Table I.

TNF-α makes up to 1 to 3% of the protein content secreted by the activated macrophages, indicating its importance *in vivo*. It is considered to play a major role in the regulation of inflammation (31-36), tissue repair (37-39) and host immunity in response to parasitic (40-46), fungal, bacterial (18, 47-50) and viral (51) infections. Besides its protective function the protein is also one of the major mediators of endotoxin shock and has been shown to cause chronic wasting or cachexia (16, 52).

The amino acid sequences of mouse and human TNF-α are known. Human TNF-α is secreted as an unglycosylated 157 amino acid polypeptide, whereas mouse protein is considered to be glycoprotein in nature containing a potential N-glycosylation site at asparagine-7 (Asn-Ser-Ser) (22). In fact murine TNF-α has been shown to contain sialic acid and galactosamine. In addition both human and mouse TNF-α contain two cysteine residues at position 69 and 100/101 that may act as sites for disulfide linkages.

TABLE I

Tumor necrosis factor-alpha (TNF-α)

Historical Background

W. B. Coley ___ 1893 (5)

Treatment of human cancer with bacterial 'toxins'

M. Shear *et al.* ___ 1943 (4, 5)

Isolation of LPS from *Serratia marcescens*

E. A. Carswell, L. J. Old *et al.* ___ 1975 (10, 30)

Discovery of TNF-α and the demonstration of anti-tumor effects of BCG primed mouse serum

S. Green *et al.* ___ 1976 (5)

Partial purification of murine TNF

B. Beutler, A. Cerami *et al.* ___ 1985 (53)

Purification of murine cachectin

B. B. Aggarwal *et al.* ___ 1985 (54)

Purification and characterization of human TNF-α

D. Pennica *et al.* ___ 1985 (55)

Cloning and expression of gene coding for murine TNF-α

T. Shirai *et al.* ___ 1985 (56)

A. M. Wang *et al.* ___ 1985 (5)

Cloning and expression of gene coding for human TNF-α

B. Beutler, A. Cerami *et al.* ___ 1985 (57)

Identification of TNF-α with cachectin

Both human and mouse TNF-α are synthesized as precursor (223 amino acids for human) molecules that are cleaved to give the mature protein (157 amino acids in humans 156 in mice). The human and murine TNF-α molecules are highly conserved showing 79% homology between the precursor polypeptides (22, 58). The N-terminal amino acid sequence that is cleaved is conserved in both species indicating that it is involved in secretion of TNF-α. This is borne out by the fact that both peptides have a long hydrophobic sequence that could serve as a transmembraneous domain.

TNF-α shows 28% homology with the lymphokine TNF-ß (lymphotoxin), and the two cytokines have similar biological and physical properties (Table II). The genes for TNF-α and TNF-ß are closely linked and lie within the major histo-compatibility complex (MHC) proximal to the D locus on chromosome 17 in mice (59, 60). In humans the genes are arranged in tandem between the D related and A human leukocyte antigens (HLA-DR and HLA-A respectively). This location points to the important physiological role these proteins play in host immune regulation and surveillance (61, 62).

TABLE II
Comparison of TNF-α and lymphotoxin (TNF-ß)

TNF-α and TNF-ß are two closely related cytokines with similar functions. Both lyse L929 target cells, necrotize a variety of human tumors by intravenous, intramuscular, intraperitoneal or intralesional administration and synergize with interferon-gamma.

TNF-α (Cachectin)	**TNF-ß (Lymphotoxin)**
Molecular weight (native protein): 17 kDa	25 kDa
Isoelectric point: 5.8	5.3
Source: Predominantly monocytes or macrophages, natural killer and monocytic leukemic cells	Predominantly lymphocytes, human myeloma cells and B lymphoblastoid cells
Amino Acid Sequence:	It is 35% identical and has 51% homology with TNF-α
156 (human) 157 (murine) Valine amino terminal and two cysteine residues (amino acids 69 and 101)	155 (human) 171 (murine) Leucine or histidine N-terminal and no cysteine residues
Glycosylation: Human TNF-α is not glycosylated but murine may be	It is usually glycosylated
Gene: The human TNF-α gene is located on chromosome 6, between HLA-DR and HLA-A, and has 3 introns and a 3′ AT rich region.	The human TNF-ß gene is also located on chromosome 6 and is similar in structure.

TNF-α biosynthesis appears to be controlled at both the transcriptional and post-transcriptional levels (63, 64, 65). LPS, phorbol myristate acetate, GM-CSF, IL-2 and IFN-gamma are all known to induce TNF-α production (5, 24, 66). Ligands, like LPS, that bind Ia MHC molecules, induce macrophage/monocyte TNF-α secretion probably by a pathway involving protein kinase C (PKC) and protein tyrosine kinase (PTK) (67). TNF-α synthesis is inhibited by gluco-corticoids, *p*-toluenesulfonyl-L-arginine methyl esters (TAME) (68) and activated protein C (APC) by mechanisms not entirely clear.

TNF-α is detected *in vitro* by its cytotoxic actions on murine L929 cells and *in vivo* tumor necrosis activity in Meth A sarcoma tumor model (5). The *in vitro* biological assay is dependent on TNF-α killing of a murine target cell line sensitized by treatment with actinomycin D or mitomycin C (69). Various modifications of this assay exist (70-75) as do enzyme immunoassays and radioimmunoassays (76) for measurement of TNF-α.

At the molecular level TNF-α is thought to be active in multimeric (possibly trimeric) form (77-79). It exists essentially as a ß sandwich formed by two antiparallel ß-pleated sheets with the hydrophobic residues clustered in the core of the molecule.

The effects of TNF-α are thought to be receptor mediated (80, 81) and antibodies against the soluble form of TNF receptor exhibit TNF like activities (82). TNF-α and TNF-ß bind either receptor competitively (83) and N-terminal amino acids are currently considered to be involved in receptor binding (84, 85). The synthesis of TNF receptors appears to be closely regulated (86) and is enhanced by interferon-gamma (IFN-γ) (86-90).

TNF-α receptors have been found on both tumor and normal cells and in the circulation (82, 91, 92). Current research favors two types of TNF receptors, type A and type B (83-103). Type A receptors have been identified on many myeloid cell lines like HL60 and U937. Type B receptors are found on cells of epithelial origin. Some cell lines like HL60 have both receptor types (103). Type A receptor binding leads to a 100 kDa and the type B to a major 75 kDa and a minor 95 kDa band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It has been shown that the 75-kDa band contains the cross-linked TNF-α monomer and the 95-kDa cross-linked TNF-α dimer (104).

Receptor binding leads to quick activation of a transcription factor, the nuclear factor *k*B (NF-*k*B), via a pathway that is not mediated by adenosine-3′,5′-monophosphate (cyclic AMP) or protein kinase C (105, 106).

Maximal induction of the transcription factor requires only a small fraction (20-25%) of TNF receptors to be occupied (105). In addition TNF-α also induces phosphorylation of a messenger ribonucleic acid cap binding protein (mRNA CBP), the eukaryotic initiation factor 4E. These factors bind the 7-methylguanosine cap structure found on 5′ end of all eukaryotic mRNAs and help initiate protein synthesis (107).

TNF-α is a pleiotropic cytokine with a wide range of *in vivo* and *in vitro* functions (5, 13, 17, 20-24, 30-39, 52) (Table III and Table IV). It has also been implicated in many diseases (40-51) (Table V).

Some of the effects of TNF-α are synergistic with IFN-γ (58, 108) and other cytokines like interleukin-1 (IL-1), interleukin-6 and interleukin-8 (IL-8). The relationship between TNF-α and granulocyte colony stimulating factor and granulocyte-macrophage colony stimulating factors is unclear (109, 110), but all these cytokines seem to be part of a network that work in cohesion to regulate the immune response.

TNF-α is cytotoxic for tumor cells *in vitro* and *in vivo* without killing normal cells. (5, 24,36, 80, 111-114). Some species specificity has been observed for the cytotoxic activities of human and murine TNF-α, each being more cytotoxic on cell lines from homologous species (104).

TABLE III

In vitro functions of TNF-α

General Effects
Suppression of lipoprotein lipase (LPL)
Cytolysis of transformed and/or tumor cell lines (cervical, lung cancer and malignant melanoma)
On endothelial cells
Augmentation of MHC class I antigen expression
Augmentation of ICAM-1 expression that helps in binding of leukocytes to endothelial surfaces
Promotes secretion of IL-1
Down regulation of thrombomodulin expression
On neutrophils
Enhances hydrogen peroxide and superoxide anion production
Increases phagocytosis and ability to kill certain organisms
Causes degranulation
Stimulates adhesion to endothelial cell monolayers
On adipocytes
Suppression of LPL,
Suppression of glycerophosphate dehydrogenase (a fatty acid binding protein)
Suppression of adipsin, a serine protease
Activation of hormone sensitive lipase

TABLE IV

In vivo functions of TNF-α

Hemorrhagic necrosis of tumors
Weight loss, cachexia and profound wasting
Leukopenia
Fever
Wound healing
Induction of reactive oxidant species (ROS)

TABLE V

Diseases associated with increased serum TNF-α

Infectious Diseases
Bacterial
Gram-negative septicemia
Meningococcal septicemia
Parasitic
Malaria (including cerebral malaria)
Trypanosomiasis
Viral
Acquired immunodeficiency syndrome (AIDS)
Autoimmune Diseases
Rheumatoid arthritis
IgG immune complex lung injury
Miscellaneous Diseases
Adult respiratory distress syndrome
Alcoholic hepatitis

The histidine residue at position 15, arginine at position 32 and alanine at positions 14 and 84 have been shown to play an important role in the antitumor and receptor binding activity of TNF (84, 85). However, it is still unclear whether or not receptor binding is necessary for cytotoxicity (115-119).

Although TNF-α has promising non-specific anti-tumor activity its clinical use has been limited because of serious side effects. Most subjects are unable to tolerate the 400 μg/kg doses that are necessary in human carcinoma therapy. Additionally the molecule has a very short biological half life (6 minutes) and is taken up by TNF-α receptors on neutrophils (120, 121). Recent research has focused on gene transfer therapy utilizing the TNF-α gene (114) and the development of mutant TNF peptides that have increased receptor binding and anti-tumor activity with little or no side effects for the patient (84, 85).

The Food and Drug Administration (FDA) recently approved gene therapy for cancer employing the TNF-α gene. The FDA approved protocol was developed by Dr. Steven A. Rosenberg, at the National Cancer Institute (NCI), and is the first therapeutic gene transfer utilized for the treatment of cancer. The aim is to use activated tumor-infiltrating lymphocytes (TIL) as carriers for the TNF-α gene. These cells are isolated from the patient's tumors,

are activated by interleukin-2 (IL-2) and exposed to re-engineered harmless mouse leukemia retroviruses, that have been endowed through DNA recombinant techniques with the human gene for TNF-α. It is hoped that TNF-α will improve the therapeutic efficacy demonstrated by TILs alone in metastatic malignant melanoma. It is expected that using the TIL delivery method would be much safer than administering TNF-α directly into the circulation. Additionally, it would also allow a relatively large amount of TNF to be delivered directly to a cancer site (122).

Studies have shown TNF-α to be one of the primary endogenous mediators of the lethal effects of endotoxin and it evokes changes that essentially duplicate the pathological effects of endotoxin administration (7). When injected in large amounts TNF-α causes a fatal state of shock with all the signs of acute Gram-negative septicemia including hypotension, profound metabolic acidosis, biphasic changes in blood glucose concentration (an initial phase of hyperglycemia followed by a late phase of profound hypoglycemia), hemoconcentration, hyperkalemia, disseminated intravascular coagulation (DIC), hemorrhage and necrosis (1, 3, 4, 22). Tissue damage includes mesenteric ischemia, acute renal tubular necrosis, pancreatic and adrenal hemorrhage and interstitial pneumonitis associated with pulmonary vascular leukostasis (123-128).

Some of these effects may be produced by TNF-α induced release of Platelet-activating Factor (PAF), prostaglandins and other endogenous inflammatory mediators, like interleukin-1 (129, 130). Toxic oxygen derived radicals released by TNF-primed macrophages may also play a role in the cellular damage.

PAF, an endogenous phospholipid, is thought to produce its ischemic bowel necrosis effect by activation of the complement system as C5 deficient (B10.D2/OSNJ, C5D) mice are protected from endotoxin and TNF-α induced shock (129). These effects are also prevented by PAF antagonists (SRI 63-019). Some studies have shown that dextran sulfate inhibits the PAF mediated pulmonary hemodynamic response to TNF (129).

The role of TNF-α in endotoxin shock has been suggested by both *in vitro* and *in vivo* studies (7-9, 13, 16, 17, 19). *In vitro* it has been shown that LPS stimulates monocytes, macrophages and certain other cell lines to produce TNF-α (9, 131). The lipid A component of LPS has been implicated in eliciting this response (132-135).

In vivo it was found that endotoxin sensitive strains of mice (C3H/HeN) exhibited TNF-α secretion and lipoprotein lipase (LPL) suppression when injected with LPS while endotoxin resistant strains (C3H/HeJ) did not (136, 137). After injection of a lethal dose of 20 mg/kg of LPS into

C3H/HeN mice, intravenously, the circulating TNF-α rises rapidly and reaches a maximum within 2 hours and then rapidly declines to almost undetectable levels at 4-5 hours (8, 9, 24). The endotoxin resistant mice can, however, develop LPL suppression when injected with serum derived from endotoxin sensitive mice (20).

Transplantation studies done by Kawakami and Cerami demonstrated that bone marrow from endotoxin sensitive C3H/HeN mice infused into endotoxin resistant C3H/HeJ strains leads to endotoxin sensitivity in the latter. Further experiments revealed that the macrophages from C3H/HeJ or endotoxin resistant mice do not produce TNF-α, and that they are more susceptible to Gram-negative infection than normal mice (8, 9, 11, 13, 22).

In vivo injection of LPS induces TNF-α production and the cytokine can be detected in the serum minutes after LPS treatment (138-140) and the serum levels of the peptide correlate with the severity of the septicemic shock (141). The serum concentration of TNF-α in rabbits in endotoxin shock has been found to be approximately 0.3 micromolar (13, 23, 142-144). Administration of TNF-α in quantities similar to those produced endogenously in response to endotoxin, cause the same effects as observed in endotoxin shock or following LPS administration. After injection TNF-α appears in circulation in about a minute and reaches a peak level in

about 2 hours after which there is a rapid decline in its concentration. The half-life of the hormone is about 10 to 30 minutes (7).

Galactosamine and lead acetate have been found to lower the LD_{50} of LPS by five orders of magnitude and to enhance the sensitivity of mice to TNF-α (145, 146). Subcutaneous injection of LPS can 'prepare' normal skin to become sensitive to the induction of hemorrhagic necrosis by TNF-α injected at the same site after 24 hours. Similarly *in vitro* LPS treated fibroblasts can cause hemorrhagic necrosis *in vivo* when injected together with TNF-α (11).

Beutler and his colleagues found that mice passively immunized against TNF-α were protected against the lethal effects of endotoxin or LPS (147). Similar results were obtained by use of polyclonal antibodies in other animals (148, 149). Intravenous injection of antibody to TNF-α in rabbits reduced endotoxin induced fever especially in the latter half of the febrile response (149). Some studies indicated that the effect of anti-TNF-α antibodies may be mediated via reduction of IL-1 production (150, 151). All these studies indicate that LPS is required for induction of TNF-α release and that strong synergism between LPS and TNF-α may be part of a primitive defence mechanism against infections, independent of T and B cell immunity.

To further evaluate the role TNF-α plays in endotoxin shock and to exclude the possibility of other related cytokines causing LPS induced TNF-α actions, it is necessary to study the effect of anti-TNF-α neutralizing antibodies *in vitro* and *in vivo*. Such an antibody would be useful in detecting serum TNF-α levels prior to the onset of systemic shock and it could also be tested in clinical trials for treatment of Gram-negative septicemia.

Several anti-TNF-α anti-sera are available but there are theoretical reservations about these polyclonal sera (152). Polyclonal serum refers to all the immunoglobulin subclasses (IgM, IgG, IgE, IgD and IgA) present in the animal. They lack precise specificity, require high (10-20 mg/kg) doses and high flow venous delivery to be effective. In addition the polyclonal antibodies are not useful for study of domains of complex antigens, as they recognize multiple epitopes and the amount of antibody obtained is dependent on the life of the immunized animal.

These problems with polyclonal antibodies can be overcome by the production of large quantities of homogeneous antibodies of defined specificity (153). Antibodies or immunoglobulins (Ig) that recognize specific antigenic epitopes or antigenic determinants, are homogeneous and are developed from a single clone are called monoclonal antibodies (mAb).

Monoclonal antibodies have revolutionized biological research since the publication of a relatively simple procedure by Milstein and Köhler for which they were awarded the Nobel prize in Physiology and Medicine in 1984 (153-155). These mAb are produced by cellular hybridization of splenocytes from immunized animals with immortal myeloma cells, employing polyethylene glycol (PEG) (155-156). Selection of the hybrid cells is made possible by use of selective hypoxanthine-aminopterin-thymidine (HAT) medium (157). This medium contains hypoxanthine and thymidine together with aminopterin, a folic acid analogue, which blocks *de novo* synthesis of purines and pyrimidines. The spleen cells do not survive for long under tissue culture conditions and the parent myeloma cells are killed by the presence of aminopterin.

Based on a modified Milstein and Köhler procedure, mAb against human recombinant TNF-α were produced at Wadley Institutes (158-161). Since these antibodies were produced by fusion of mouse splenocytes with a mouse myeloma cell line none of the established clones produced a neutralizing antibody against mouse TNF-α. To produce such a neutralizing antibody I proposed the production of a mouse-rabbit hybridoma secreting anti-murine TNF-α monoclonal antibody.

Mouse proteins, as expected, are poor immunogens for mice but produce high titers in other species. In comparison, rabbits produce high titers of high-affinity antibodies when hyperimmunized with most murine soluble antigens (162). In addition rabbits are easier to inject and repeated blood collection from the marginal ear vein is relatively simple. The rabbit antibodies are also easier to characterize as so far no subclasses of rabbit IgG have been described.

As myelomas are unknown in rabbits, a mouse myeloma cell line would be used to perpetuate the antibody producing hybrid. This rabbit-mouse fusion has been attempted previously with limited success (162-164). If successful the aim would be to produce a neutralizing anti-murine TNF-α monoclonal antibody and to test it *in vitro* and in mouse models. The aims of the project are outlined in Table VI.

TABLE VI

Aims of the study

1. Purification of mouse TNF-α
2. Production of anti-TNF-α mAb
3. *In vitro* characterization of mAb
4. Prevention of LPS induced endotoxin shock by mAb

In order to achieve the aims of the project mouse TNF-α would be produced by mitogen stimulation of mouse monocyte-macrophage cell lines (10, 27, 53). The culture supernatants would be collected, pooled and subsequently used to purify murine TNF-α by affinity chromatography, a powerful one-step protein purification technique (165, 166).

In affinity chromatography goat anti-sera against TNF-α, coupled to a suitable matrix, would be employed. Diluted culture supernatant would be passed through an affinity column to allow binding of TNF-α to the antibody ligand. Finally the TNF-α would be nonspecifically eluted by change in buffer salt concentration and pH.

Affinity purified TNF-α would be used to hyperimmunize rabbits, and rabbit anti-murine TNF-α mAb would be prepared using conventional hybridoma fusion techniques. Antibody secreting clones will be identified by biological cytotoxicity assays (73, 75) and enzyme linked immunosorbent assays (ELISA) (167). TNF-α neutralizing, antibody secreting, hybrid cells would be selected and cloned by limiting dilution, and propagated in culture or by induction of ascites in pristane treated mice (168). The antibody would be purified by protein A affinity chromatography (165) and characterized *in vitro* employing TNF-α biological cytotoxicity assays (73), immunoprecipitation (169), Ouchterlony (170), ELISA and radioimmunoassays (167, 171).

The mAb would then be utilized in BALB/c mouse models to test for prevention of LPS induced endotoxin shock. If successful such experiments would indicate that TNF-α is one of the major mediators of LPS induced shock. Anti-TNF-α antibody may also be used in detecting serum TNF-α levels prior to onset of systemic shock and anti-human TNF-α antibodies could also be tested in clinical trials for treatment of Gram-negative septicemia, opening new avenues for treatment of septicemic shock.

CHAPTER 2

MATERIALS AND METHODS

Cell Lines. Cell lines used in the study were purchased from American Type Culture Collection (ATCC, Rockville, MD). Mouse monocyte-macrophage cell lines PU5-1.8 (PU5-1R, ATCC TIB 61), obtained from a spontaneous BALB/c mouse lymphoid tumor, and RAW 264.7 (ATCC TIB 71), established from ascites induced in male mice by injection of Abelson leukemia virus (A-MuLV), were used for the production of TNF-α. Both cell lines have detectable amounts of lysozyme and are capable of antibody-dependent lysis of both sheep erythrocytes and tumor targets.

NCTC clone 929 strain L (L929, ATCC CCL 1), a mouse fibroblast cell line, was used in TNF-α cytotoxicity assays. L929 was one of the first cloned cell strain to be established in continuous culture. The parent L strain was derived from normal subcutaneous areolar and adipose tissue of a 100 day old male C3H/An mouse.

A non-secreting mouse myeloma cell line Sp2/0-Ag14 (ATCC CRL 1581) was employed as fusion partner during production of monoclonal antibodies. The cell is a clone of Sp2/HL-Ag cell line derived from a hybrid between a BALB/c spleen

cell, with anti-sheep red cell activity, and a myeloma cell. Sp2/0-Ag14 does not synthesize or secrete any immunoglobulin chains and is resistant to 8-azaguanine at 20 μg/ml and does not survive in HAT containing medium.

WISH (ATCC CCL 25) an epithelial cell line derived from human amnion tissue was used in interferon cell cytotoxicity assays. These cells support the growth of vesicular stomatitis (Indiana strain) virus (VSV) that is employed in the assay.

Cytokines. Human recombinant TNF-α (Wadley Institutes of Molecular Medicine, Dallas, TX) was used as a control and standard solution in the cytotoxicity assay. Human TNF-α cDNA was constructed from Sendai virus induced human peripheral blood lymphocytes (PBL). The cDNA library was screened with a 17 base TNF probe and a clone encoding the mature protein was selected for expression in an *E. coli* expression vector. The nucleotide sequence of mature TNF-α cDNA clone isolated from human PBLs was identical with the cDNA isolated from the HL-60 cell line and a genomic TNF-α clone. The expression vector contained a Shine-Dalgarno sequence, a P_L promoter and a gene for a temperature sensitive cI repressor. The TNF cDNA contained in this vector was induced at 42°C to generate a 17,300 dalton polypepetide.

The TNF-α was purified by affinity chromatography using mouse anti-human recombinant TNF-α mAb column. The cytokine was 95% pure as determined by SDS-PAGE. The human recombinant TNF-α had a specific activity of 1.5 X 10^7 units/mg of protein measured on 24 and 72 hours L929 bioassays. This TNF-α standard was compared with recombinant human TNF-α (1 X 10^7 units/mg) obtained from Amgen Biologicals (Thousand Oaks, CA).

Other cytokines employed included recombinant mouse TNF-α, recombinant human granulocyte colony stimulating factor (G-CSF), recombinant human macrophage colony stimulating factor (M-CSF), recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF), recombinant human interleukin-3 (Multi-CSF, IL-3), recombinant human ß-Interleukin-1 (p17.0) (Genzyme, Boston, MI), recombinant human interleukin-2 (Hoffmann La-Roche Inc., Nutley, NJ) human leukocyte α-interferon, lymphotoxin (TNF-ß) (Wadley Institutes of Molecular Medicine, Dallas, TX) and interferon-gamma (IFN-τ) (Immuno Modulators, Stafford, TX).

Antibodies. Goat anti-human recombinant TNF-α polyclonal antibody (Wadley Institutes, Dallas, TX) was used for purification of mouse TNF-α by affinity chromatography. Mouse mAb against recombinant human TNF-α (WI-AT-1 and WI-AT-4), produced at the institute, were employed as

controls and for purification of human recombinant TNF-α. Other mouse antibodies (30.10.E and WI-MN-1) produced at Wadley Institutes were used as controls in animal studies. Heavy and light chains specific goat anti-mouse IgG, sheep anti-rabbit IgG, rabbit anti-goat IgG and peroxidase conjugated, affinity purified, goat anti-mouse IgG were used in enzyme linked immunosorbent (ELISA) and immuno-precipitation assays (Cappel Organon Teknika Corp., West Chester, PA). Mouse whole serum, used as carrier in immunoprecipitation assays, was also obtained from the same source. Peroxidase labeled goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) was utilized in ELISA assays employing rabbit IgG.

Mitogens. *E. coli* Serotype 055:B5 trichloroacetic acid extracted LPS (Sigma Chemical Co., St. Louis, MO) was used for *in vitro* production of mouse TNF-α and for *in vivo* studies of LPS induced endotoxic shock in animal models. Purified phytohaemagglutinin (PHA) (Wellcome Research Lab., Beckenham, England), Concanavalin A (Con A) and phorbol 12-myristate 13-acetate (PMA/TPA) (Sigma Chemical Company, St. Louis, MO) were used in conjunction with LPS to study induction of murine TNF-α by adherent cell lines. *Salmonella typhimurium* mitogen (RIBI Immunochem Research, Hamilton, MO) was used as a B-cell stimulant to promote growth of mouse-rabbit hybridoma.

Buffers. The chemical compounds used in the preparation of buffers were all purchased from Sigma Chemical Company, unless stated otherwise. The buffers were all prepared with double-distilled, deionized water (Milli-Q water purification system, Millipore Corporation, Bedford, MA). The pH was monitored using Fisher Accumet pH meter, model 260 with Accu-pHast electrode (Fisher Scientific Company, Fair Lawn, NJ). All buffers were filtered through sterile, 0.22 μm bottle top filters (Becton Dickinson Labware, Lincoln Park, NJ) into autoclaved glass bottles and stored at 4°C or room temperature (172).

Phosphate-buffered saline (PBS) pH 7.2-7.4 was prepared from a ten times concentrated (10X) autoclaved stock solution containing 20.5 g monobasic, anhydrous sodium phosphate (NaH_2PO_4), 95.5 g dibasic, anhydrous sodium phosphate (Na_2HPO_4) and 701.3 g sodium chloride (NaCl) in 8 liters of double-distilled water. The stock solution was diluted 1:10 and filtered prior to use to give a final buffer of 0.01 M phosphate and 0.15 M NaCl.

A 0.05 M phosphate buffer, pH 7.5 was used in the radio-iodination of TNF-α by lactoperoxidase method. It was prepared by dissolving separately 8.9 g Na_2HPO_4 in 1 liter and 1.95 g NaH_2PO_4 in 200 ml distilled water and adjusting the pH by adding the latter to the former.

A 1 M stock solution of N-2 hydroxy ethyl piperazine-N'-2 ethane sulfonic acid (HEPES) was diluted in Roswell Park Memorial Institute (RPMI) 1640 culture medium to give the desired concentration (2-25 mM) of the buffer.

Sorenson's buffer, used in L929 assays, was prepared by mixing solution A (61.2 ml) and solution B (38.8 ml) in 95% ethanol (100 ml) (Quantum Chemical Corporation, Pittsburg, PA). Solution A was prepared by adding 21 g citric acid (Matheson, Coleman and Bell, Norwood, OH) to 200 ml 1 N sodium hydroxide (Fisher) and bringing the volume up to 1 liter with water. Solution B contained 10 ml 0.1 N hydrochloric acid (HCl) in 120 ml water.

Stock solutions for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared as follows and stored at 4°C. The acrylamide stock solution contained 30% w/v acrylamide and 0.8% w/v N, N'-methylene-bis-acrylamide (Bio-Rad, Richmond, CA) in water. The upper (stacking) gel stock solution, pH 6.8, contained 6.06g tris(hydroxymethyl) aminomethane (tris base) and 0.40 g SDS (Bio-Rad) in 100 ml double-distilled water to give a final stock solution containing 0.5 M tris base and 0.4% w/v SDS. The lower (resolving) gel buffer stock solution, pH 8.8, contained 18.17 g tris base and 0.40 g SDS in 100 ml double-distilled water to give a final stock solution containing

0.5 M tris base and 0.4% w/v SDS. The chamber or electrode buffer, pH 8.3, contained 0.025 M tris base, 0.192 M glycine and 0.1% w/v SDS. The sample buffer, pH 6.8, contained 10% w/v sucrose (Fisher), in order to underlay the sample in the well without mixing, 5% w/v 2-mercaptoethanol (2-ME) (J. T. Baker Chemical Company, Phillipsburg, NJ), to disrupt disulfide linkage, 2% w/v SDS, 0.625 M tris base and 0.001% w/v bromophenol blue (Matheson Coleman and Bell) as dye front. The gel fixing solution contained 17.3 g sulphosalicylic acid and 57.5 g trichloroacetic acid (Fisher) in 500 ml distilled water and the destaining solution contained 500 ml ethanol and 160 ml acetic acid made upto 2 liters with distilled water. The staining solution contained 0.460 g Coomassie brilliant blue R250 (Bio-Rad) in 400 ml destaining solution (500 ml ethanol with 160 ml acetic acid in 2l distilled water). The solution was filtered through Whatman #1 filter paper (Whatman Limited, England) before use. Some gels were stained using Bio-Rad silver stain kit.

Stock solutions for Western (immuno-) blotting and the subsequent immunological detection of transferred proteins included the transfer buffer, tris buffered saline (TBS), and Tween-PBS. The transfer buffer, pH 8.3, contained 25 mM tris base, 192 mM glycine and 20% v/v methanol (Fisher) in double-distilled water. The TBS stock solution, pH 7.6,

contained 100 mM tris base and 2.5 mM NaCl. This stock solution was diluted 1:5 in distilled water to give a final TBS solution containing 20 mM Tris and 0.5 M NaCl. A 0.5% v/v Tween-20 (Bio-Rad) solution in PBS was used for blocking and washing the blotting paper and for antibody dilution.

For trypsinization a solution containing 0.1% trypsin (Difco Laboratories, Detroit, MI) and 0.3% ethylene diamine tetraacetic acid (EDTA) was prepared. The solution was filtered into sterile, non-pyrogenic evacuated containers (Travenol Laboratory Incorporated, Deerfield, IL) and stored at 4°C.

Culture Medium. RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 5-15% v/v heat inactivated (56°C, 0.5 h) fetal calf/bovine serum, FCS, (Ultraserum, Salzman Corp., Davenport, IO; or J. R. Scientific, Woodland, CA), 1 mM L-glutamine (Flow Laboratory Incorporated, McLean, VA), 2 mM sodium pyruvate (Hazleton Biologics Inc., St. Lenexa, KS), 5-20 mM hepes and 0.1% v/v gentamicin (Flow) was used for all cell cultures. For washing cells prior to stimulation with mitogens Hank's balanced salt solution (HBSS) was used (GIBCO).

Supplemented Dulbecco's modified Eagle's medium (DMEM) (Sigma) was utilized for hybridoma production and propagation. It was also used in the preparation of the

selective hypoxanthine-aminopterin-thymidine (HAT) and hypoxanthine-thymidine (HT) medium. HAT medium contains hypoxanthine and thymidine together with aminopterin, a folic acid analogue, which blocks *de novo* synthesis of purines and pyrimidines. Aminopterin blocks dihydrofolate reductase, an enzyme that promotes the reduction of dihydrofolate to tetrahydrofolate using NADPH as the reductant. This in turn blocks pyrimidine synthesis by inhibiting thymidylate synthase, an enzyme that converts deoxyuridine 5′-monophosphate (dUMP) to deoxythymidine 5′-monophosphate (dTMP). The methyl donor in this reaction is a folic acid derivative (N^5, N^{10}-methylenetetrahydrofolate) that also serves as an electron donor. Purine biosynthesis is blocked by an activated folic acid derivative, N^{10}-formyltetrahydrofolate, that furnishes the C-2 and C-8 parts of the purine nucleotides. The conversion of 5′-phosphoribosyl-glycinamide to 5′-phosphoribosyl-N-formylglycinamide is inhibited as is the latter conversion of 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole to 5′-phosphoribosyl-4-carboxamide-5-formamidoimidazole.

Supplemented DMEM contained glucose (4.5 g/liter), sodium bicarbonate (2.8 g/liter), hepes buffer (5-25 mM) L-glutamine (2 mM), sodium pyruvate (1 mM), gentamicin (0.1% v/v), and the appropriate percentage of heat inactivated FCS

(5-10% v/v) and/or normal rabbit serum, NRS (Sigma), (7.5% or 15% v/v). HT medium consisted of supplemented DMEM containing hypoxanthine (1 X 10^{-4} M) and thymidine (1.6 X 10^{-5} M). HAT medium consisted of HT supplemented DMEM containing 15% NRS or 15% FCS and 4 X 10^{-7} M aminopterin, a folic acid analogue. Stock solutions of HT and HAT were obtained from GIBCO or Hazleton.

Animals. Determination of the LD_{50} for LPS (the dose that is lethal to 50% of the animals) and the study of the suppression of endotoxin shock, using monoclonal antibodies, was carried out in BALB/c mice. Female BALB/c mice (Harlan Sprague Dawley, Indianiapolis, IN) were used in all experiments. Additional mice were bred at the Wadley Institute animal facilities. Three white New Zealand female rabbits (Kings Wheel Rabbitry, Mt. Vernon, OH) were immunized with mouse TNF-α and used for generating monoclonal and polyclonal antibodies. All animals were housed in an air conditioned room on a light dark schedule, (illumination 7.00 a.m. to 7.00 p.m.), with food and water available *ad libitum*.

Cell Cultures. All cell lines were maintained in supplemented RPMI 1640 in sterile, nonpyrogenic, 75 cm^2 polystyrene tissue culture flasks (Corning Glass Works, Corning, NY) and kept in a humidified Steri-cult 200

incubator (Forma Scientific, Marietta, OH) at 37°C in 4-5% carbon dioxide (CO_2) atmosphere (173). Some hybridoma cell lines were maintained in 75 cm^2 vented culture flasks (Costar, Cambridge, MA) with 0.2 μm vented filter cap. The cells were harvested and subcultured twice a week at a density of 1 X 10^4 to 1 X 10^5 cells/ml. Generally adherent cell lines were grown in 5% FCS and nonadherent cells in 10% FCS. Some L929 and WISH cell cultures were grown in medium containing 5% FCS and 5% horse serum (GIBCO). Hybridoma cultures were initially maintained in supplemented DMEM containing 15% rabbit serum, subsequently in supplemented DMEM containing 7.5% rabbit and 7.5% FCS and eventually in RPMI 1640 supplemented with 10% FCS.

Cell Harvesting. Adherent cells were harvested by trypsinization, or scrapping, and centrifugation, and non-adherent cells by centrifugation. Adherent cell lines PU5-1R and RAW 264.7 were harvested by treatment with 3 mM solution of EDTA in PBS, pH 7.2-7.4, or by use of disposable cell scrapers (Costar).

Trypsinization of adherent cells (L929 and WISH) was carried out by adding 5 ml prewarmed trypsin solution, filtered through 0.22 μm syringe filter (Corning), to confluent cell cultures. After 3-5 minute incubation the trypsin was diluted by adding 5-10 ml of supplemented RPMI

1640 and the cell suspension was transferred into disposable, sterile 15 ml modified polystyrene, or 50 ml polypropylene, plug seal cap, centrifuge tubes (Corning). The tubes were balanced and centrifuged at 2000-3000 rpm for 5-10 minutes in a IEC Centra-7 centrifuge (International Equipment Company, Needham Heights, MA). The supernatant was discarded by vacuum aspiration, using a Pasteur pipette, and the cell pellet resuspended in 2-5 ml supplemented RPMI 1640, and subcultured at a density of 10^4 to 10^5 cells/ml.

Nonadherent cell lines and hybridomas were harvested by transfer of culture flask contents into centrifuge tubes and centrifugation at 2000-3000 rpm for 2-5 minutes to collect the cell pellet. All tissue culture work was performed under sterile conditions in a BBL biological cabinet (Becton Dickinson, Mountain View, CA).

Cell Counting. Cell suspensions in supplemented RPMI 1640 were appropriately diluted in 0.9% saline (usually 1:10) and counted in the white cell count area of a 0.1 mm deep hemacytometer or Neubauer chamber (American Optical, Buffalo, NY) using trypan blue exclusion to distinguish between viable and nonviable cells. Viable cells exclude the dye and can be differentiated from the blue-stained nonviable cells. An equal amount of 0.4% trypan blue stain (GIBCO) was added to the diluted suspension and using a

Gilson Pipetman (Rainin instrument Company, Emeryville, CA) the cell suspension was loaded under the coverslip taking care that the hemacytometer grid area was not over filled. After staining the count was performed within 3 minutes, as after that time the viable cells also take up the dye. The viable cell count was obtained by the formula:

$$\text{cells/ml} = \text{average viable cells (in large WBC square)} \times 10^4/\text{ml} \times 1/\text{dilution}$$

$$\% \text{ viable cells} = \frac{\text{number of viable cells}}{\text{number viable + number dead cells}} \times 100$$

Cryopreservation of Cells. Cell lines and hybridomas that were to be preserved were harvested and resuspended in RPMI 1640 supplemented with 33% FCS and 10% (v/v) dimethyl sulfoxide, DMSO, (J. C. Sales, Bogota, NJ) at a density of 2 X 10^6 to 6 X 10^6 cells/ml. The cell suspension (1 ml) was transferred into each polypropylene, cryogenic vial (Corning or Nalge Company, Rochester, NY). The tubes were placed in a foam insulated box, allowed to gradually cool overnight in an ultralow (-70°C) freezer (Revco, West Columbia, SC) and finally stored at -196°C in a 35 VHC liquid nitrogen container (Union Carbide Corporation, Danbury, CT).

Frozen cells were recovered by defrosting the tubes in a 37°C waterbath with constant moderate agitation, disinfecting the vial and transferring the contents into a sterile culture flask containing supplemented RPMI 1640. Viability was determined after 24 hours incubation at 37°C.

TNF-α Cytotoxicity Assays. In vitro study of TNF-α activity, immunoprecipitation and neutralization of the antigenic murine and human recombinant TNF-α by anti-TNF-α mAb was carried out by L929 biological cytotoxicity assays (73). Initially 3 day biological cytotoxicity assays were carried out but during the screening of the monoclonal hybridomas a 1 day assay was developed and used in subsequent studies (71).

In the 1 day assay confluent L929 cells were harvested by trypsinization and centrifugation, counted in a hematocytometer, using trypan blue exclusion, and resuspended in supplemented RPMI 1640 (5-10% FCS) at a density of 3 X 10^5 cells/ml. The suspension was transferred into a reagent basin (Flow) and 0.1 ml of cell suspension was added to each well of sterile, flat bottom, 96-well tissue culture plates (Costar) using a Titertek-12 channel pipette (Flow). This gave monolayers of 3 X 10^4 target (L929) cells per well. After allowing 2-4 hours for the cells to adhere to the plate, TNF-α standard and test samples, appropriately diluted with RPMI 1640, were added to the appropriate columns of the plate. The standard was added to column 3, column 1 acting as a monolayer control (100% viability and 0% cytotoxicity) and column 2 as blank (0% viability or 100% cytotoxicity).

Each plate carried its own internal TNF-α standard, consisting of a human recombinant TNF-α preparation of predetermined activity (usually 200 units/ml). This preparation was stored at -70°C. One aliquot was removed on a given day, diluted in supplemented RPMI 1640 (1:4 or 1:8), and used as a standard.

After addition of the standard and test samples a two fold serial dilution of samples and standard was carried out from top to bottom, 0.1 ml of actinomycin D (Sigma) solution (5 μg/ml) was added to each well and the plate incubated overnight at 37°C. Cell viability was checked after 18-24 hours by measuring either neutral red or gentian violet dye uptake. The relative ease and quickness of the latter method made it the method of choice in later assays.

For neutral red staining the spent medium was discarded by inverting and tapping the culture plate and 0.15 ml of pre-warmed 1% w/v neutral red (J. T. Baker) solution was added to each well except those of the second column which were left blank so as to simulate 100% cytotoxicity. The plate was incubated at 37°C for 1 hour after which the dye was drained off and washed with 0.9% physiological saline solution to get rid of excess dye. After air drying the plates, 0.1 ml of Sorenson's buffer was added to each well to extract the dye.

In gentian violet staining, the spent culture medium was discarded and the cells stained for 2-5 minutes with a 0.5% solution of Gram crystal violet (Difco) dissolved in a 1:5 v/v mixture of methanol:water. The staining solution (0.1 ml) was added to each well, except those in the second column. The staining was stopped by decanting the stain from the plates. The plates were washed 2-3 times with distilled water and allowed to dry before spectrophotometry.

After staining the plates were read on a Multiskan Titertek spectrophotometer at 540 nm. The spectrophotometer, with vertical light path, read one column at a time and was interphased with a computer and the data analyzed by a program written in Fortran 4 (Goddard Computer Science Institute, Dallas, TX). The results were stored and displayed immediately on the screen and a hard copy obtained from the printer.

The percent cytotoxicity was determined by calculating the percent reduction in the amount of dye uptake as measured by the optical density. There is a good correlation between sample dilution and percent cytotoxicity (coefficient of correlation =0.98-0.99) and the computer program utilized these parameters in calculating the TNF-α activity (73). The computer transformed the percent cytotoxicity into probit values and converted the sample

dilutions to their natural logarithms. The probit values helped increase the linearity of the curve. A linear regression analysis performed on the data and slope of the curve was calculated and plotted and from the regression curve the natural log of the dilution which gave 50% cytotoxicity was determined. The anti-log of this value represented the TNF-α titer. The titer of the internal standard (Column 3) was determined by the test and this value was corrected to the known titer for the standard (200 units/ml). The correction factor (CF) was calculated for each plate by the following formula.

CF = Standard Titer(Column 3)/exp[(5 - y-intercept)/slope]

This factor was applied to the titers of the test samples to obtain the corrected values. The program excluded regression curves that did not correspond to within 13% and 95% cytotoxicity. In addition any data containing a correction factor of more than 3.0 or less than 0.1 was disregarded.

In the 3 day assay confluent L929 cells were harvested and resuspended at a density of 2 X 10^5 cells/ml in supplemented RPMI 1640 (5-10% FCS), containing mitomycin C (0.5 μg/ml). The suspension was seeded onto 96 well tissue culture plates and incubated at 37°C. After overnight incubation the TNF-α standard and test samples,

appropriately diluted with RPMI 1640, were added to the appropriate columns of the plate, as described above, and a two fold serial dilution of samples and standard was carried out from top to bottom. In experiments utilizing actinomycin D, 0.1 ml of actinomycin D (5 μg/ml) was added to each well after diluting the samples and mitomycin treatment was omitted. Cell viability was checked after 18-24 hours by measuring either neutral red or gentian violet dye uptake as outlined above.

Cytokine Production. Mitogens, substances that evoke significant cellular proliferation on a nonimmune basis, were used to induce TNF-α production (53, 57, 66). In preliminary studies *E. coli* Serotype 055:B5 trichloroacetic acid extracted LPS, PHA, and PMA were used for *in vitro* production of mouse TNF-α by PU5-1.8 and RAW 264.7 cell lines. Each cell suspension, containing 1 X 10^6 cells/ml, was seeded on sterile, disposable, tissue culture treated, flat bottom, polystyrene 6 or 12 well plates (Costar) in supplemented RPMI 1640 (2 ml). The mitogens were added immediately after seeding. For each cell line LPS was added at a final concentration of 0, 1, 10, 25, 50 and 100 μg/ml, and PMA and PHA at a final concentration of 0, 5, 50, 500,

1000 and 2000 ng/ml. The experiments were replicated and the cell cultures were incubated at 37°C. The spent culture medium was collected at different times and tested for TNF activity by L929 cell cytotoxicity assays.

For mass production of crude mouse TNF-α, RAW 264.7 cell line was chosen as it consistently produced high levels of TNF-α on LPS or PMA stimulation. The cell were cultured in 150 cm^2 tissue culture flasks at a density of 1 X 10^5 cell/ml supplemented RPMI 1640. On reaching confluency the spent medium was discarded and the cells washed with HBSS. After 2 hours at 37°C 10-25 μg LPS/ml was added to the cultures and the cultures incubated at 37°C overnight. The culture supernatant was collected the next day, centrifuged at 3000-4000 rpm to get rid of cell debris, pooled and stored at 4°C till purification. The activity of crude TNF-α was tested by TNF cytotoxicity assays.

Affinity Chromatography. Mouse TNF-α produced by LPS stimulation of RAW 264.7 cells was purified using affinity chromatography (165). A column of cyanogen bromide activated sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ) coupled with goat anti-human recombinant TNF-α polyclonal antisera, that cross reacted with mouse TNF-α, was utilized for this purpose.

Cyanogen bromide activated Sepharose 4B was used as the matrix support. The cyanogen bromide reacts with hydroxyl groups on sepharose and converts these to imidocarbonate groups which act as nucleophiles. These activated groups react with primary amino groups of the protein ligand to form isourea linkages. This multipoint attachment prevents hydrolysation of ligand from matrix and the activation procedure also cross links sepharose to enhance its chemical stability.

For coupling the antibody 0.5 g of sepharose 4B was swollen by incubation with 20 ml of 1 mM HCl for 30 minutes and subsequently washed with the same solution (200 ml/g) in order to preserve the activity of the reactive groups which hydrolyze at alkaline pH. After a final wash with coupling buffer the gel was immediately transferred to a solution of the ligand (purified goat anti-human recombinant antisera). The antibody was also dissolved in the 0.1 M sodium bicarbonate coupling buffer, pH 8.3, containing 0.5 M NaCl at a concentration of 5 mg/ml gel. The high NaCl content minimized protein-protein adsorption caused by the polyelectrolyte nature of the proteins. A gel:buffer ratio of 1:2 was used for coupling and the reaction was carried out overnight at 4°C in an end-over-end Hema-tek sample mixer (Miles Incorporated, Elkhart IN). The remaining

active groups were blocked by treating the gel with 0.2 M glycine buffer (pH 8.0), for 2 hours at room temperature, and the excess adsorbed protein removed by washing alternatively with coupling and acetate buffer (0.1 M acetate, 0.5 M NaCl, pH 4.0). This procedure reduced the possibility of free ligand remaining ionically bound to the immobilized ligand. After a final wash with coupling buffer the gel was transferred into a 0.7 cm Bio-Rad Econo-column and stored at 4-8°C, in PBS containing 0.1% (w/v) sodium azide, till use. Murine TNF-α was purified by passing pooled supernatant from LPS stimulated RAW 264.7 cell line through the affinity column. Before passage the supernatant was diluted with an equal volume of starting buffer, pH 7.20 (0.05 M tris, 0.15 M NaCl). After loading the sample, the immobilized ligand was washed with starting buffer (6 ml/ml gel), followed by a wash with washing buffer (0.1 M tris, 0.75 M NaCl, pH 8.5). Finally, the TNF-α was eluted with 0.1 M tris, 0.5 M potassium chloride elution buffer (pH 10.8). The pH of the eluate was adjusted between 7.2-7.6 with 2 N HCl added prior to collection. The protein content of the elution fractions was determined by Bradford's method and the fractions with the highest protein contents were pooled and stored at 4°C. The column was regenerated with starting buffer and stored at 4°C in PBS (0.1% sodium azide).

Protein Quantitation. Quantitation of affinity eluate, crude and purified TNF-α, was done by Bio-Rad's colorimetric assay which is based on a method developed by Bradford (174). The dye reagent contains Serva Blue G in phosphoric acid and methanol. In the presence of proteins the absorbance maximum of the dye in an acidic solution shifts from 495 to 595 nm. The shift is due to stabilization of the anionic form of the dye by hydrophobic and ionic interactions involving arginine, histidine, lysine, tyrosine, tryptophan and phenylalanine residues in the protein.

The assay was carried out in 96 well tissue culture plates. Briefly, 0.05 ml PBS was added to all wells of the plate. An equal amount of the protein solution and bovine serum albumin (Standard II) or bovine gamma globulin (Standard I) standards (Bio-Rad), diluted to 0.8 mg/ml, were added to the top row and a two-fold serial dilution was made. Finally 0.150 ml of diluted dye reagent was added to all wells, including the blank, and the plates were read after 10-15 minutes on a titertek at 595 nm. The absorbance at 595 nm was plotted against the amount of reference standard and the sample protein concentration was computed from the standard curve.

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out in Mini-PROTEAN II dual slab gel apparatus (Bio-Rad) for determination of molecular weights and purity (175). The 15% running gel was usually prepared a day before by assembling the mini-gel casting stands and preparing a running gel containing 1.5 ml/gel lower gel buffer, 3.0 ml/gel acrylamide/bis-acrylamide solution and 1.5 ml water. This mixture was degassed and 5 μl TEMED and 10 μl freshly prepared 10% (w/v) ammonium persulfate solution were added immediately before casting. A glass pipette was used to pour the gel mixture in between the glass plates taking care no leaks occurred and that no air was trapped within the gel. The gel was poured till a centimeter below a point, marked before the start of the procedure, representing the size of the comb. The gel was layered with water to prevent evaporation from the top and in order to make an even interface between the resolving and stacking gels. Once the gel was set (usually 40-60 min) it was kept in at 4°C or used the same day.

The stacking gel was prepared on the day the gel was supposed to be run. It was prepared by adding 0.75 ml upper gel stock buffer, 0.5 ml acrylamide/bis stock solution and 1.75 ml water. The mixture was degassed and 0.003 ml TEMED and 0.025 ml ammonium persulfate were added and the gel

poured on top of the running gel using a glass pipette. The formation of wells was accomplished by use of a comb.

Once the stacking gel was set, the gel sandwiches were removed from the casting stand. The inner cooling core of the minigel was laid flat on a bench top and the gel sandwich was fixed onto it such that the inner glass plate of the gel sandwich faced the cooling core. After loading the gel sandwiches onto the inner cooling core, the core was placed in lower buffer chamber of the Mini-PROTEAN II cell. The upper buffer chamber formed by the inner cooling core and the gel plate sandwiches was filled till the buffer level reached a level halfway between the short and long sandwich plates. The lower chamber was filled with electrode buffer so that at least the bottom 1 cm of the gel sandwich was covered. Air bubbles were removed by using a Pasteur pipette.

The samples and the molecular weight standards (Bio-Rad) to be run were appropriately diluted and mixed with sample buffer and placed in boiling water for 2-5 minutes. On cooling 0.020-0.030 ml of diluted sample was added into the wells under the electrode buffer using a Pipetman and Gel-Well capillary pipette tips (Rainin). The tips were placed 1-2 mm from the well bottom before discharging the sample so as to avoid air bubbles. The lid was placed on top of the lower buffer chamber to fully enclose the cell

and the correct orientation made by matching the colors of the plugs on the lid with the jacks on the inner cooling core. The leads were attached to a power source with proper polarity. With the current and power on full the voltage was set at 80 volts and electrophoresis started. In some cases an initial run with the voltage set at 40 volts was also preformed. In the latter instance the voltage was increased to 80 volts once the samples reached the resolving gel.

Electrophoresis was stopped when the dye front migrated to within 1 cm of the bottom of the gel sandwich. With the electric leads disconnected, the cell lid was removed and the inner cooling core pulled out of the lower chamber, after pouring the upper buffer. The gel sandwich was removed and the gel placed in fixative for staining or in transfer buffer solution for immunoblotting.

Gel Staining. For Coomassie brilliant blue staining the gel was placed in fixative solution (containing 17.3 gm sulpho-salicylic acid and 57.5 gm trichloroacetic acid in 500 ml distilled water) for 0.5-1 hour. Subsequently the gel was stained by immersing the gel in staining solution, that had been pre-heated to 60°C for 10-30 min. The excess stain was removed by immersing the gel in destaining solution overnight with frequent changes at the beginning.

Silver staining was done to detect protein bands of lower concentrations. The Bio-Rad silver staining kit was used for this purpose. The gel was placed in fixative solution containing 40% (v/v) methanol and 10% (v/v) acetic acid for 30 minutes and then transferred into a fixative solution containing 10% (v/v) ethanol and 5% (v/v) acetic acid for 30 minutes, adding fresh fixative after 15 minutes. Next the gel was placed in a freshly prepared oxidizer solution for 3 minutes. The solution contained 20 ml oxidizer concentrate in 180 ml deionized water. The gel was then washed in three changes of deionized water, or left overnight at room temperature in deionized water in order to reduce background staining. Freshly prepared silver reagent was then added to the dish containing the gel. This was prepared by mixing 20 ml of silver reagent concentrate with 180 ml water. The gel was washed with deionized water for a minute and the color developed by adding developer solution. Frequent changes were made in this solution, the first after 30 seconds, the next after 5 minutes. After sufficient color development the reaction was stopped by adding 5% acetic acid stop solution for 5 minutes. The gel was then placed in water till it was dried.

Gel Drying. SDS-PAGE gels were preserved by drying the gels on Bio-Rad gel slab dryer (model 224). The gels were sandwiched between pre-wetted layers of filter paper and

cellophane membrane. After application of vacuum, the heat was turned on and the gels dried for 1-2 hours. Before removal the gels were allowed to cool with the vacuum on, so as to prevent curling.

Isolation of Splenocytes. Mouse spleen cells and thymocytes were employed as feeder cells for mouse-rabbit hybridomas (176, 177). BALB/c mice were killed by cervical dislocation, or CO_2 inhalation, dipped in a beaker containing 70% (v/v) ethanol, to reduce airborne dander and hair, and secured on sterile paper towels placed on a styrofoam surface. A cut was made in the loose skin on the left dorsal side and the skin pulled off to expose the peritoneal wall. The spleen was identified as a reddish, flat tissue overlying the left kidney and bordering the greater curvature of the stomach. After flooding the peritoneal surface with 70% ethanol to remove any loose hair, the peritoneal flap was lifted with sterile toothed forceps and a cut was made around the spleen. The spleen was gently lifted with the forceps and separated from the blood vessels and connective tissue with scissors and blunt dissection and transferred to a Petri dish containing serum free RPMI 1640. The spleen cells were gently teased through a stainless steel grid, using forceps or the plunger of a sterile disposable syringe, and the cell suspension was

transferred into a 15 ml graduated conical tube and centrifuged at 2000-3000 rpm for 5-10 minutes. The cells were washed in serum free RPMI 1640 2-3 times and red cells were removed by lysis with tris-buffered ammonium chloride (0.16 M ammonium chloride, 0.17 M tris). This was accomplished by resuspending 0.1 ml packed spleen cells/ml tris-ammonium chloride, incubating the cell suspension at 4°C for 2-5 minutes, and centrifugation at 3000 rpm for 10 minutes.

Isolation of Thymocytes. BALB/c mice (3-4 weeks old) were killed by CO_2 inhalation, placed on their back, on a styrofoam pad, and immobilized by taping their stretched limbs to the pad. The neck region was washed with 70% ethanol and an incision was made in the midline, extending from the xiphoid process to the submandibular region. The skin edges were reflected laterally and the rib cage was removed on either side of the midline and reflected upwards to expose the thymic lobes. The thymus was lifted gently proceeding from the lower pole towards the upper pole and placed in a Petri dish containing sterile RPMI-1640. The cells were teased with forceps, suspended in culture medium, centrifuged at 3000 rpm for 5-10 minutes, rewashed, counted and resuspended in supplemented RPMI 1640 for use. Approximately 2 X 10^8 thymocytes were obtained per mouse.

Monoclonal Antibody Production. Purified mouse TNF-α was used as antigen for the production of mAb in rabbits (156, 162, 164, 178). Two female New Zealand White rabbits were hyperimmunized with purified murine TNF-α and one with a combination of murine and human recombinant TNF-α.

The rabbits immunized with murine TNF-α received an initial intramuscular injection of 100 μg of the purified protein. The TNF-α (5 X 10^6 units/mg and 1 X 10^6 units/ml) was dissolved in 5 ml PBS containing 0.1% v/v albumin for stabilization and mixed with an equal amount of Complete Freund's Adjuvant (CFA) just prior to injection. The mixture was injected at multiple sites on the back of the animal (179). After 14 days, weekly injections of purified mouse TNF-α (80-240 μg/week) mixed with an equal volume of Incomplete Freund's Adjuvant (IFA) were given subcutaneously till rabbit serum (obtained from blood collected from the marginal ear vein) showed high antiserum titers in L929 biological neutralization assays. Once high antiserum titers were obtained the rabbit was given a final TNF-α booster dose and sacrificed by terminal bleeding three days later.

The rabbit spleen was removed and placed in a petri-dish containing 8-10 ml serum free RPMI-1640. The splenocytes were teased out with sterile forceps and the

cell suspension was transferred from the dish into a graduated conical tube and centrifuged at 2000-3000 rpm for 10-15 minutes. The supernatant was removed and the cells resuspended and washed two more times in serum free RPMI 1640. In the meantime a cell suspension of Sp2/0-Ag14, was prepared in serum free medium.

Rabbit spleen cells (5×10^8) were mixed with 5×10^7 Sp2/0-Ag14 myeloma cells (10:1) in a 50 ml centrifuge tube. The mixture was centrifuged at 3000-3500 rpm for 10-15 minutes, at room temperature, to form a tight cell pellet. The supernatant was removed and the tube placed in a makeshift waterbath at 37°C. Subsequently, 1 ml of pre-warmed 43% w/v polyethylene glycol 4000 (PEG 4000) (Merck, Rahway, NJ) was added to the cell pellet, slowly over a one minute period, gently stirring the pellet with the tip of the pipette. Stirring was continued for another minute to expose cells to PEG while maintaining as much cell contact as possible to promote fusion. Using the same pipette 2 ml of serum free medium was added slowly over 2 minutes to the clumped cells in order to gradually dilute the PEG without lysing the cells. Finally 7-10 ml serum free medium was added to the tube over 2-5 minutes using continuous stirring motions.

The suspension was centrifuged at 3500-4000 rpm for 10 minutes, the supernate discarded and the pellet resuspended in 10 ml supplemented DMEM containing HT and 15% NRS or 15% FCS. The suspension was transferred into a culture flask and an additional 150 ml supplemented medium added to it. The flask was swirled, to suspend the contents, and 0.1 ml of cell suspension (3 X 10^6 cells) was dispensed into each well of sixteen 96-well tissue culture plates, referred to as the master plates. These plates have raised well rims, recessed areas between wells and special lid rings to reduce cross-contamination.

Each master plate contained BALB/c thymocytes and splenocytes feeder cells, mixed in a 1:1 ratio, seeded at 10^6 cells per well. The plates were placed in a humidified sterile culture chamber stored in a 5% CO_2-in-air incubator at 37°C. The day of fusion was referred to as day 0. On day 1, the day after fusion, 0.1 ml of HAT medium was added to each well. The aminopterin in the medium blocked *de novo* nucleotide bio-synthetic pathways forcing the mammalian cells to rely on alternate pathways and exogenous purines (hypoxanthine) and pyrimidines (thymidine) for deoxyribo-nucleic acid (DNA) synthesis. These alternate nucleotide bio-synthetic pathways are termed the salvage pathways. The myeloma cells Sp2/0-Ag14 and NS-1 being deficient in the

enzyme, hypoxanthine-guanine phosphoribosyl transferase (HGPRT), cannot catalyze the formation of inosine monophosphate (IMP/inosinate) from hypoxanthine and 5′-phosphoribosyl-1′-pyrophosphate (PRPP). These cells cannot utilize hypoxanthine from the HAT medium for the synthesis of purines and so they die within a couple days of culture in such medium. The hybrids between the $HGPRT^-$ mouse myeloma cell lines and the normal antibody producing splenic lymphocytes can grow in the HAT selective medium because they contain the enzymes HGPRTase and thymidine kinase. Thymidine kinase enables these cells to take up the exogenous thymidine and convert it into deoxythymidine monophosphate (dTMP) utilizing ATP. There is no positive selection against the growth of normal spleen cells or spleen-spleen cell hybrids in this selection and therefore it is often called half selection. Passive selection takes place because normal spleen cells have a limited growth potential. They do not survive for long under tissue culture conditions and die off by two weeks in culture.

On days 2, 3, 5, 8 and 11 half of the medium was aspirated from each well by Pasteur pipette, attached to a vacuum outlet, and 0.1 ml HAT medium added to each well. After day 11 the culture fluid aspiration and replacement with fresh HAT medium was carried out at intervals of 3-4

days. This was continued till preliminary screening and selection of the required clone was accomplished. Between day 28-42 the remaining cells were cultured in HT medium in order to dilute aminopterin and to prepare them for growth in normal medium. The cultures were fed regularly with half the medium replaced every 3 days till their transfer into other plates. The clones were named according to the alpha numeric codes inscribed on the 96 well plates and subsequently depending on their growth and stability after limiting dilution. Contamination was combatted by rinsing the contaminated well three times with 6 N sodium hydroxide, or by discarding the plate. Between days 14-28, the spent culture supernates were harvested, using individual pippette tips for each culture plate well, and tested undiluted for production of neutralizing antibody using initially ELISA and subsequently L929 bioassays. Spent culture supernates from the second fusion were tested only by L929 bioassays.

After determining which wells were making the antibody of interest the cells were expanded in fresh 96 well tissue culture plates using BALB/c thymocytes and splenocytes as feeder cells. All initial cultures were carried out in HT and normal rabbit serum supplemented DMEM. Selected hybrid cells were subsequently renamed mouse-rabbit hybridoma followed by a number (MRH.#), stored in liquid nitrogen and characterized one at a time.

Once stabilized in 96 well plates the hybrid cells were gradually expanded into 48 (Costar), 24, 12 and finally 6 well tissue culture plates (Corning). Rabbit mAbs for further study were produced by adapting the appropriate MRH lines to grow in DMEM supplemented with HT, 7.5% NRS and 7.5% FCS and finally the MRH cells were weaned onto RPMI 1640 supplemented with 10% FCS and grown in the absence of NRS. Rabbit antibody was purified from these MRH cultures.

Limiting Dilution. Cloning by limiting dilution was carried out on selected, positive, stabilized clones (155, 156). A suspension of 10^7 BALB/c thymocytes per ml supplemented DMEM (7.5% NRS and 7.5% FCS) was prepared and viable hybridoma cells were collected by centrifugation and diluted such that 460 live hybrid cells were suspended in 4.6 ml of thymocyte containing cloning medium. After dispensing 0.1 ml of this mixture in 36 wells of a 96-well culture plate (10 cells/well) an additional 4 ml thymocyte containing medium was added to the remainder. Another 0.1 ml of cell suspension was added to 36 more wells (5 cells/well). Finally 1.4 ml of feeder cell medium was added to the remaining 1 ml of cell suspension and 0.1 ml dispensed in to the last 24 wells (1 cell/well). Supplemented DMEM was added (0.1 ml) to each well on day 5 and subsequently the spent medium was aspirated and replaced every 3-4 days.

Indirect Antibody ELISA. Indirect antibody ELISA was set up for rapid preliminary screening of culture supernatants from growing hybridoma cells, following fusion (167). Purified murine or human recombinant TNF-α was coated onto 96 well, polyvinyl chloride treated, flat bottomed, ELISA plates (Dynatech Laboratories, Alexandria, VA). An antigen solution of 50 ng/0.1 ml of 0.1 M sodium carbonate (Na_2CO_3) buffer was added to each well along with 0.01 ml of carbodiimide solution (1 mg/ml) in the same buffer and the plates incubated overnight at 4°C. After overnight incubation at 4°C the coated plates were washed with PBS and incubated for 0.5 hour with 0.1 M ammonium chloride at room temperature to bind the residual groups. The plates were then blocked with gelatin or bovine serum albumin containing diluent/ blocking solution (Kirkegaard and Perry Laboratories (Kpl), Gaithersburg, MD) and stored at 4°C in PBS containing 0.05% sodium azide. Some plates were blocked again just before use.

On the day of use the PBS was discarded and the plate washed once with PBS. Spent culture supernatant (0.1 ml) from 96 well plates was added to each well and allowed to react for 1 hour at room temperature. After incubation the plate was emptied and washed 3-6 times using 0.3 ml of PBS The wash solution was added to each well and the plate

rapidly inverted and tapped on paper towels to remove residual liquid. After the final wash, 0.05-0.1 ml of peroxidase conjugated goat anti-mouse, or sheep anti-rabbit IgG, was added to each well and allowed to react at room temperature for 1 hour. The plates were then washed repeatedly as outlined above.

After the final wash each well was soaked for 5 minutes and the residual liquid was then thoroughly removed from the plate by tapping. Color development was observed by adding 0.05-0.1 ml of enzyme substrate solution (Kpl) to each well. For peroxidase color development either a one component ABTS (2,2′-azino-di-3-ethyl-benzthiazoline sulfonate) or a two component TMB (3,3′,5,5′-tetramethylbenzidine) substrate system was used. The latter had to be mixed with an equal amount of hydrogen peroxide just prior to use. After sufficient color development which usually took 10-15 minutes 0.1 ml of stop solution was added to each well. PBS with 0.5% SDS was used as stop solution for ABTS and 1 M phosphoric acid for TMB. The plates were then read either visibly or in a titertek microplate reader using a 405 nm filter for ABTS and 450 nm filter for TMB (180).

TNF-α Neutralization Assays. The specificity of selected mAb was determined by neutralization and immunoprecipitation (161). Confluent L929 cells were harvested and resuspended in supplemented RPMI 1640, transferred into a 96 well plate

at a density of 3 X 10^5 cells/ml, and incubated at 37°C for 2-4 hours. In the meantime, appropriate dilutions of purified or crude TNF-α and antibody were prepared. During screening, the hydridoma culture supernatants were used undiluted and mixed in a 1:1 ratio with TNF-α (200-5000 units/ml). The ascitic fluid was diluted 1:1000 and the purified antibody used in a concentration of 10-100 μg/ml. All antigen antibody dilutions were made in supplemented RPMI 1640.

The antigen antibody mixtures were placed at 37°C for 2 hours and subsequently at 4°C for 0.5 hour, to reduce nonspecific binding. After incubation 0.1 ml of the mixture was added to the appropriate columns of the 96-well L929 plate to assess residual activity. Each plate carried its own internal TNF-α standard and control. After addition of the standard and test samples a two fold serial dilution of samples and standard was carried out from top to bottom, as discussed earlier. Cell viability was checked after 18-24 hours by measuring gentian violet uptake on a Multiskan Titertek spectro-photometer at 540 nm and the data analysed by the computer (73).

TNF-α Immunoprecipitation Assays. L929 target cells (3 x 10^4 cells/well) were added to 96-well culture plates as described earlier. In the meantime equal amounts of

appropriately diluted TNF-α and antibody were mixed and incubated at 37°C for 2 hours, following which 0.01 ml of carrier serum and 0.05 ml of goat anti mouse, or sheep anti-rabbit IgG, were added to the TNF-α and antibody mixture. After incubation for 1-2 hours at 37°C the mixture was kept at 4°C for 0.5 hour to reduce nonspecific binding and then centrifuged in a table top ultracentrifuge for 10-15 minutes. The supernatant was diluted, if necessary, and added to the appropriate columns of the 96-well L929 plate along with the internal TNF-α standard and controls. A two fold serial dilution of samples and standard was carried out from top to bottom, as discussed earlier and cell viability was checked after 18-24 hours by measuring gentian violet uptake on a Multiskan Titertek spectrophotometer at 540 nm and the data analysed by the computer (73).

Antibody Purification: Monoclonal or polyclonal anti-serum was purified by utilizing Bio-Rad's Affi-Gel Protein A monoclonal antibody purification system (MAPS II) kit (165). The system utilized purified *Staphylococcus aureus* protein A coupled to cross-linked agarose beads (2 mg/ml gel) via chemically stable amide bonds.

Protein A selectively binds the F_C region of antibody heavy chain and tyrosine residues in the F_C region of IgG

are involved in the interaction. The protein A agarose was packed in a 1 X 10 cm Econo-Column chromatography column and equilibrated with 5 bed volumes of binding buffer (0.1 M tris, 0.25 M NaCl, pH 8.5). The flow rate was adjusted to 0.6 ml/minute.

The ascitic fluid or rabbit serum was filtered through 0.8 μm syringe filter and diluted with an equal amount of binding buffer and applied to the column. After loading the sample the column was washed with 15 bed volumes of binding buffer and the antibody eluted with 5 bed volumes of elution buffer, pH 3. The eluate was collected in batches of 2 ml each and neutralized immediately after elution with 6 N sodium hydroxide (70 μl/2 ml eluate). The pH was checked with pH indicator sticks (E. Merck, Darmstadt, Germany).

The column was eluted with an additional 10 volumes of elution buffer to ensure total removal of immunoglobulin and subsequently washed with 5 bed volumes of regeneration buffer and stored in PBS containing sodium azide. The protein was quantified using Bradford's method and then dialyzed overnight against water and finally against 0.9% physiological saline or PBS (pH 7.6) using a cut off point of 6000-8000 daltons. The antibody purity was checked by SDS-PAGE using 15% resolving gels. A stock solution of 1.6 mg/ml of purified mAb (MRH.3) was used in the animal studies.

Antibody Sandwich ELISA. Antibody sandwich ELISA was used to test the specificity of the mAb and to exclude its binding to related cytokines (167, 180). Rabbit anti-TNF-α polyclonal antisera was purified from serum obtained from an immunized rabbit and used as the first antibody to capture the cytokines. A stock solution of 30 μg antibody/60 ml (500 ng/ml) 0.1 M sodium carbonate buffer (pH 9.5) was prepared and used to coat polyvinyl chloride treated ELISA plates. The plates were coated with the protein by dispensing 0.1 ml (50 ng) of antibody solution/well. Carbodiimide activation and blocking was carried out as outlined previously. The plates were stored at 4°C in PBS containing 0.05% sodium azide.

On the day of use the PBS was discarded and the plate washed once with PBS. A number of different cytokines, appropriately diluted, were added to each well and allowed to react for 1 hour at room temperature. After incubation the plate was emptied and washed 3-6 times as described earlier and then rapidly inverted and tapped on paper towels to remove residual liquid. Appropriately diluted mAb MRH.3 was added to each well (0.1 ml/well) and allowed to react for 1-2 hours at room temperature. The plate was subsequently washed 3-6 times and after the final wash, 0.05-0.1 ml of peroxidase conjugated sheep anti-rabbit IgG

was added to each well and allowed to react at room temperature for 1 hour. The plates were then washed repeatedly and after the final wash each well was soaked for 5 minutes in wash solution and the residual liquid thoroughly removed from the plate by tapping. Color development was observed by adding 0.05-0.1 ml of ABTS enzyme substrate solution (Kpl) to each well. After sufficient color development 0.1 ml of stop solution (PBS with 0.5% SDS) was added to each well and the plates were then read either visibly or in a titertek plate reader using a 405 nm filter.

Preparation of Virus Stocks. Vesicular stomatitis (Indiana strain) virus (VSV) was employed in biological assay for IFN-τ (181, 182). The virus was provided by Dr. K. Cantell (Finnish Red Cross Blood Transfusion Centre, Helsinki, Finland). VSV is a rhabdovirus containing a lipid enclosed single stranded RNA. It infects a wide range of mammalian cells and is easy to grow. Virus stocks were prepared by infecting confluent cultures of WISH cells in 75 cm^3 culture flasks with a low multiplicity of infection. The low input multiplicity helps circumvent the generation of defective-interfering viral particles. After allowing adsorption for 1 hour at 37°C the monolayers were rinsed with warm HBSS and incubated overnight at the same temperature. Next day the

flasks were frozen rapidly at -70°C and thawed at 37°C and the culture medium collected. The culture medium was centrifuged in sterile tubes at 2000 g for 10 minutes under aseptic conditions and the supernatant, containing VSV, was saved and tested for its cytopathic effect (CPE) on WISH cells. The CPE is quantified by determining gentian violet uptake by spectrophotometry.

Viral suspensions that were thousand times the concentration, required for inducing 50% CPE in 24 hours, were transferred into polypropylene tubes and stored at -70°C. It was determined that a 1:10 dilution of this virus, when added to a confluent monolayer of WISH cells, produced 100% CPE in 24 hours.

Interferon Assays. In order to exclude the possibility of the mAb (MRH.3) neutralizing IFN-τ, interferon cytotoxicity assays were set up (181). On day 1 confluent WISH cultures were treated with 5 ml trypsin solution and after 3-5 minutes they were washed with RPMI 1640 medium supplemented with 5% FCS and 5% horse serum. The cells were collected by centrifugation, plated at a density of 2 X 10^5 cells/ml and incubated overnight at 37°C.

The test samples were added on day 2. To test for neutralization 0.1 ml of IFN-τ (5000 units/ml) and 0.1 ml of MRH.3 (100 μg/ml or 25 μg/ml) were added to microcentrifuge

tubes, incubated at 37°C for 2 hours, followed by incubation at 4°C for 30 minutes. Next 0.15 ml of the mixture was added to the top well of a 96 well plate and 1:2 serial dilution made down the line. The plate was incubated overnight at 37°C. To test coprecipitation of IFN-τ equal volumes (0.15 ml) of IFN-τ and mAb (100 μg/ml) were mixed and the mixture incubated at 37°C. After 2 hours 0.06 ml of whole serum and 0.240 ml of sheep anti-rabbit IgG antibody was added to each sample mixture and the samples reincubated for 1 hour at 37°C. The mixture was next placed at 4°C for 0.5 hour and plated after appropriate dilution. A 1:2 serial dilution was made down the column and the plate was placed in an incubator overnight.

On day 3 a VSV suspension was thawed and diluted 1:10. The spent culture medium was discarded by inverting the plate and the viral containing medium added to all wells except lane 1 that acted as a positive control simulating 100% cell viability. The plates were incubated overnight to allow cell lysis. After cell lysis had occurred the plate was removed from the incubator, the medium discarded and 0.1 ml gentian violet solution was added to each well. After 2-5 minutes incubation at room temperature the plates were washed with tap water and dried overnight. The plates were read the next day at 540 nm on a titertek microplate reader.

Western Blotting. In order to test specificity of mAb against TNF-α immunoblotting was carried out using a Bio-Rad Mini Trans-Blot transfer cell (183, 184). The samples along with the pre-stained standards were run on SDS-PAGE and the gel placed in 50 ml transfer buffer for 30 minutes for equilibration. The gel holder, sponge, filter paper and nitrocellulose paper, cut to the size of the resolving gel were also saturated with transfer buffer for at least 30 minutes prior to transfer. The blotting medium was dipped in transfer buffer and allowed to wet by capillary action.

The gel holder was opened by sliding the latch and placed in a trough containing transfer buffer so that the clear panel (anode) was at an angle against the wall of the vessel and the gray panel (cathode) flat on the bottom of the vessel. A sponge pad was put on the gray panel of the gel holder and a saturated filter paper on top of the sponge pad. The pre-equilibrated gel was kept on top of the filter paper and the pre-wetted nitrocellulose transfer sheet on top of the gel. This was done by holding the sheet at opposite ends allowing the sagging center portion to contact the gel first and then gradually lowering the ends. All air bubbles were removed and excess liquid was displaced by roller pin exclusion using a glass pippette. The sandwich was completed by placing a saturated filter on top of the transfer membrane and a saturated sponge pad on top of the

filter paper. The gel holder was closed by holding it firmly while securing the latch and placed in Trans-Blot tank containing transfer buffer, so that the gray panel of the holder was on the gray side of the tank. A magnetic stirrer and the Bio-Ice cooling unit were placed in the buffer tank which was then filled with transfer buffer to just above the level of the top row of circles on the gel holder cassette. The apparatus was placed on top of a magnetic stirrer and the lid was put in place making sure that the appropriate pins of the electrode module were attached correctly to the electrode wires, black to black (cathode) red to red (anode). Normal transfer polarity was from cathode to anode. The unit was plugged into the power supply and with power set on full, current was set at 150 mA and voltage at 100 volts. Transblotting was continued for 1-2 hours or until the temperature of the buffer system approached 20°C and the power supply was turned off after completion of the transfer. The nitrocellulose paper, showing pre-stained standards, was quenched with two successive 30 minute washes using 0.5% Tween 20 in PBS and incubated with MRH.3 antibody mixed in the same buffer. The filter was allowed to react with the antibody for 2 hours at room temperature, or overnight at 4°C. The excess unbound antibody was removed from the membrane by washing it three

times (5-15 minutes each) with PBS containing Tween 20. Subsequently 1:50-1:200 dilution of horse-radish peroxidase-labeled (HRP-labeled) antibody was made in approximately 30 ml PBS-Tween 20 and the blot was incubated at room temperature for 2 hours with this solution. The blot was washed 2-3 times in PBS-Tween 20 and once with TBS-Tween for 10-15 minutes. In the meantime the HRP color developing reagents (Bio-Rad) were prepared. Immediately before use 0.6 ml of HRP color reagent B (hydrogen peroxide) and 20 ml of HRP color reagent A (4-chloro-1-naphthol) were added to 100 ml of HRP color development buffer (TBS) at room temperature. The mixture was protected from light, if not used immediately. After the final wash the blot was drained and immersed in the color development solution (30 ml/blot) protected from light. The color was allowed to develop at room temperature and color development was stopped by transferring the blot into water for 10 minutes. The filter was dried in air or in the drying apparatus as outlined earlier and stored between polyester sheets, protected from light to minimize fading.

In case no stained bands were apparent Ponceau S (Gelman Instrument Company, Ann Arbor, MI) stain solution was employed to see if bands had transferred (178). This step could also be done prior to blocking. Briefly the membrane was washed with 6 M urea for 3 minutes, then 5

times with water (4 minutes each), and stained in Ponceau S solution for several minutes. Staining was stopped by washing in water for 2 minutes. As an alternative, amido black staining (Allied Chemical Corporation, New York, NY) was employed in some cases. Presence of protein bands on the blot indicated a successful transference. If the transferred bands were not visible on the blot following use of antibodies, the substrate and HRP conjugated antibody were added together (2:1) and the color development was observed. Color development indicated a workable enzyme-substrate system, and the absence of bands on the blot indicated a failure, in recognition, or binding, of the first antibody to the transferred protein.

Iodination of TNF-α. To carry out radioimmunoassays (RIA) and to test specificity of mAb, peroxidase-catalyzed iodination of TNF-α was carried out (185, 186). The peroxidase halogenation was used because it preserves biological activity and has fewer side reactions than halogenation employing other reagents because the oxidant , peroxide, is used in very low concentrations. It also has the advantage that under the proper experimental conditions the peroxidase catalyzed halogenation is very selective. Only two amino acids, tyrosine and histidine form stable derivatives as a result of peroxidase-catalyzed iodination.

Purified TNF-α containing 40 μg protein/ml (20,000 units/ml) PBS was prepared and labeled with a carrier-free solution of sodium iodide ($Na^{125}I$), utilizing lactoperoxidase and hydrogen peroxide (H_2O_2). Radiolabeling was carried out by adding 0.015 ml (1.5 mCi) of the stock solution of $Na^{125}I$ (100 mCi/ml 0.1 N NaOH) (ICN Radiochemicals, Irvine, CA) and 0.01 ml of lactoperoxidase (1×10^{-5} M) to the TNF-α solution in polypropylene tubes. Hydrogen peroxide (30% v/v) was freshly diluted approximately 1 to 1000 in 0.05 M phosphate buffer, pH 7.4, to produce a 1×10^{-2} M solution, and then introduced (0.01 ml) with gentle stirring, and the mixture incubated at room temperature for 0.5 hour.

The iodinated TNF-α was isolated from contaminating lactoperoxidase and excess iodide by passage through a Sephadex G-25 M gel filtration column (Column PD-10, Pharmacia). Prior to loading the sample, the column was equilibrated with 25 ml of 0.05 M phosphate buffer and the sample was diluted with the same buffer before application to the column. The eluate with the highest radioactivity and protein content were tested by L929 biological assay for presence of TNF-α biological activity and used for the thymidine uptake assay.

Thymidine Uptake Assay. LPS induced cellular proliferation of BALB/c spleen cells was performed in the presence or absence of mAb, in order to exclude the possibility of the antibody neutralizing LPS (187). The cellular proliferation was quantitated by assay of DNA synthesis, utilizing incorporation of exogenous radiolabeled thymidine, [methyl-^{3}H] (ICN Radiochemicals) that was measured by scintillation counting. Spleen cells suspended in supplemented RPMI 1640 were plated at a final concentration of 5-6 X 10^5 cells/0.1 ml/well. Various concentrations of LPS, and/or mAb, diluted in sterile, supplemented RPMI 1640 were added to each well (0.1 ml/well). Only supplemented RPMI 1640 was added to control suspensions. Control and experimental cultures were run in triplicate. The cells were incubated overnight in a 95% humidified incubator at 37°C in an atmosphere of 5% CO_2 in air. Next day 1 μl of radiolabeled thymidine (specific activity 1.54 Ci/mmole) was added to each well and the plates incubated for 4-6 hours at 37°C. The cells were harvested using an automated MASH II sample harvester unit (Whittaker Bioproducts Incorporated, Walkersville, MD) for 96 well plates and aspirated onto 12 X 1.5" glass microfibre filter strips (Whatman, Clifton, NJ). The wells were thoroughly washed with PBS. Individual filter strips, representing each well, were cut and placed in labeled

scintillation vials and allowed to air dry overnight at room temperature. After addition of 3 ml of biodegradable Ecolite scintillation fluid (WestChem, San Diego, CA) to each vial the counts per minute (cpm) were determined in a Delta 300 beta scintillation counter system (Tracor Analytic, Elk Grove Village, IL).

Animal studies. Various concentrations of LPS were injected into the mice in the presence or absence of purified rabbit anti-murine TNF-α monoclonal antibody as detailed elsewhere. The effect of the antibody on the mouse fatality were evaluated and analysed.

Where necessary the appropriate statistical analysis were carried out by parametric and nonparametric tests using Statgraphics 4.0 software (Statistical Graphics Corporation, Rockville, MD). Graphics were made using SigmaPlot scientific graphing software 4.1 (Jandel Scientific, Corte Madera, CA).

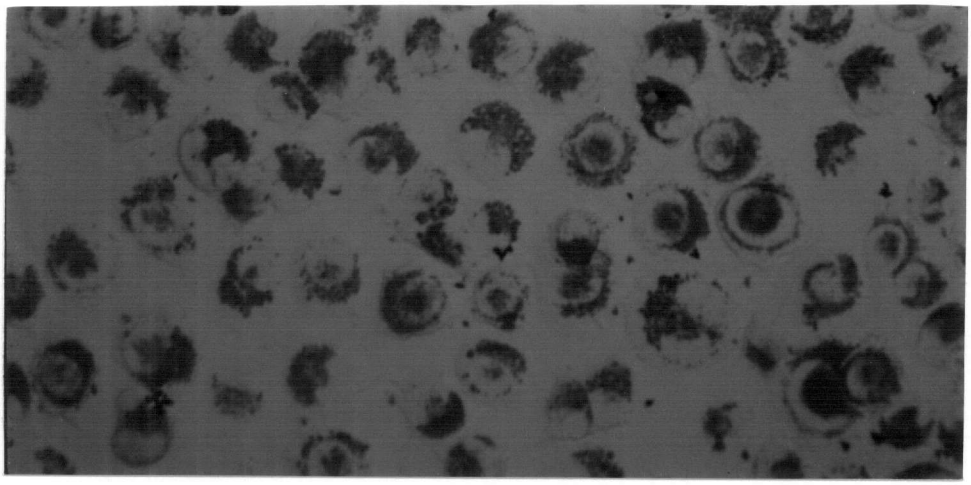
CHAPTER 3

RESULTS

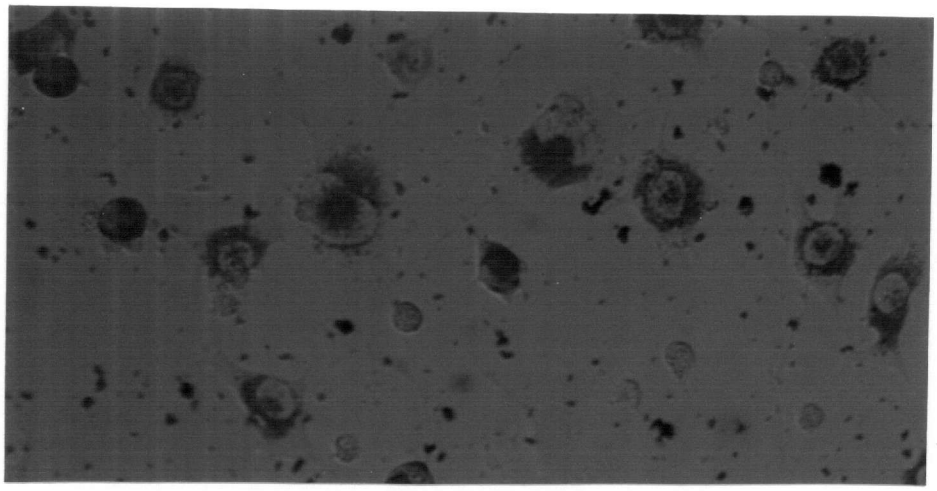
The LD_{50} (the dose that kills 50% of animals tested) for LPS was calculated by employing normograms of Litchfield and Wilcoxon (188). Female BALB/c mice (4-6 weeks old) were injected intraperitoneally (i.p.) with varying concentrations of LPS. The LPS was obtained by trichloro-acetic acid (TCA) extraction from *E. coli* serotype 055:B5. Ten mice per group were injected with the LPS and their survival was charted over 7 days (Table VII). The expected percent effect was calculated by plotting LPS dosage and observed percent alive onto a logarithmic-probability paper (Keuffel and Esser). The results were then plotted onto a nomograph to obtain a Chi-square (X^2) value. The treated mice groups were not significantly heterogeneous (X^2 0.975, 5 = 7.73) as determined by the nomograph. The LD_{50} for intraperitoneally injected *E. coli* LPS in female BALB/c mice was 738 ± 162 μg/mouse.

Figure 1

Murine L929 target cells stained with neutral red before (1A) and after (1B) exposure to TNF-α. The L929 cells were seeded onto 96 well culture plates at a density of 2 X 10^4 cells/well in supplemented RPMI 1640 containing 10% FCS and 0.5 μg/ml mitomycin C. After overnight incubation at 37°C, murine TNF-α (200 units/ml) was added to each well, except the control (1A) and a two-fold serial dilution was carried out from top to bottom. Cell viability was observed microscopically after 18-24 hours by measuring neutral red dye uptake. The photomicrograph was taken after incubation of the plate for 1 hour at 37°C following the addition of 1% w/v neutral red solution in RPMI 1640 to each well, except the blank. There is a decrease in the number of viable target cells after exposure to TNF-α, as demonstrated by the reduced number of cells that take up neutral red (1B).



1 A



1 B

TABLE VII

Determination of LD_{50} for LPS in female BALB/c mice

LPS Dose (μg/mouse)	S/T	Percent Survival		O-E	X^2
		OBSERVED(O)	EXPECTED(E)		
100	10/10	100	99.99	0.01	0.010
200	10/10	100	99.20	0.80	0.008
400	9/10	90	86.00	4.00	0.012
500	7/10	70	75.00	5.00	0.013
800	8/10	80	42.00	38.00	0.600
1000	1/10	10	26.00	16.00	0.120
1500	0/10	0	99.99	0.01	0.010

In order to determine TNF-α levels in serum of LPS treated mice 1.5 mg of TCA extracted LPS was injected i.p. into five female BALB/c mice (6 weeks old) weighing 18-21 gm each. Blood was collected by eye puncture at the indicated intervals (Table VIII) and serum TNF-α activity determined by 72 hours L929 biological cytotoxicity assays, utilizing mitomycin C and neutral red staining as outlined in materials and methods. No TNF-α activity was observed in serum obtained from control mice injected with 0.9% saline (Fig. 1). TNF-α activity was not apparent until 6 hours following LPS treatment. Another much higher peak was

obtained around 24 hours following LPS injection. These findings correlated with the development of adverse reactions in the animals. By 6 hours the mice developed shivering and diarrhea. They huddled together and became anorexic. By 24 hours the mice had become extremely weak and had limited movements. Their fur became discolored and they died between 24-48 hours. The mice that died had higher TNF-α titers in their serum compared to the two that survived. No TNF-α was detectable in serum of the surviving mice (Table VIII, Fig. 2).

TABLE VIII

Serum TNF-α levels (units/ml) in female BALB/c mice after intraperitoneal injection of LPS

Hours after LPS injection (1.5 mg/mouse)				
0	6	12	24	48
0	18.33	0	24.97	Dead
0	9.07	0	64.86	Dead
0	17.49	0	69.72	Dead
0	12.55	16.33	24.97	0.04
0	17.15	18.89	37.21	0.96

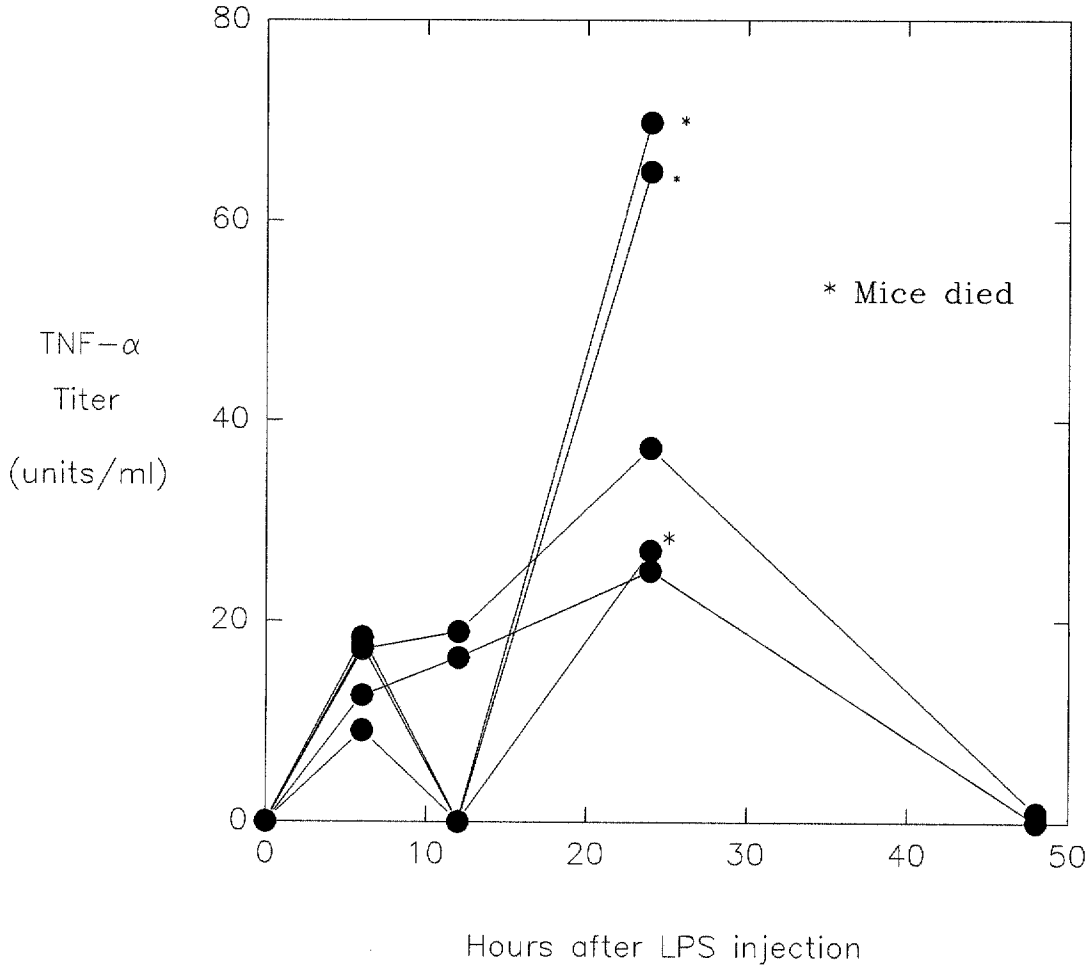


Figure 2

Serum TNF-α levels in female BALB/c mice following LPS injection

Once confirmation of TNF-α secretion by LPS treated mice had been obtained mouse monocyte-macrophage cell lines RAW 264.7 and PU5-1.8 stimulated with mitogens were used for *in vitro* production of murine TNF-α (53, 54). Results from biological assays indicated that LPS induced RAW 264.7 cell lines produced the maximal amount of the cytokine as determined by 3 day L929 cytotoxicity assays. TNF-α production by PU5-1.8 and RAW 264.7 cells stimulated with phytohemagglutinin and phorbol ester was not consistent and the sample size was too small to conduct statistical analysis.

RAW 264.7 cells (1 X 10^6/ml) were stimulated with 1, 10, 25, 50 and 100 μg/ml LPS (*E. coli* 055:B5) and incubated at 37°C for 4 hours. Culture medium was collected after 4 hours and tested for TNF-α activity employing the 3 day L929 assay and neutral red staining. The plates were read on the Multiskan Titertek spectrophotometer interfaced with a computer. Corrected TNF-α titer values (units/ml) were obtained from the computer output.

The results indicated that TNF-α production by murine RAW 264.7 cells was significantly different among the various LPS concentrations (Kruskal-Wallis one-way analysis of variance (AOV), $H = 16.5$, $p = 2.4 \times 10^{-3}$). A non-parametric one-way (AOV) was carried out because the group

variances were highly significantly different (Bartlett's test statistic = 6.58, $p = 4.6 \times 10^{-11}$) (Table IX). Tukey's parametric multiple range test showed that the LPS induced RAW 264.7 TNF-α titers were separated into three statistically distinct groups, which are represented by the horizontal lines (Table IX). LPS concentrations of 100 μg/ml produced higher TNF-α titers as compared to 10, 25 and 50 μg LPS/ml, which in turn produced significantly greater TNF-α when compared to 1 μg LPS/ml (Fig. 3, Fig. 4). The multiple range comparisons were hampered by the heteroscedasticity of variances and the relatively small and unequal sample size.

The results showed that maximum murine TNF-α production by macrophage cell line (RAW 264.7) required LPS concentrations of 10, 25 or 50 μg/ml. All these concentrations produced statistically similar amount of TNF-α. This LPS concentration was much higher than reported in earlier studies (53, 55) but the discrepancy could be explained on the basis of different sources of LPS and different experimental conditions.

TABLE IX

TNF-α production by LPS stimulated RAW 264.7 cell line

LPS	1	10	25	50	100	μg/ml
Mean	102.03	2091.34	2261.8	1943.9	4284.3	units/ml
± SD	± 82.2	± 1004.8	± 1117.3	± 827.0	± 4712.5	
	75.59	3324.02	2108.15	2421.73	11516.87	
	84.81	1291.42	1855.84	1153.94	8836.16	
	26.16	1014.54	1386.20	1056.15	935.96	
	54.56	2919.33	956.45	2110.74	261.45	
	36.93	1907.39	928.71	2976.95	1415.27	
	230.93		2812.64		2740.24	
	205.24		2487.98			
			2083.04			
			1775.64			
			3065.31			
			4822.61			
			3687.22			
			1433.67			
	——1——					
		————2————				
					——3——	

Horizontal bars represent 3 statistically distinct groups

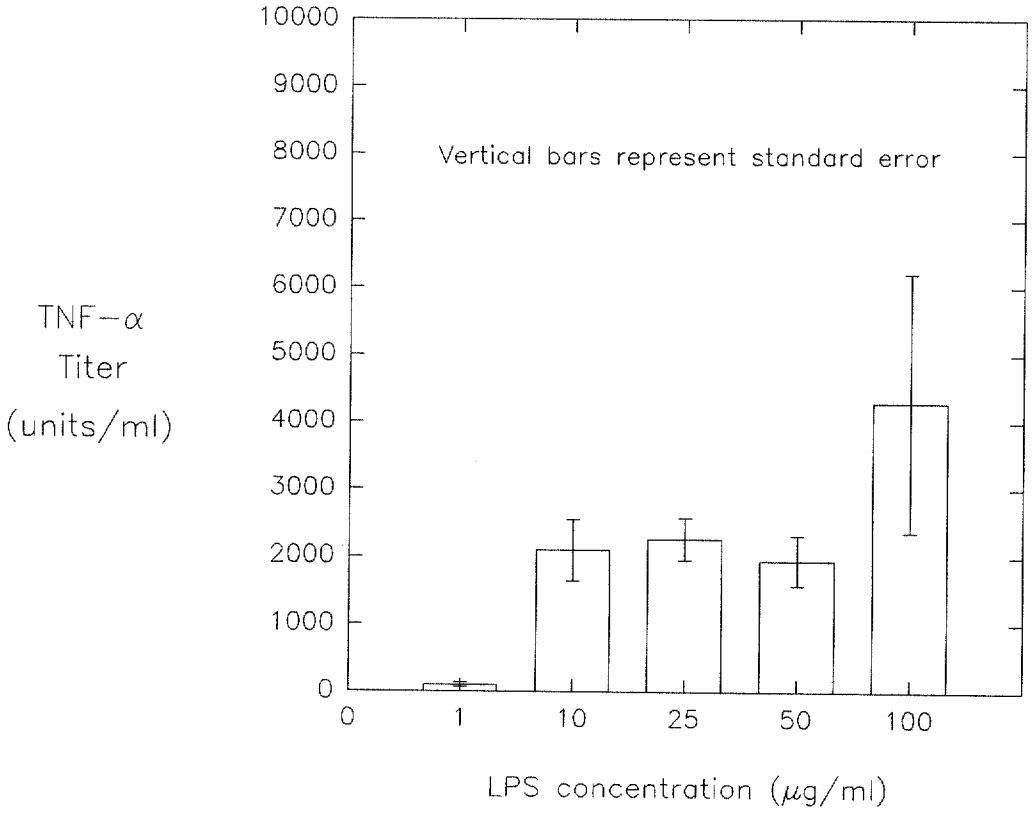


Figure 3

TNF-α production by LPS stimulated RAW 264.7 cells

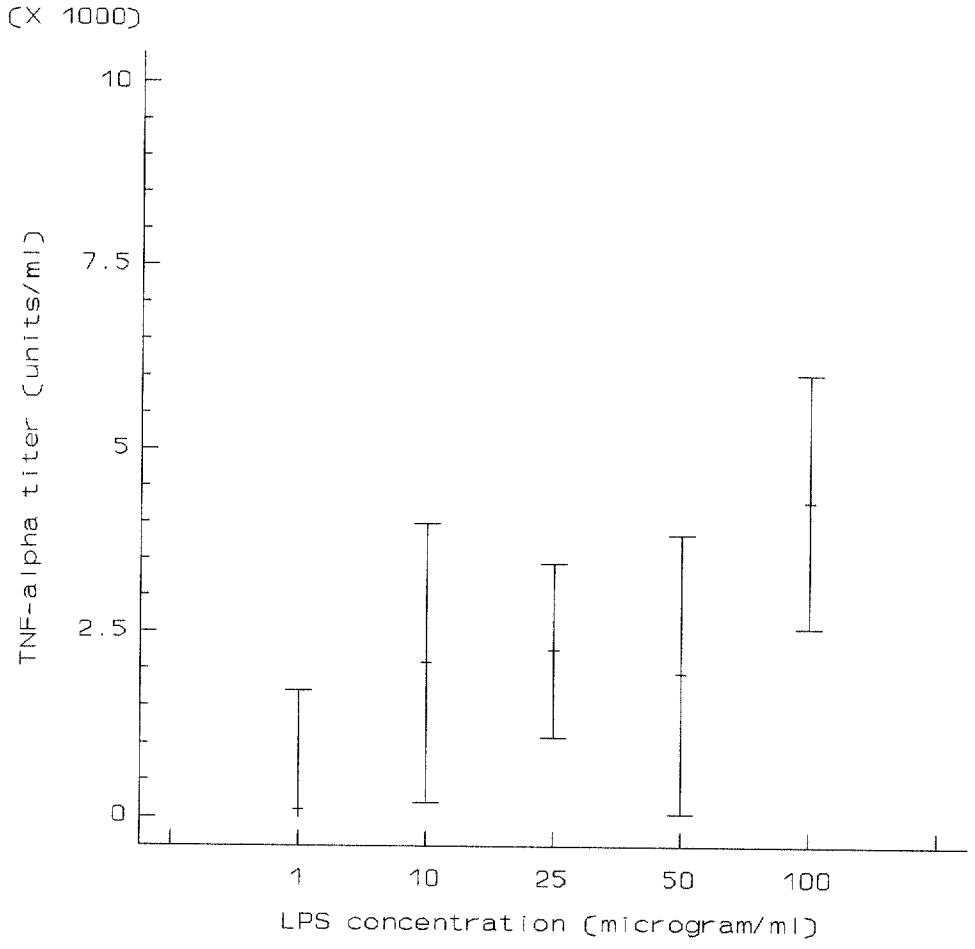


Figure 4

TNF-α production by LPS stimulation

(Vertical bars represent standard deviation)

The RAW 264.7 cell line was selected for mass production of murine TNF-α. Confluent cell cultures of RAW 264.7 in supplemented RPMI 1640 were washed with HBSS and incubated overnight at 37°C in fresh RPMI 1640 supplemented with 10-25 μg LPS per ml. The culture supernatant was then collected, centrifuged to remove cell debris, pooled and stored at 4°C till purification. Filtration of spent culture supernatants through a 0.22 μm bottle top filter was discontinued after it was found that there was loss of TNF-α biological activity after filtration, as determined by L929 biological cytotoxicity assays (Table X). A significant difference was observed in mean TNF-α titers between filtered and unfiltered culture supernatants (independent t test, $t = -2.4$, $p = 0.04$).

Murine TNF-α was purified from the pooled culture supernatants by affinity chromatography utilizing a column of cyanogen bromide activated sepharose 4B coupled with goat anti-TNF-α polyclonal antisera (Table XI). This antibody was shown to immunoprecipitate murine TNF-α. Diluted, pooled supernatants from LPS stimulated cell cultures were passed through the 15 cm X 0.7 cm affinity column containing 2.5 ml of gel with 16 mg of goat anti-TNF-α polyclonal antibody attached to it. The flow rate was maintained at 0.5-1.0 ml/minute employing a Econo-column pump (Bio-Rad).

TABLE X

TNF-α activity (units/ml) in filtered and unfiltered RAW 264.7 culture supernatants as determined by L929 biological cytotoxicity assays

Filtered	Unfiltered
0.35	58.71
43.20	107.13
43.86	91.28
61.35	66.17
1.76	38.31
22.90	34.15

TABLE XI

TNF-α activity before and after affinity chromatography

	Before	After
Volume (ml)	6000	14
Specific activity (units/mg)	500	5×10^6
TNF-α Titer (units/ml)	1600	1×10^6
Total TNF-α (units)	9.6×10^6	1.4×10^7
Protein (mg/ml)	3.2	0.2
Total Protein (mg)	19,200	2.8

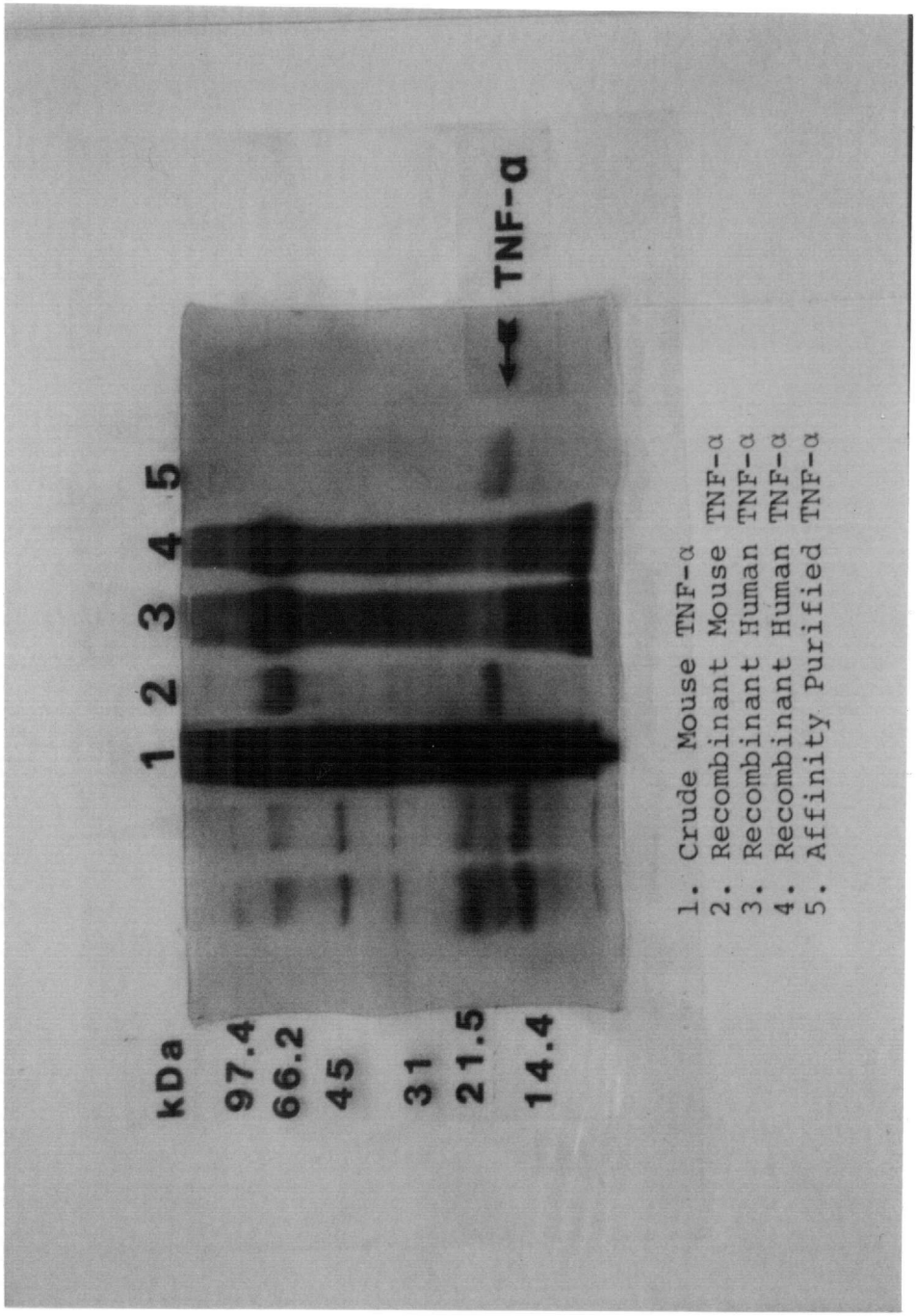
The affinity column had a saturation capacity of 0.3 mg and was used several times without loss of efficiency (Table XI). Elution of TNF-α was carried out by tris buffer (pH 10.8). The pH of the eluate was immediately adjusted to 7.2-7.6 so as to prevent denaturing and loss of biological activity. The eluates showing the highest protein concentrations were pooled and stored at 4°C until use.

The purification factor, which is a comparison between the specific activity before and after purification, was 10,000 and the efficiency of the affinity column was 100%. Specific activity of any protein is a measure of its biological activity per mg.

The purity of the eluate was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Both Coomassie blue and silver staining techniques were employed to detect the TNF-α bands (Fig. 5). The molecular weight of the monomeric murine TNF-α was 17,000 daltons, which collaborated with earlier studies (53, 55).

Figure 5

SDS-PAGE analysis of affinity purified TNF-α. Crude and affinity purified TNF-α samples were run on a 20% polyacrylamide gel and visualized by silver staining as described in materials and methods. The position of TNF-α is indicated by the arrow. Multiple bands were visible in the lanes containing crude mouse TNF-α (lane 1). Recombinant mouse TNF-α was obtained from Genzyme and contained 0.1% bovine serum albumin as stabilizer (lane 2). The albumin band was also seen in lanes containing human recombinant TNF-α. Standards of known molecular weights were obtained from Bio-Rad. The proteins used as molecular weight standards included rabbit muscle phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), hen egg white ovalbumin (45 kD), bovine carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD) and hen egg white lysozyme (14.4 kD). For silver staining the standards were diluted 1:100 in sample buffer, heated at 95°C for 5 minutes. Approximately 5-15 μl/well of standard was loaded onto the mini-gel.


kDa
97.4
66.2
45
31
21.5
14.4
1 2 3 4 5
TNF-α
1. Crude Mouse TNF-α
2. Recombinant Mouse TNF-α
3. Recombinant Human TNF-α
4. Recombinant Human TNF-α
5. Affinity Purified TNF-α

Purified mouse TNF-α was injected into two female New Zealand White rabbits to produce an immune response, as outlined in the procedure for monoclonal antibody (mAb) production. The aim was to generate a hybrid cell line between immune rabbit splenocytes and mouse myeloma cell line (Sp2/0-Ag14) that produced antibody against murine TNF-α.

In order to screen hybridoma supernatants quickly for presence of TNF-α neutralizing antibodies a one day L929 biological assay for TNF-α was developed. The assay replaced mitomycin C with actinomycin D, a stable analogue that intercalates between the DNA strands and disrupts mammalian gene transcription. In addition gentian violet staining of viable cells was instituted as it was less time consuming and simpler than neutral red staining. Comparison of TNF-α titers in neutral red and gentian violet stained viable L929 target cells (Table XII, Fig. 6) revealed that the staining technique had no significant effect on mean TNF-α titers in 24 hours L929 biological assays utilizing 3 X 10^4 target cells per well (two tailed independent t test, $p = 0.67$).

TABLE XII

Comparison of TNF-α titers in L929 biological cytotoxicity assays using neutral red and gentian violet staining

STAINING TECHNIQUES	TNF-α TITER (units/ml)	
1. Neutral Red Staining	(NRed)	
	1464994	
	1780771	
	1546501	Mean 1647877.67
	1844030	± SD ± 267277.61
	1267488	
	1983482	
2. Gentian Violet Staining	(GViol)	
	1342219	
	1517748	
	1191992	Mean 1571085.83
	2150417	± SD ± 334036.41
	1512118	
	1712021	

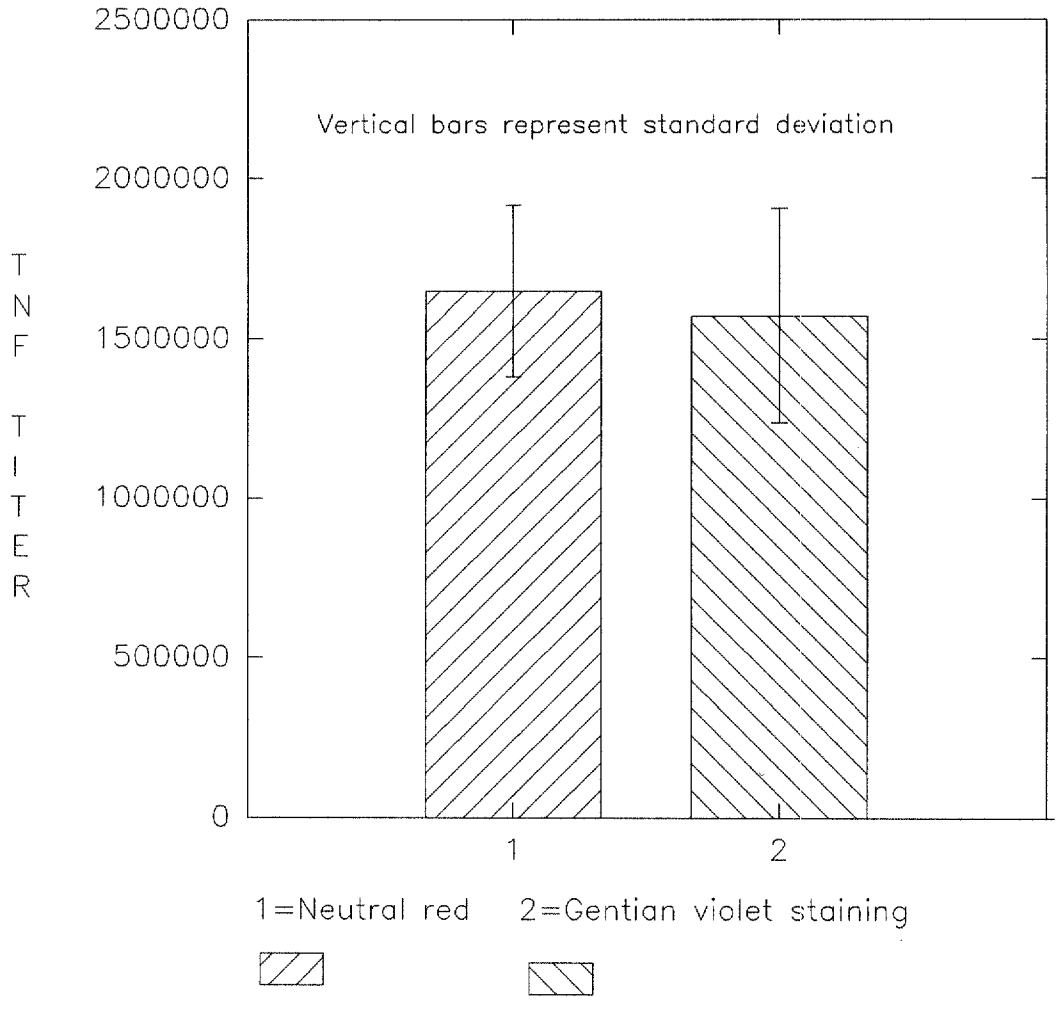


Figure 6

Comparison of TNF-*α* titers (units/ml)

in L929 target cells using different staining techniques

The one day assay was dependent on target cell (L929) density. The assay became less sensitive as the cell density was increased (Table XIII, Fig. 7). The TNF-α titers were significantly different among the different cell densities (Kruskal-Wallis one-way AOV, $H = 21.9$, $p = 2.1 \times 10^{-4}$). A nonparametric Kruskal-Wallis one-way AOV was carried out as according to Bartlett's test group variances were not homoscedastic.

The one day assay employed for screening the hybridoma supernatants utilized 8×10^5 L929 cells/well as it was shown that such a cell density gave consistent (although 2.5 times lower) results with regards to the laboratory human recombinant TNF-α standard titer (Table XIII). Subsequent to screening all one day assays employed 3×10^5 target cells/ml.

Production of antibody secreting hybridoma cell line employing immunized rabbit splenocytes and non-secreting mouse myeloma cell line (Sp2/0-Ag14) was carried out as detailed earlier. Briefly spleen cells obtained from an immunized rabbit were fused with mouse myeloma cells (Sp2/0-Ag14) using polyethylene glygol (PEG 4000). Mouse-rabbit hybridomas secreting intact rabbit immunoglobulin (IgG) have been successfully produced by such modification of Milstein's and Köhlers's techniques (153, 162, 164).

TABLE XIII

Effect of L929 cell density on TNF-α activity (units/ml)

CELL DENSITY	TNF-α TITER	Mean ± SD
2 X 10^5/ml (72 hours assay)	2328900	
	528300	
	647700	2136366.7
	4046900	± 1921221.7
	456900	
	4809500	
3 X 10^5/ml (24 hours assay)	1991480	
	2465735	
	1201984	1555155.7
	1293635	± 551021.7
	1325800	
	1052300	
4 X 10^5/ml (24 hours assay)	4925200	
	4794800	
	757769	2127262.7
	922807	± 2119082.5
	652700	
	710300	
6 X 10^5/ml (24 hours assay)	85439	
	81310	
	177763	110954.7
	111159	± 35699.4
	117453	
	92604	
8 X 10^5/ml (24 hours assay)	450005	
	472479	
	457722	432543.0
	432500	± 37781.4
	415745	
	366807	

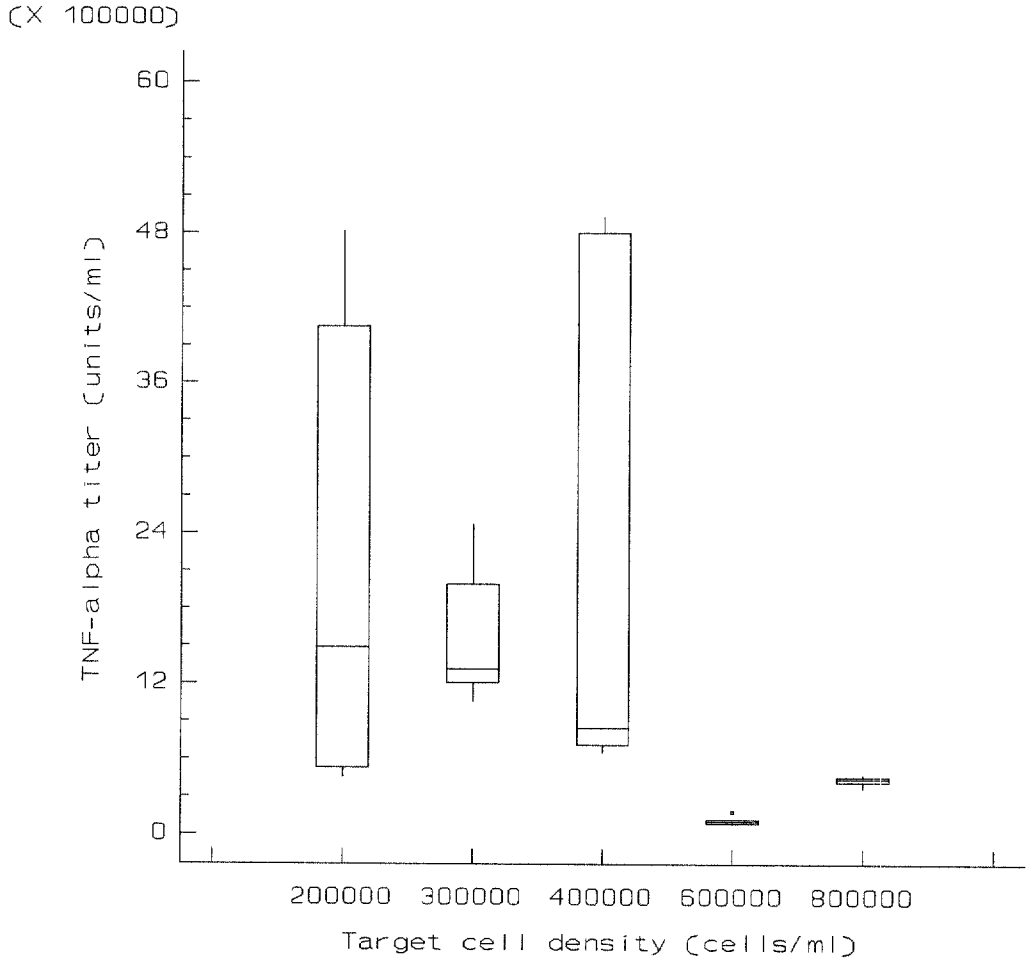


Figure 7

Effect of L929 cell density on TNF-α titer

(Box and whisker plot)

As shown in Table XIV, fusion II was successful. A few of the hybrid cells survived and there was minimal fibroblast growth. In addition to the change in medium supplement, feeder cells consisting of thymocytes and spleen cells from BALB/c mice, mixed in a 1:1 ratio, were added to each well at a density of 5 X 10^5 cells per well. Raybould and Takahashi have reported successful production of stable rabbit-mouse hybridomas secreting monoclonal IgG employing the same technique (164). Although these scientists reported better results using peritoneal exudate cells as feeder layer this could not be confirmed as all the plates utilizing these feeder cells became contaminated.

Due to the overwhelming number of ELISA positive wells in the first fusion it was decided to drop the ELISA and to directly observe neutralization of murine and human recombinant TNF-α employing the one day L929 biological assay. Stabilized hybrids that secreted antibody neutralizing both human and mouse TNF-α were selected and cloned by limiting dilution as outlined in materials and methods (Fig. 8). This was carried out prior to freezing the clones as the cell growth was extremely slow and this increased the chances of fungal contamination.

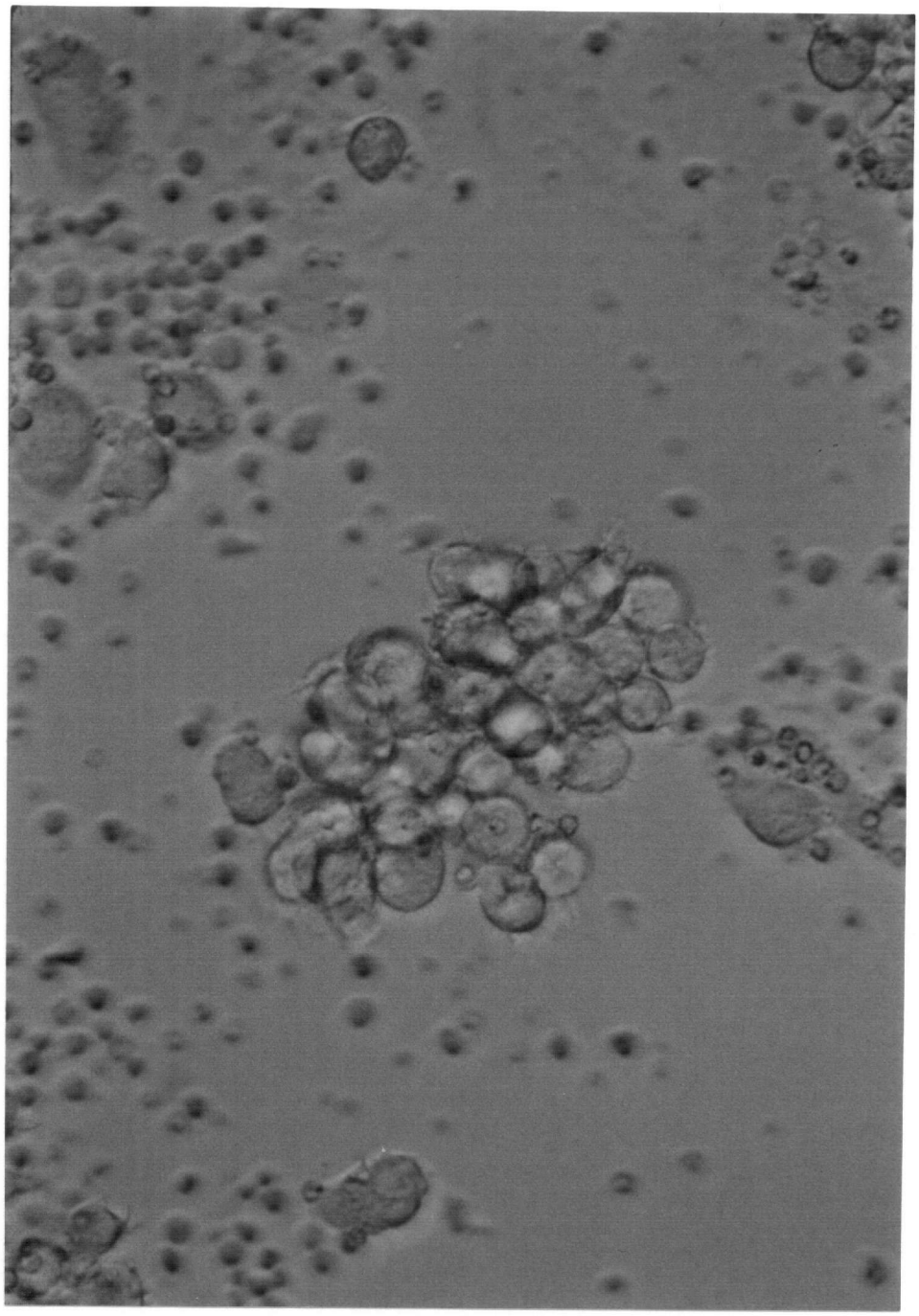
TABLE XIV

The effect of serum and feeder cells on the outcome of fusion between hyperimmune rabbit splenocytes and mouse myeloma cells

Feeder Cells	Appearance of cultures on day 7	ELISA positive wells	Neutralizing Clones (L929 Assay)	Outcome
Fusion I				
The HAT medium consisted of supplemented DMEM (15% FCS)				
No	Multiple	502/1542	294/1542	-
Fusion II				
The HAT medium consisted of supplemented DMEM (7.5% NRS and 7.5% FCS)				
Yes	Multiple	ND	306/1056	Few

Figure 8

Photomicrograph of a mouse-rabbit hybridoma that secretes rabbit IgG. The photomicrograph shows a clump of cells in the center that are growing in supplemented HAT culture medium (DMEM). The fusion between immunized rabbit spleen cells and a non-secreting, mouse myeloma, cell line (Sp2/O-Ag14) was mediated by PEG 4000 as detailed in materials and methods. Some fibroblast and thymocyte feeder cells are visible in the background.



Stabilized clones were frozen and characterized one by one. The first two clones that were revived did not adapt to regular growth medium (supplemented RPMI 1640) and were discarded. The third clone (MRH.3) was grown initially in supplemented DMEM and gradually weaned onto RPMI 1640 supplemented with 10-15% FCS (Table XV). Growth in DMEM required loosely closed flasks to allow adequate CO_2 exchange.

Growth of the hybrid cell line did not require any elaborate growth factors like *Salmonella typhimurium* mitogen (STM) or 2-ME. The former is a water-soluble proteinaceous extract prepared from the cell walls of mutant bacteria and is considered a potent and specific stimulator of B lymphocytes. The STM effect is a result of its synergistic interaction with thymocytic or splenic feeder cells. This may involve secretion of cytokines that effect B-cells proliferation.

The clone MRH.3 was subsequently recloned by limiting dilution again and then expanded. Antibody secretion by the clone was reconfirmed and the clone frozen in liquid nitrogen. It has since undergone two freeze thaw cycles with no effect on its antibody production.

TABLE XV

MRH. 3 growth medium evaluation

Medium	Culture Plates				Culture Flasks	
	96	48	24	6	10 mm^3	25 mm^3
1.	+	+	+	+	+++	+
2.	+	+	+	+-	-	-
3.	-	-	-	-	-	-
4.	+	+	+	+	ND	ND
5.	+	+	+	+	+	ND
6.	ND	ND	ND	-	+-	ND
7.	ND	ND	ND	+	+++	++
8.	ND	ND	ND	ND	++	++
9.	ND	ND	ND	ND	+	+

ND (Not Done) - No growth + Fair ++ Good +++ Excellent

1= DMEM + 15% FCS (Ultraserum) + 25 mM HEPES

2= DMEM + 15% FCS (Ultraserum)

3= DMEM + 15% FCS (Ultraserum) +30 μM 2-ME

4= DMEM + 15% FCS (J R serum^{500}) + 25 mM HEPES + 30 μM 2-ME

5= DMEM + 15% FCS (J R serum^{500}) + 30 μM 2-ME

6= DMEM + 15% FCS (J R serum^{500}) + 25 mM HEPES

7= RPMI + 15% FCS (Ultraserum) + 4 mM HEPES

8= RPMI + 10% FCS (Ultraserum) + 4 mM HEPES

9= DMEM + 15% FCS (Mixture) + 2.5 μg STM + 15 μM 2-ME + HT

Media 1 - 6 were also supplemented with HT

2-ME = 2-mercaptoethanol

STM = *Salmonella typhimurium* mitogen

To check the class of immunoglobulin produced by the hybridoma cell line Ouchterlony assays (170) were carried out using Ouchterlony plates containing 1.5 % agarose in phosphate buffer (Meloy, Springfield, VA). Briefly 0.02 ml of anti-mouse immunoglobulin (IgG) was added to the central well and hybridoma cell supernatant, ascitic fluid or affinity purified antibody was added to each of the surrounding wells and the plates incubated at 37°C. The plates were inspected after 4-24 hours for formation of precipitin arc.

The antibody produced by the cell line was found to be IgG type. Surprisingly the antibody crossreacted with both unconjugated anti-rabbit and anti-mouse IgG (Table XVI). However, the antibody did not react with anti-mouse IgG subtypes when using murine IgG subclass monoclonal kit (The Binding Site, San Diego, CA). Rabbit anti-sera was used as control in all Ouchterlony assays. No subclasses of rabbit IgG are known at present because of the difficulty in inducing myelomas in this species.

TABLE XVI

MRH.3 mAb characterization by Ouchterlony

Unconjugated	MRH.3 reactivity with Anti-rabbit IgG	Anti-mouse IgG
MRH.3 (purified)	+ (6)*	+ (6)
(ascites)	+ (4)	+ (3)
(supernatant)	+ (5)	+ (5)

* The number in parenthesis indicates the number of times the experiment was repeated.

Binding affinity of MRH.3 mAb against a fixed amount of ^{125}I-TNF-α was tested by incubating varying concentrations of mAb with 2.5 nM of radiolabeled TNF-α (Table XVII). Equal amounts (0.025 ml) of mAb and radiolabeled TNF-α were mixed together and incubated at 37° C for 2 hours in the presence of excess amount (25 nM) of unlabeled cytokine. Subsequently 0.01 ml of carrier serum and 0.04 ml of anti-IgG antibody was added to the mixture. After 1 hour at 37°C and 0.5 hour at 4°C the mixture was centrifuged for 15-20 minutes and the supernatant aspirated. The radioactivity in the pellet was counted in a gamma counter.

TABLE XVII A

Binding of mAb MRH.3 to radiolabeled TNF-α

mAb Concentration (μg/ml)	^{125}I-TNF-α Bound cpm	Free cpm	B/F	Average B/F
100	749070	228663	3.28	
	689195	288538	2.39	3.25
	785598	192135	4.09	
10	391277	586456	0.67	
	413011	564722	0.73	0.68
	377711	600022	0.63	
1	55938	921795	0.06	
	64966	912767	0.07	0.07
	61150	916583	0.07	
0.1	20351	957382	0.02	
	26012	951721	0.03	0.03
	28802	948931	0.03	
0.01	21302	956431	0.02	
	21367	956366	0.02	0.02
	24832	952901	0.03	
0.001	25481	952252	0.03	
	22994	954793	0.02	0.02
	21298	956435	0.02	
	-	984400		
	-	969200		
	-	979600		
100*	9382	968351	0.01	
	10022	967711	0.01	0.01
	8921	979600	0.01	

*Mouse immunoglobulin used as a control

TABLE XVII B

Mean concentration of bound and free radiolabeled TNF-α

mAb concentration (μg/ml)	Bound cpm (nM)	Free cpm (nM)
100	741288 (1.43)	236445 (1.07)
10	393400 (0.76)	583733 (1.74)
1	60685 (0.12)	917048 (2.38)
0.1	25055 (0.05)	952678 (2.45)
0.01	22500 (0.04)	955233 (2.46)

The affinity constant (K_D) for the antigen antibody reaction was calculated from the plot of bound ligand (*b*) versus the antibody concentration (Fig. 9). This plot follows Michaelis-Menten kinetics and the dissociation constant (K_D) is calculated from the point where half of the ligand is bound to the antibody (1/2 $[B]_{max}$). At this point the antibody concentration equals K_D (189). The K_D of mAb (MRH.3) for 2.5 nM (0.4 μg) radiolabeled TNF-α was 67 nM (Fig. 9).

Selectivity of the selected mAb was tested by neutralization and immunoprecipitation assays. The results are presented in Table XVIII, Table XIX and Fig. 10.

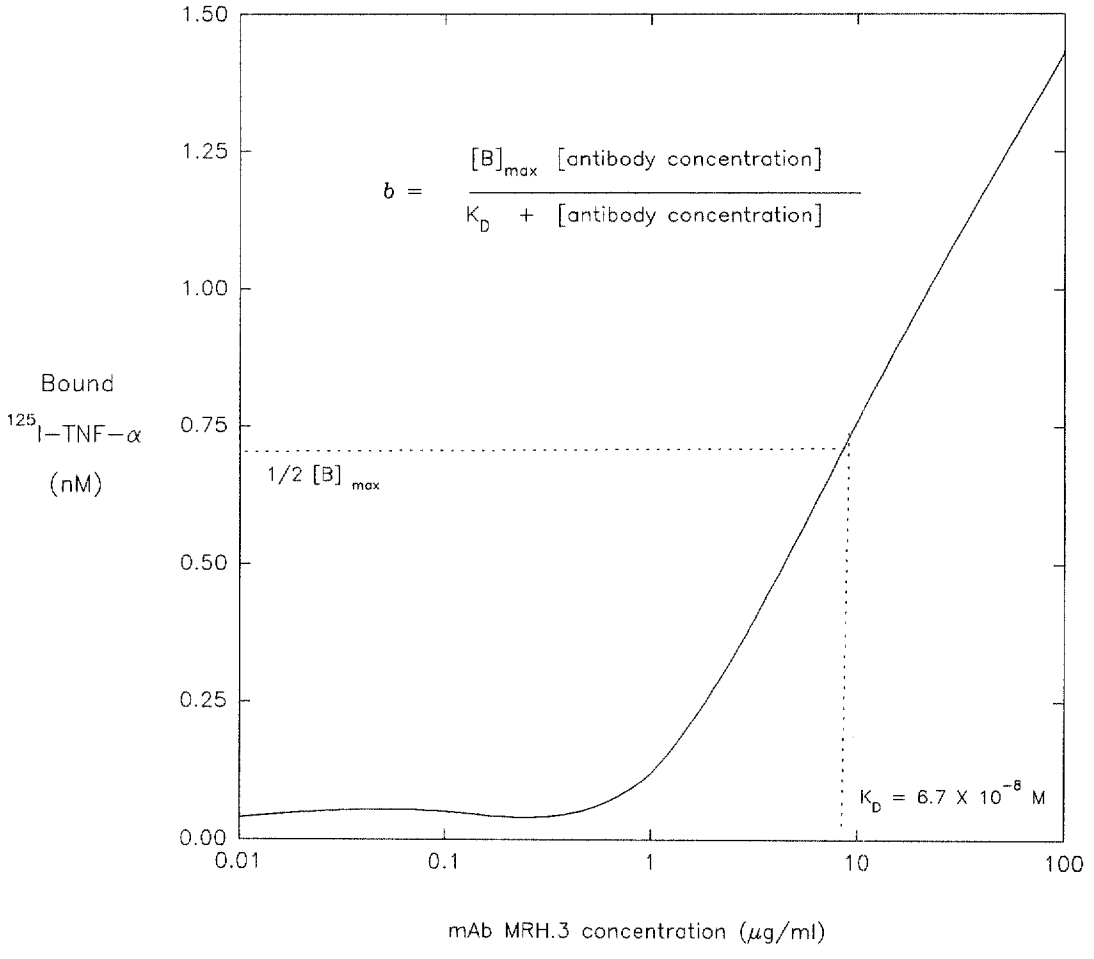


Figure 9

Calculation of MRH.3 dissociation constant (K_D)

TABLE XVIII

Immunoprecipitation of TNF-α by mAb MRH.3

(One day L929 biological cytotoxicity assay)

mAb concentration (μg)	TNF-α Activity (units/ml)	Percentage Precipitation	Average %
Control	5224.35	-	
	7155.81	-	0
	5335.77	-	
mIgG*	4014.42	-	0
mIgG	7185.07	-	
mIgG	4850.76	-	
10	0.00	100.0	
	2.58	100.0	100.0
	0.00	100.0	
5	27.33	99.5	
	75.75	98.7	99.4
	0.00	100.0	
1	433.26	92.7	
	228.03	96.1	92.5
	665.23	88.7	
0.1	3853.08	34.8	
	4194.90	29.0	27.9
	4736.30	19.8	
0.01	4614.52	21.9	
	4249.84	28.0	23.3
	4729.37	19.9	
0.001	4855.01	17.8	
	4337.67	26.6	22.2

* mIgG (mouse immunoglobulin) was used as control

$$\text{Percentage Precipitation} = \frac{\text{(Control Av - Experimental Av)}}{\text{Control Average (Av)}} \times 100$$

TABLE XIX

Neutralization of TNF-α by mAb MRH.3

(One day L929 biological cytotoxicity assay)

mAb concentration (μg/ml)	TNF-α Activity (units/ml)	Percentage Neutralization	Average %
Control	5426.25	-	
	3002.89	-	0
	4600.11	-	
10	388.00	91.1	
	451.40	89.6	91.6
	257.68	94.1	
5	417.87	90.4	
	424.24	90.2	92.0
	206.22	95.3	
1	321.03	92.6	
	455.39	89.5	92.4
	214.96	95.1	
0.1	2134.27	50.9	
	2306.60	46.9	54.0
	1549.37	64.3	
0.01	3349.07	22.9	
	3101.23	28.6	30.5
	2606.01	40.0	
0.001	4461.70	0.0	
	5431.63	0.0	0.0
	4340.83	0.0	

mIgG was used as control

$$\text{Percentage Neutralization} = \frac{\text{(Control Av - Experimental Av)}}{\text{Control Average (Av)}} \times 100$$

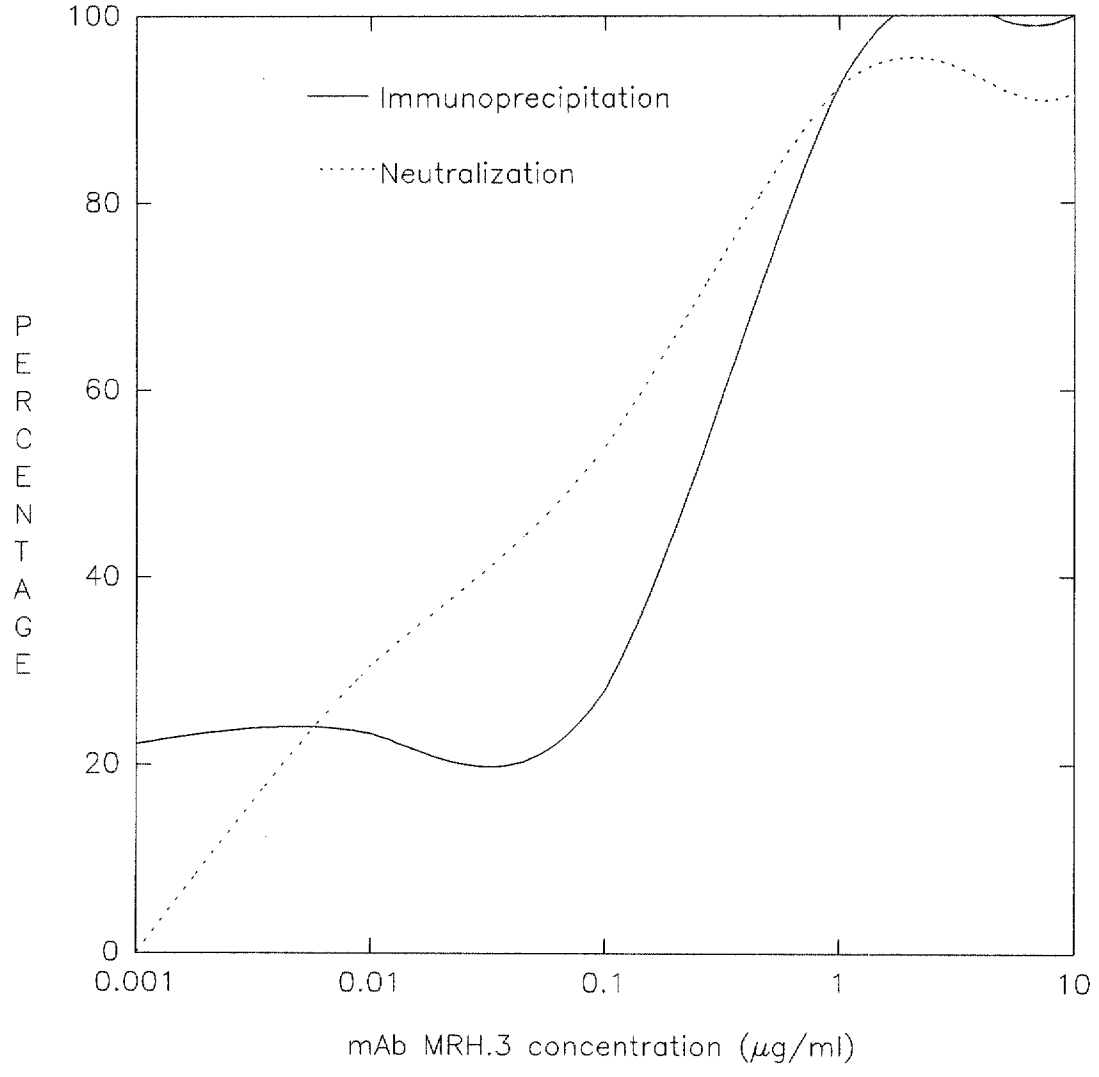


Figure 10

Percentage immunoprecipitation and neutralization of TNF-α by MRH.3

The results indicated an increase in antigen binding parallel to an increase in antibody concentration. An antibody concentration of 1 μg/ml neutralized 250-500 ng (5000 units/ml) of TNF-α. There was a close association between neutralization and immunoprecipitation of TNF-α at various antibody concentrations. However, at lower antibody concentrations (<0.01 μg/ml) there was about 20-30% precipitation but no neutralization.

Immunoprecipitation and neutralization of TNF-ß by mAb MRH.3 was also measured by one day cytotoxicity assays using L929 target cells. Briefly an equal amount of TNF-ß (1000 units/ml) was incubated with various amounts of antibody MRH.3. After 2 hours at 37°C and 30 minutes at 4°C the mixture was appropriately diluted and 0.1 ml was added to the top well in 96 well culture plates seeded with L929 target cells, as discussed earlier. The plates were incubated overnight at 37°C after addition of actinomycin D (5 μg/ml) and cell viability was checked by gentian violet staining. Each experiment was run in triplicate and the percentage immunoprecipitation and neutralization for each antibody concentration were obtained from corrected TNF-ß titers after computer assisted analysis (73). MRH.3 did not immunoprecipitate or neutralize TNF-ß (Tables XX and XXI and Fig. 11).

TABLE XX

Immunoprecipitation of TNF-ß by mAb MRH.3

(One day L929 biological cytotoxicity assay)

mAb concentration (μg)	TNF-ß Activity (units/ml)	Average ± SD	Percentage Precipitation
Control	954.71 900.79 871.99	909.16 ± 42.00	
20000 ng/ml	875.39 840.57 766.35	827.44 ± 55.69	8.99
2000 ng/ml	849.31 848.54 616.65	771.50 ± 134.10	15.14
200 ng/ml	981.87 907.71 777.35	888.98 ± 103.54	2.22
20 ng/ml	849.39 915.91 729.23	831.51 ± 94.62	8.54
2 ng/ml	765.97 1030.33 738.87	845.06 ± 161.02	7.05

For the assays 1000 units of TNF-ß were incubated with various concentrations of mAb MRH.3 for 2 hours at 37°C and 30 minutes at 4°C to reduce nonspecific binding. The antigen antibody mixture was immunoprecipitated as outlined elsewhere and the lymphotoxin activity measured by L929 assay using gentian violet staining.

TABLE XXI

Neutralization of TNF-ß by mAb MRH.3

(One day L929 biological cytotoxicity assay)

mAb concentration (µg)	TNF-ß Activity (units/ml)	Average ± SD	Percentage Precipitation
Control	1099.21 946.31 1113.17	1052.90	
20000 ng/ml	1126.76 940.28 998.57	1021.87 ± 95.40	3.00
2000 ng/ml	1060.26 850.72 906.36	939.11 ± 108.54	10.81
200 ng/ml	1109.39 960.07 948.62	1006.03 ± 89.70	4.45
20 ng/ml	1260.62 1004.98 1065.80	1110.47 ± 133.55	0
2 ng/ml	1386.33 995.41 1248.39	1210.04 ± 198.26	0

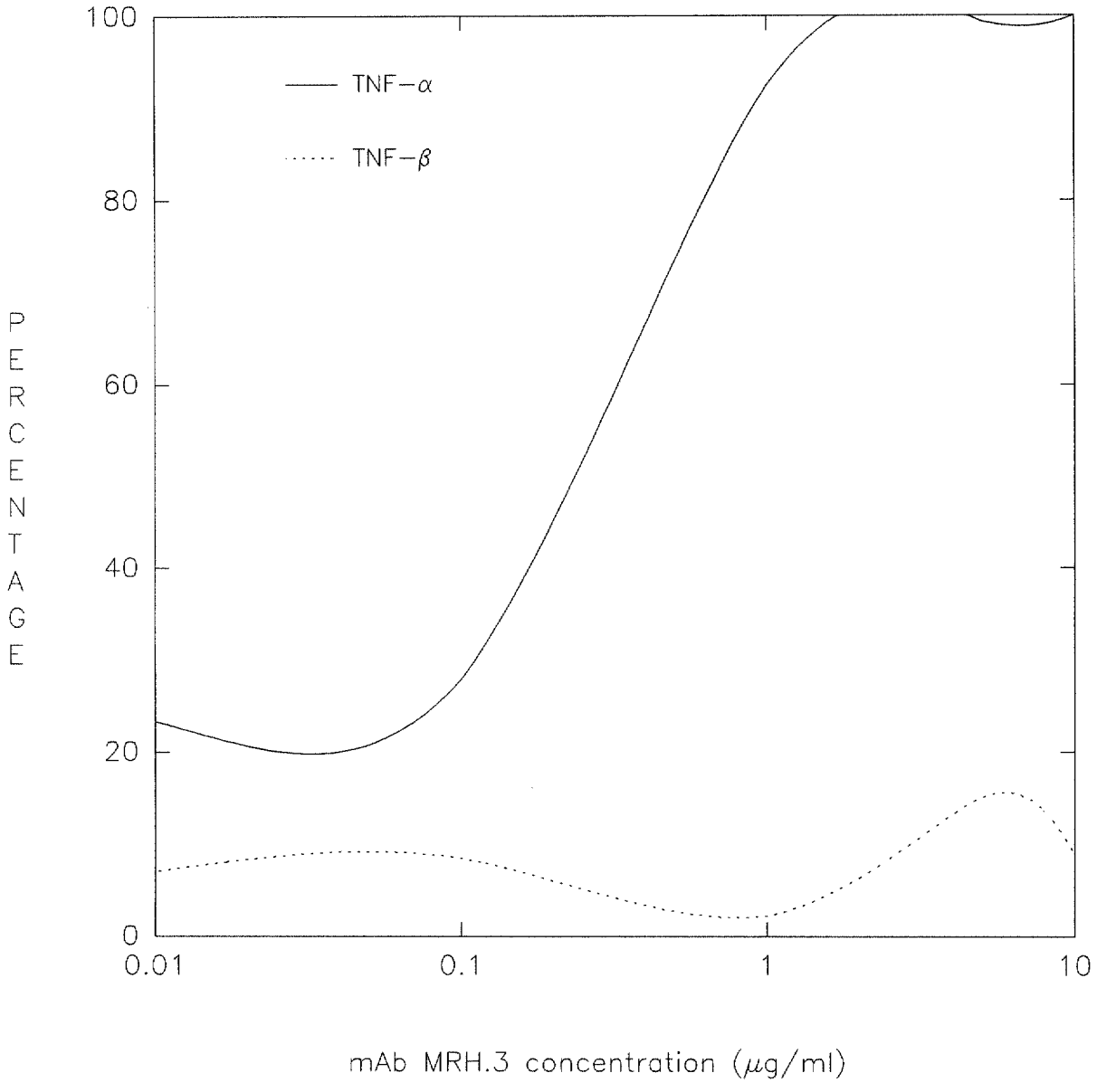


Figure 11

Comparison of percentage immunoprecipitation of

TNF-α and TNF-ß by mAb MRH.3

The results presented in Table XX and Table XXI, indicated a 11% neutralization and a 16% immunoprecipitation of TNF-ß, which could be attributed to non-specific binding (TABLE XX). Comparison of TNF-α and TNF-ß immunoprecipitation by different concentrations of MRH.3 are illustrated in Fig. 11.

In order to exclude the possible neutralization of LPS by the mAb (MRH.3), LPS induced cellular proliferation of BALB/c spleen cells in the presence or absence of mAb MRH.3 was performed. Cellular proliferation was quantified by measurement of exogenous radiolabeled thymidine uptake by the splenocytes obtained from two female BALB/c mice (187). The results (Table XXII, Fig. 12) indicated that the mAb MRH.3 did not effect the LPS induced cellular proliferation of BALB/c spleen cells.

The mean thymidine uptake by splenocytes obtained from the first mouse (Experiment 1) were highly significantly different among the groups (one-way parametric AOV, $F = 138.28$, $p < 0.0001$) and were separated into three statistically significant groups (Tukey's MRT, $\alpha = 0.05$). Control mean was less than the mean thymidine uptake by non-stimulated spleen cells in the presence of mAb (MRH.3) only.

TABLE XXII

Effect of mAb (MRH.3) on thymidine uptake by LPS stimulated BALB/c mouse spleen cells

Control (cpm)	MRH.3	LPS	LPS+mIgG	LPS+MRH.3
Experiment 1:				
316	1034	3090	3450	3322
770	1139	3411	3150	2826
327	906	3401	3395	2966
Mean 471.0 <	1026.3 <	3300.7 =	3331.7 =	3038.0
(Tukey's MRT, α = 0.05)				
Experiment 2:				
220	359	1887	1627	1734
216	524	1900	2097	2021
231	757	1267	1971	1867
Mean 222.3 =	546.7 <	1684.7 =	1898.3 =	1874.0
(Tukey's MRT, α = 0.05)				

MRH.3 = MRH.3 only (10 μg/ml)

LPS = LPS only (1 μg/ml)

LPS+mIgG = LPS (1 μg/ml) + mouse IgG (10 μg/ml)

LPS+MRH.3 = LPS (1 μg/ml) + MRH.3 (10 μg/ml)

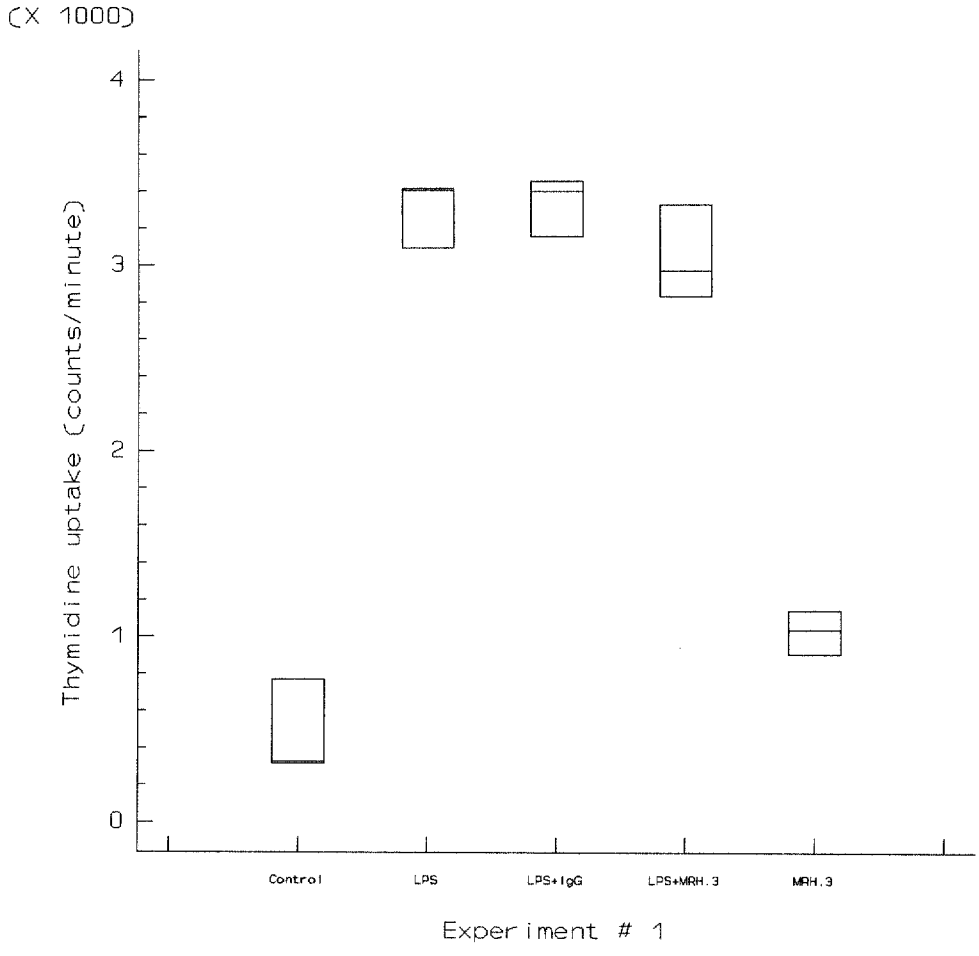


Figure 12 A

Effect of MRH.3 on thymidine uptake by LPS stimulated BALB/c splenocytes

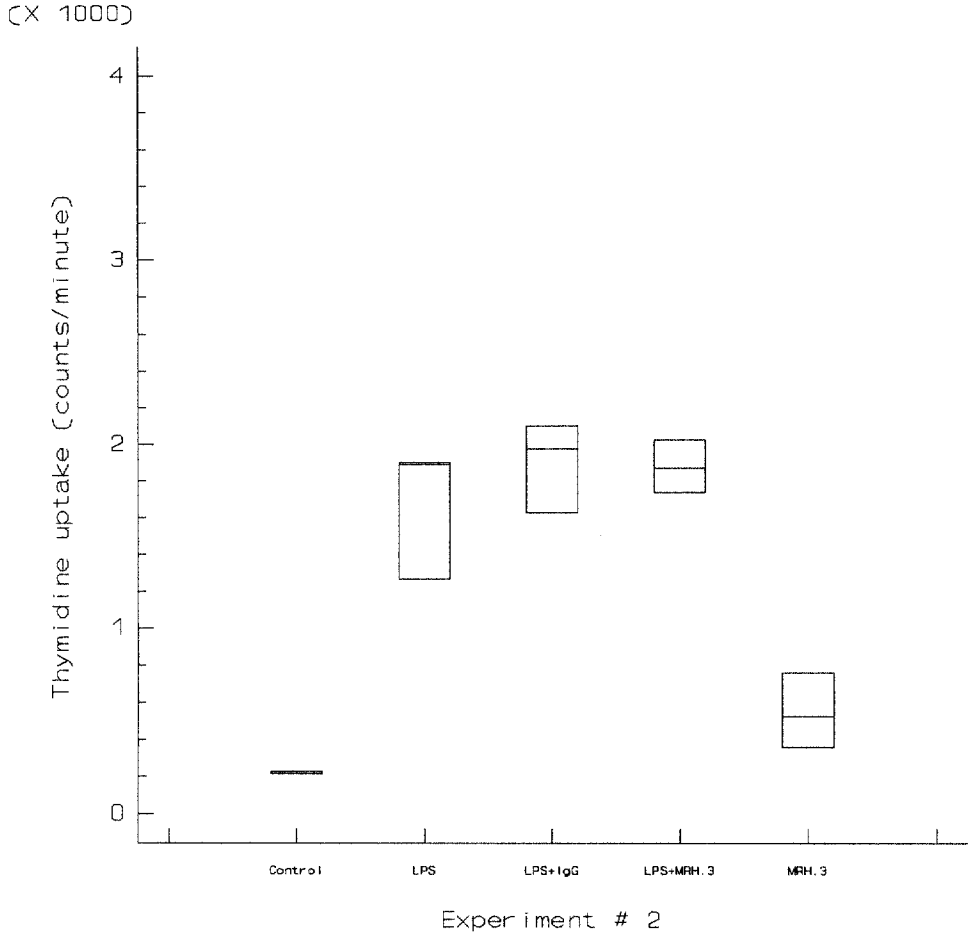


Figure 12 B

Effect of MRH.3 on thymidine uptake by LPS stimulated

BALB/c splenocytes

Both means were significantly less than the mean thymidine uptake in the presence of LPS and there was no significant difference among the mean thymidine uptake of LPS stimulated splenocytes alone, or in the presence of mouse IgG (LPS + mIgG), or MRH.3 (LPS + MRH.3) (Fig. 12A).

In the second mouse (experiment 2, Fig. 12B) the mean thymidine uptake by BALB/c splenocytes were highly significantly different among the groups (one-way parametric AOV, $F = 38.13$, $p < 0.0001$) and were separated into two statistically significant groups (Tukey's MRT, $\alpha = 0.05$). Control and mean thymidine uptake by non-stimulated spleen cells, in the presence of MRH.3 only were significantly less than the mean thymidine uptake in the presence of LPS and there was no significant difference among the mean thymidine uptake of LPS stimulated splenocytes alone, or in the presence of mouse IgG (LPS + mIgG), or MRH.3 (LPS + MRH.3).

The difference between the mean thymidine uptake in the presence of mAb alone can be explained on the inherent variation of the immune response to mitogens in different individuals. Foreign proteins elicit mitogenic responses in hypersensitive individuals and treatment of mouse spleen cells with rabbit mAb can elicit such a response.

Antibody sandwich ELISA was used to test the specificity of MRH.3 and to exclude its binding to related cytokines. ELISA plates coated with rabbit anti-TNF-α

antisera were employed in the assay. Binding of the mAb was tested against human and mouse recombinant TNF-α, native TNF-ß, recombinant G-CSF, M-CSF, GM-CSF, IL-1ß, IL-2, IL-3, native α-interferon and IFN-τ. The ELISA was positive for native and recombinant human and mouse TNF-α and it also gave a weakly positive reaction to IFN-τ. None of the other cytokines gave a positive color reaction after addition of the substrate. This indicated that the mAb is very specific and does not exhibit cross reactivity with closely related cytokines like TNF-ß, interleukins (IL-1 and IL-2) and colony stimulating factors.

Interferon cytotoxicity assays were set up to exclude the possibility of the mAb neutralizing IFN-τ. To test for neutralization equal amounts of IFN-τ (5000 units /ml) and MRH.3 (100 μg/ml) were incubated together and added to the WISH target cells growing in 96 wells culture plates on day 2. IFN-τ diluted with equal amount of supplemented RPMI was used as a control. All samples were serially diluted and the plates incubated overnight at 37°C. The VSV suspension was added to the plate on day 3 and the cytopathic effect observed by gentian violet staining. The results show (Table XXIII) that the mAb does not neutralize IFN-τ, although it does bind the cytokine as demonstrated by immunoprecipitation.

TABLE XXIII

Neutralization and immunoprecipitation of IFN-τ by mAb MRH.3

Control (units/ml)

5160.88 5009.39 4938.63 Mean ± SD = 5036.30 ± 113.54

	IFN-τ Activity (units/ml)	Neutralization (Percentage)	IFN-τ Activity (units/ml)	Immunoprecipitation (Percentage)
1.	5321		3443	
	4836	0.00%	4810	13.90%
	5140		4753	
	5099.67 ± 245.03		4336.08 ± 773.43	
2.	4770		2040	
	4371	5.67%	3863	36.56%
	5110		3681	
	4750.85 ± 369.84		3195.14 ± 1004.07	
3.	4812		3761	
	4316	10.62%	3236	32.58%
	4375		3188	
	4501.50 ± 270.85		3395.55 ± 32.58	

TABLE XXIV A

MRH.3 mAb prevents LPS induced endotoxic shock

(24 hours survival)

Time of Injection	Amount of mAb MRH.3 Injected 250 µg/mouse				500 µg/mouse			
	Alive	Dead	T	%S	Alive	Dead	T	%S
-20 hrs	5	0	5	100	3	2	5	60
0 hrs	3	2	5	60	3	2	5	60
3 hrs	5	0	5	100	2	3	5	40
Controls								
LPS Only	0	4	4	0				
mAb Only	3	0	3	100	3	0	3	100

In preliminary animal experiments each female BALB/c mouse (weighing 16-20 gm), except mAb controls, was injected with 1.5 mg *E. coli* LPS (serotype 055:B5 TCA extracted) i.p. at 0 hour. This was prepared from a stock solution of 5 mg LPS/ml. The LPS was diluted in 0.9% bacteriostatic saline.

LD_{50} for female BALB/c mice as previously determined was 738 ± 162 μg/mouse for LPS and the dose chosen was greater than the lethal dose. The aim was to give a dose that was capable of killing all animals and to observe whether the mAb has any effect on the animal survival. Mouse survival was measured at 24 and 48 hours and 7 day intervals. There was no difference in 48 hours and 7 day survival in any of the groups. All the LPS controls died within 48 hours.

In the initial study the effect on percentage survival of the test animals of two different doses of the mAb MRH.3 (250 and 500 μg/ml/mouse) were compared (Table XXIV A, Table XXIV B). The mAb was purified by affinity chromatography using a Bio-Rad Affi-Gel protein A column. The eluate was subsequently dialyzed against 0.9% physiological saline.

The preliminary animal experiments showed that the mAb MRH.3 by itself was non-toxic and that it did increase the survival chances from LPS induced endotoxemia. The 24 hours survival was comparable between the two different doses of mAb, but a greater percentage of mice receiving the higher (500 μg/mouse) mAb dose survived after 48 hours (40-60% as compared to 20%). The study also demonstrated that the chances of survival were improved if the mAb was injected at the same time as the LPS (0 hrs) as compared to 20 hours before (-20 hrs) or 3 hours later (3 hrs).

TABLE XXIV B

MRH.3 mAb prevents LPS induced endotoxic shock

(48 hours survival)

Time of Injection	Amount of mAb MRH. 3 Injected 250 μg/mouse				500 μg/mouse			
	Alive	Dead	T	%S	Alive	Dead	T	%S
-20 hrs	1	4	5	20	0	5	5	0
0 hrs	1	4	5	20	3	2	5	60
3 hrs	0	5	5	0	2	3	5	40
Controls								
LPS Only	0	4	4	0				
mAb Only	3	0	3	100	3	0	3	100

In the next stage percentage survival of female BALB/c mice was compared after injection of 500 μg of mAb MRH.3 at the same time or 3 hours after injection of a lethal dose of LPS (Table XXV). As before each female BALB/c mouse, except mAb controls, was injected with 1.5 mg LPS i.p. at 0 hour. A mouse mAb (30.10.E) against human lymphokine activated killer (LAK) cell surface antigen was used as a control (Group 3). Groups 1 and 2 received mAb MRH.3 injection at the same time or 3 hours after they were exposed to LPS.

TABLE XXV

BALB/c survival from LPS induced endotoxic shock

Group	Alive	Dead	Total	%Survival	X^2	p[b]
24 hours survival						
1. 0 hrs (MRH.3)	15	5	20	75%	20.42	6.2×10^{-6}
2. 3 hrs (MRH.3)	2	18	20	10%	0.36	0.55
3. 0 hrs (30.10.E)	8	12	10	40%	7.03	8.0×10^{-3}
48 hours survival						
1. 0 hrs (MRH.3)	12	8	20	60%	13.79	2.1×10^{-4}
2. 3 hrs (MRH.3)	1	19	20	5%		
3. 0 hrs (30.10.E)	7	13	20	35%	5.63	0.02
CONTROLS						
LPS Only	1	19	20	5%		
MRH.3	10	0	10	100%		

X^2 values compared to control in a 2 X 2 contingency test.

b: probability relative to LPS control group

At 24 hours 75% of mice in group 1 survived and by 48 hours the percentage of surviving mice was reduced to 60% (Table XXV). Only 5% of the mice survived in the group that received the antibody 3 hours after exposure to a lethal dose of LPS (group 2). As shown in Table XXV groups 1 and 3 are significantly different from LPS control group at 24 hours (2 X 2 X^2 contingency test, $X^2 = 20.42$, $p = 6.2 \times 10^{-6}$ and $X^2 = 7.03$, $p = 8.0 \times 10^{-3}$ respectively), indicating that death following intraperitoneal LPS injection was highly significantly contingent upon the use of mAbs MRH.3 or 30.10.E. No improvement in mice survival was observed in group 2 which received mAb injection 3 hours after the lethal dose of LPS (X^2 contingency test = 0.36, p = 0.55).

The discovery of improved survival outlook after i.p. injection of mouse mAb 30.10.E against LAK cells was unexpected. This antibody was intended as a negative control to exclude the possibility that any mAb could alleviate LPS induced shock. At 24 hours there was a significant difference in mice survival between groups 1 and group 3 (X^2 2 X 2 contingency test = 5.01, p = 0.025). However, no statistical difference was observed at 48 hours between 0 hour treatment with 30.10 E (group 3) or MRH.3 (group 1) (X^2 contingency test = 2.54, p = 0.11).

Keeping in view the results from the early animal experiments an extensive animal study was carried out using many controls (Table XXVI). Eight groups of 6-8 weeks old female BALB/c mice (weighing 18-22 gm each) were housed in separate cages and given food and water *ad libitum*. Groups 1-7 containing 20 mice/group were injected with LPS (1.5 mg/mouse) i.p. at 0 hour. The ten mice in group 8 were injected with MRH.3 only (500 μg/ml/mouse).

TABLE XXVI A

Effect of MRH.3 mAb on LPS induced endotoxic shock (24 hours survival)

Time of LPS Injection

Group	Alive	Dead	Total	%Survival
1. LPS Control	5	15	20	25%
2. 0 hrs (MRH.3)	20	0	20	100%
3. 3 hrs (MRH.3)	5	15	20	25%
4. 0 hrs (30.10.E)	15	5	20	75%
5. 0 hrs (MRH.3/30.10.E)	20	0	20	100%
6. 0 hrs (WI-AT-1)	18	1	19	95%
7. 0 hrs (WI-MN-1)	5	15	20	25%
8. MRH. 3 Control	10	0	10	100%

Groups 1-4 were injected 1.5 mg LPS/0.5ml/mouse. Groups 5-7 were injected with 1.5 mg LPS/0.3ml/mouse. The LPS was dissolved in bacteriostatic saline, just prior to use. The reduction in the injected fluid volume was done in order to reduce the discomfort felt by the mice on injecting large fluid volumes i.p. As before the LPS controls (Group 1) died within 48 hours (Table XXVI B).

Groups 2, 4, 6, and 7 were injected with 500 μg of selected mAb i.p. at the same time as LPS injection (0 hour). No mAb was injected in group 1 mice and group 3 recieved the mAb (MRH.3) injection 3 hours after the LPS injection. Mice in group 5 recieved 250 μg each, of two selected antibodies (30.10.E and MRH.3).

Groups 2 and 3 received mAb (MRH.3) against TNF-α. Other groups (except group 1) were injected with antibodies, produced at Wadley Institutes. These antibodies were used as controls. Group 4 recieved mAb developed against human LAK cells (30.10.E). This mouse mAb against human LAK cell surface antigen was used as a control, but the preliminary results showed a statistically significant survival in mice injected with this antibody (Table XXV). Mice in group 5 were injected with 30.10.E and MRH.3 (250 μg of each mAb/mouse) at the same time as LPS injection. This was done in order to study whether any benefit could be gained by use of a combination of antibodies, both of which improve mouse

survival after exposure to a lethal dose of LPS. Mice in group 6 were injected with mouse mAb (WI-AT-1) against human recombinant TNF-α (161), and mouse immunoglobulin against human malignant melanoma (WI-MN-1) was used as a control (Group 7).

TABLE XXVI B

Effect of MRH.3 mAb on LPS induced endotoxic shock (48 hours survival)

Time of LPS Injection

Group	Alive	Dead	Total	%Survival
1. LPS Control	0	20	20	0%
2. 0 hrs (MRH.3)	19	1	20	95%
3. 3 hrs (MRH.3)	3	17	20	15%
4. 0 hrs (30.10.E)	8	12	20	40%
5. 0 hrs (MRH.3/30.10.E)	2	18	20	10%
6. 0 hrs (WI-AT-1)	0	19	19	0%
7. 0 hrs (WI-MN-1)	0	20	20	0%
8. MRH. 3 Control	10	0	10	100%

The results indicate a significant improvement in short and long term mouse survival in the presence of mAb against TNF-α (Table XXVII). All the mice injected with mAb MRH.3 survived after 24 hours and the survival rate was 95% at the end of 48 hours (X^2 = 24, p = 9.6 X 10^{-7} and X^2 = 36.2, p = 1.8 X 10^{-9} respectively). No additional fatality was observed in any of the groups after 48 hours.

None of the mice that were injected with mAb against human recombinant TNF-α (WI-AT-1) survived after 48 hours (group 6, Tables XXVI B and XXVII B). At 24 hours the mice survival in this group was significantly different than LPS controls (X^2 = 19.6, p = 9.6 X 10^{-6}) but by 48 hours the survival rate of this group was similar to that of the LPS and mouse IgG (WI-MN-1) controls (group 1 and group 7). This indicated that antibody against human recombinant TNF-α did not neutralize the mouse TNF-α *in vivo*.

To be effective the mAb has to be injected at the same time or just prior to LPS exposure, as a delay of 3 hours proves fatal (group 2 vs group 3). The survival in mice receiving MRH.3 at the same time as LPS was 95% compared to the 15% survival in mice recieving the mAb 3 hours after LPS exposure. The survival of mice injected with mAb (MRH.3) 3 hours after LPS exposure was not statistically distinct from the LPS control group (X^2 = 3.2, p = 0.07).

TABLE XXVII A

Statistical analysis of the effect of MRH.3 mAb on LPS induced endotoxic shock

24 hours survival (Data from TABLE XXVI A)

Group	Alive	Dead	X^2 value	p	
1. Control	5	15			
2. (MRH.3)	20	0	24.0	9.63×10^{-7}	S
3. (MRH.3)	5	15	ND		NS
4. (30.10.E)	15	5	10.0	1.57×10^{-3}	S
5. (MRH.3 + 30.10.E)	20	0	24.0	9.63×10^{-7}	S
6. (WI-AT-1)	18	1	19.6	9.62×10^{-6}	S
7. (WI-MN-1)	5	15	ND		NS

The X^2 values were obtained by comparison of each group with LPS control group (group 1) in a 2 X 2 X^2 contingency test and the probability was relative to the LPS controls. S or NS denotes whether the groups were significant or not significant respectively.

TABLE XXVII B

Statistical analysis of the effect of MRH.3 mAb on LPS induced endotoxic shock

48 hours survival (Data from TABLE XXVI B)

Group	Alive	Dead	X^2 value	p	
1. Control	0	20			
2. (MRH.3)	19	1	36.2	1.79×10^{-9}	S
3. (MRH.3)	3	17	3.2	0.07	NS
4. (30.10.E)	8	12	10.0	1.57×10^{-3}	S
5. (MRH.3 + 30.10.E)	2	18	2.1	0.15	NS
6. (WI-AT-1)	0	19	ND		NS
7. (WI-MN-1)	0	20	ND		NS

The X^2 values were obtained by comparison of each group with LPS control group (group 1) in a 2 X 2 X^2 contingency test and the probability was relative to the LPS controls. S or NS denotes whether the groups were significant or not significant respectively.

The mAb 30.10.E is also effective in improving mouse survival after exposure to LPS and combination of the two antibodies (MRH.3 and 30.10.E) seems to work during the first 24 hours (group 5, Tables XXVI A and XXVII A). This protective effect was not significant by 48 hours (X^2 = 2.1, p = 0.15). There is no improvement in survival after injection of mAb WI-MN-1, indicating that the improved mouse survival in groups 2 was not just due to the injection of any kind of mAb.

All these results implicate TNF-α as one of the major cytokine mediators of septicemic shock and offer a possible avenue for treatment of this serious ailment. Septicemic shock is a life-threatening disease and timely intervention, possibly with immuno-reagents may prove to be the difference between life and death.

CHAPTER 4

DISCUSSION

The aim of the project was to study the role of TNF-α in endotoxin shock and to indicate whether it is the major host mediator in septicemia. This goal was achieved by study of the effects of rabbit anti-murine TNF-α mAb in mouse models with regard to its role in preventing endotoxin shock.

Septicemia ranks thirteenth among the causes of death in the United States, especially in the elderly and is one of the leading causes of death in the under developed countries, both in adults and children (190). Septic shock refers to the clinical evidence of infection associated with signs of systemic response (191). These signs include tachypnea, tachycardia, hyperthermia, hypothermia, hypoxemia, oliguria, biochemical abnormalities, altered mentation and hypotension. The septic shock is considered refractory if it lasts for more than an hour and does not respond to intravenous supportive therapy and antibiotic intervention. Endotoxin shock refers to septic shock associated with Gram-negative bacteremia.

The association between TNF-α and endotoxic shock has been the subject of many publications (5, 8, 9, 13, 14, 16, 17, 19-21, 192-194). My study supports previous reports that TNF-α is produced in response to LPS stimulation both *in vitro* and *in vivo*, and preliminary animal studies in our laboratory and from other centers suggest that TNF-α maybe the major host factor involved in septicemic shock.

The LD_{50} for intraperitoneally (i.p.) injected lipopolysaccharide (LPS), obtained by TCA extraction from *E. coli* strain 055:B5, was 738 ± 162 μg/mouse. This is much higher than earlier reported. Possible explanations for this effect could be the different sources of the LPS, the purity of the mitogen and the different routes of administration. Highly purified LPS may not be as mitogenic as crude LPS extracts, such as those obtained from Difco laboratories that were used in early studies (161). The presence of contaminants in the LPS extract may contribute to variability in the lethal doses.

Preliminary studies confirmed the production of TNF-α in response to *in vitro* stimulation by LPS and *in vivo* injection of LPS in 4-6 weeks old BALB/c females.
The serum levels of TNF-α *in vivo* correlates with the fatal outcome of LPS-induced shock and the development of adverse reactions, like shivering, diarrhea and anorexia. Loss of

weight is usually not apparent as it is a long term manifestation of exposure to TNF-α (9).

Production of natural mouse TNF-α utilized LPS stimulated mouse monocyte RAW 264.7 cell cultures. RAW 264.7 cells, stimulated with LPS at a concentration of 10 μg/ml, were found to produce consistently higher TNF-α titers. There was no statistical difference between LPS production at concentrations of 10, 25 or 50 μg/ml. Higher concentrations of LPS are toxic to the cells and increase the expense. In addition not much advantage was gained by using LPS at concentrations of 100 μg/ml in tissue cultures. The TNF-α production was erratic (4284.3 ± 4712.5 units/ml, Table IX). Some of the secreted cytokine may have been broken down by proteolytic enzymes released on cell death due to the higher LPS concentrations.

Contrary to published literature a much larger mitogen (LPS) dose was required to produce detectable levels of TNF-α. Beutler *et al.*, produced comparable TNF-α titers by using ten times less LPS for RAW 264.7 cell stimulation (53). The same LPS concentration (1 μg/ml) produced a mean TNF-α titer of 102 ± 82.2 units/ml (Table IX). One reason for this may have been use of LPS from a different manufacturer (Sigma Chemical Company vs Difco Laboratories). In addition a different biological assay was used for detection of TNF-α titers.

During mass production of mouse TNF-α it was found that the native protein produced by RAW 264.7 cells was not very stable on storage. The cytokine lost biological activity on storage at -20°C, and repeated freezing and thawing led to a remarkable drop in biological activity as detected by L929 biological cytotoxicity assays.

The biological activity was also reduced after filtration (independent t test, $p = 0.04$, Table X). Earlier studies done at our laboratory with human recombinant TNF-α had not revealed this fact. In order to maintain biological activity of TNF-α it was required that the culture supernatants be stored at 4°C, and the purified protein stabilized in albumin (0.1% w/v).

Approximately 6 liters of RAW 264.7 cell supernatant containing secreted TNF-α were passed through the affinity column and 2.8 mg murine TNF-α was purified utilizing affinity chromatography as detailed in materials and methods. The affinity column utilized goat anti-human recombinant TNF-α polyclonal antibody bound to cyanogen bromide activated Sepharose 4B. There was strong cross reactivity between the crude mouse TNF-α and the polyclonal antibody. Cytokine purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 20% resolving gel.

The affinity purified TNF-α was subsequently used for immunizations of two female New Zealand white rabbits. The aim was to eventually develop a mAb against murine TNF-α using rabbit splenocytes and mouse myeloma cells. Rabbits were chosen because soluble antigens gave good immune response in these animals. In addition immunization and repeated blood collection are relatively simple in rabbits (179). As rabbits are not known to have myeloma, a mouse myeloma cell line was used to perpetuate the hybridoma (162-164).

Prior to fusion a one day L929 biological cytotoxicity assay was perfected and standardized. The aim was rapid detection of antibody secreting hybrids so that they could be cloned as quickly as possible in order to avoid loss of clones due to contamination. In the initial fusion the hybrid cell culture supernatants were screened by indirect antibody ELISA, but this was abandoned later on in favor of the biological assay. The biological assay was preferred as it could detect antibodies that neutralized TNF-α whereas ELISA could only tell us whether the antibody recognized the cytokine or not.

The fusion of rabbit spleen cells and mouse myeloma cells was mediated by PEG 4000. Modification of conventional techniques developed by Köhler and Milstein were employed for production of stable, antibody secreting

mouse-rabbit hybrids (153, 195). Success with these techniques had been reported earlier (162, 164). Splenocytes obtained from rabbits that showed a high anti-TNF-α antisera titers were fused with non-secreting mouse myeloma cell line Sp2/0-Ag14 to develop a mAb that neutralized murine TNF-α. The results of the two fusions were presented in Table XIV. Although both fusions were initially successful, stabilized antibody producing clones were obtained only after the second attempt.

The use of normal rabbit serum supplemented medium during initial culture and the presence of feeder cells yielded stabilized monoclonal hybridoma as has been previously reported (164). These hybridomas are also more stable as regards antibody secretion compared to those cultured in the presence of fetal calf serum.

Secretion of B cell growth factors such as, interleukin-4 and interleukin-6, maybe involved in the cellular interaction. Although Raybould and Takahashi reported better results with peritoneal exudate feeder layers this could not be confirmed due to the contamination of the plates containing these feeder layers (164).

The mAb was propagated by i.p. injection of hybrid cells into BALB/c mice that were primed with pristane (168). The ascitic fluid containing large quantities of the antibody was collected after 4-5 weeks. The long duration

for development of ascites may be related to the fact that the mouse-rabbit hybrid cells were being propagated in the peritoneal cavity of mice.

The mAb was purified by affinity chromatography using a protein A affinity column (165). The purity of the antibody was checked by SDS-PAGE on a 15% polyacrylamide resolving gel.

Ouchterlony assays indicated that the selected mAb (MRH.3, mouse-rabbit hybrid 3) was a rabbit IgG. No subclasses of rabbit IgG are presently known. The observation of the mAb reaction with both unconjugated sheep anti-rabbit IgG and goat anti-mouse IgG was unexpected. The hybrid cell culture supernatant, ascitic fluid and purified mAb all gave positive precipitin arcs (Table XVI). This could be explained on the basis of cross reactivity of the rabbit IgG with both anti-rabbit IgG and anti-mouse IgG. Both these reagents were polyclonal and not species specific. The possibility of cross reactivity was also supported by the appearance of broad bands against anti-rabbit IgG.

In addition contamination of ascitic fluid with mouse blood during collection and/or the presence of mouse IgG in the mouse peritoneal cavity could not be excluded. The protein A column used for rabbit mAb purification was previously used for purification of mouse IgG and some of

that may have eluted out with the rabbit IgG. However, this should not have been a problem after a couple of runs. The possibility of antibody secretion by the mouse myeloma cell line (Sp2/0-Ag14), used as fusion partner, was, however, remote. These cells are non-secreting myeloma cells, which makes them the cell line of choice for hybridoma production (156). Use of a murine IgG subclass monoclonal kit showed that the antibody did not react with any of the anti-mouse IgG subclasses. Chromosomal analysis of the cell line may have to be carried out to identify the metacentric rabbit chromosomes and conclusively settle the argument (164).

The selectivity of the mAb MRH.3 for TNF-α was studied by employing radioimmunoassays (RIA), antibody sandwich enzyme linked immunosorbent assays (ELISA), biological cytotoxicity assays and by measurement of thymidine uptake by BALB/c splenocytes. The dissociation constant (K_D) of the mAb for TNF-α was 6.7 X 10^{-8} M (Fig. 9), indicating a high affinity. Affinity is defined as the strength of a single antigen-antibody bond and is a measure of a good fit between the antigenic determinant and the antibody binding site (159).

The mAb neutralized and precipitated 50% of the TNF-α (5000 units/ml) at a concentration of 0.1 μg/ml. More than 90% neutralization and immunoprecipitation of TNF-α

activity, as measured by one day biological assays with L929 target cells, occurred at concentrations of 1 μg/ml. The antibody immunoprecipitated TNF-α at low concentrations (0.001 μg/ml) but did not neutralize TNF-α (5000 units/ml) at this concentration. The antibody did not neutralize TNF-ß, a related cytokine product of a gene that is closely associated with the gene for TNF-α. Both genes are located near the MHC region in mice and HLA region in humans (59, 60). TNF-ß has 51% homology with TNF-α and 35% of the amino acids are identical between the two cytokines (60).

Surprisingly the antibody precipitated IFN-τ. None of the other interferons, interleukins or colony stimulating factors reacted with the mAb. The antibody bound IFN-τ, but did not neutralize its biological activity (Table XXIII). Less than 10% of IFN-τ activity (5000 units/ml) was neutralized by high concentration of mAb (100 μg/ml). Neutralization of IFN-τ by MRH.3 would have presented a problem with interpretation of data from animal studies. Both IFN-τ and TNF-α are produced by cells of the immune system and have overlapping biological effects (36, 108). IFN-τ is not known to cause septic shock but it does influence the synthesis of TNF receptors and interleukins (58).

Antibodies neutralizing TNF-α have been mapped to the linear epitopes comprising the connecting loops. These antibodies interfere with biological activity or receptor binding and have been mapped to two amino acid regions, a region between amino acids 1-15 and arginine residue at position 131 (79). It is possible that the MRH.3 interaction with murine TNF-α involves these regions.

There is very little homology between TNF-α and IFN-τ and immuno-precipitation of both these cytokines by MRH.3 may shed some light on the amino acid residues involved in the antigen antibody reaction. Two short amino acid sequences are conserved between the two molecules (Leu-Ile-Tyr-Ser, L-I-Y-S and Lys-Val-Asn, K-V-N). The amino acid sequence L-I-Y-S is located between amino acid 57-60 in TNF-α and is present in reverse orientation in IFN-τ (6-9). The K-V-N tri-peptide sequence is conserved between the two cytokines in a similar orientation. This sequence lies between amino acids 90-92 in TNF-α and 81-83 in IFN-τ. It is possible that the mAb binds either of these sequences. Since most of the amino acids are hydrophilic this part of the molecule would be accessible to the immune system. Also the K-V-N sequence lies close to amino acid 86 in TNF-α. Mutation in this amino acid residue leads to loss of biological activity of TNF-α (77). Binding of MRH.3 to one of these sequences may disrupt the three dimensional

structure of TNF-α, making it biologically inactive. These amino acid sequences are not present in TNF-ß which could explain why the mAb does not precipitate it.

The antibody does not neutralize endotoxin (LPS). This was studied by measuring thymidine uptake of LPS stimulated BALB/c splenocytes in the presence or absence of MRH.3 (Table XXII). There was no significant difference among the mean thymidine uptake of LPS stimulated splenocytes alone or in the presence of mouse IgG or MRH.3 (Fig. 12). Survival of mice injected with MRH.3, after exposure to LPS, could not be interpreted as being due to neutralization of LPS by the antibody.

Current treatment of septic shock revolves around supportive measures like intravenous hydration and use of appropriate antibiotics. The use of glucocorticoids (methylprednisolone sodium succinate) is controversial (196, 197). They have been shown to suppress TNF-α induced expression of surface adhesion proteins that may be involved in mediating the inflammatory response (198), but are not recommended for use in septic shock (196). The use of nonsteroidal anti-inflammatory drugs and cyclooxygenase inhibitors (indomethacin and ibuprofen) in septicemia is also limited. These drugs block thromboxane and prostaglandin synthesis and may effect only part of the septicemic cascade (199, 200).

Animal studies have demonstrated the efficacy of immunotherapy in septicemic shock. Tracey *et al.* demonstrated the benefits of using murine monoclonal anti-TNF-α antibody in baboons (201) and similar results have been reported by use of hamster anti-TNF-α mAb (202). The same results were obtained by use of anti-TNF-α antibodies in rabbits, BALB/c mice and humans (161, 203). Beutler and his colleagues found that mice passively immunized against TNF-α were protected against the lethal effects of LPS (8, 9, 57). Some studies indicated that the effect of anti-TNF alpha antibodies was mediated via reduction of IL-1 production and these are being evaluated at present (150).

My study corroborated the results of previous animal studies. The monoclonal anti-TNF-α antibody offered protection from the lethal effect of LPS. Mouse survival was measured at 24 and 48 hours interval. Mice surviving at 48 hours were considered protected as there were no further fatalities after day 7. After 48 hours 60-95% of the mAb MRH.3 treated mice survived the lethal LPS injection (Tables XXIV-XXVII). None of the controls survived beyond 48 hours. Death following i.p. lethal LPS injection was highly significantly contingent upon the use of MRH.3 (2 X 2 X^2 contingency test $p = 1.8 \times 10^{-9}$).

There was a discrepancy in the amount of mAb required to neutralize TNF-α activity *in vitro* and the quantity effective improving the percentage survival in LPS injected mice *in vivo*. According to the *in vitro* studies the mAb inhibited the lytic activity of TNF-α at an antibody input of 0.2 ng/unit. However, 500 μg of mAb/mouse injected i.p. was effective in extending the murine life span. Earlier experiments had shown that injection of lethal doses (1.5 mg/mouse) of LPS in five BALB/c mice produced a mean serum TNF-α titer of 44.35 units/ml within 24 hours (Table VIII). Assuming a 3 ml circulating blood volume in each mouse the total TNF-α titer was still too low to require 500 μg mAb/ mouse for a protective effect from a lethal dose of LPS.

There are many reasons for this difference between *in vitro* and *in vivo* results. The measurement of serum TNF-α levels *in vivo* at any given time may not represent the total TNF-α actually being produced in the mouse. Large amount of the cytokine may be sequestered in the interstitial fluid, and some may be in association with TNF receptors in circulation or on the cells such as granulocytes.

Not all of the i.p. injected antibody would be available for TNF-α neutralization. Some of it may have been broken down by proteolytic enzymes or taken up by

peritoneal macrophages, that have specialized receptors for IgG (F_c^r receptors), and phagocytic cells in the general circulation.

The mAb WI-AT-1 did not improve mice survival over 48 hours, although it did offer protection during the first day. This was in contrast to the earlier report which indicated a 44% survival in mice receiving WI-AT-1 (200 μg/mouse). However, those mice had received a lower amount of LPS (400 μg/mouse) that was obtained from a different manufacturer (161).

These results were not surprising because it has been observed that, despite 80% homology at the amino acid level, antibodies raised against human recombinant TNF-α do not cross react with mouse TNF-α. This may be explained by the fact that the most conserved sequences of the TNF-α molecule are the ones that form the hydrophobic core residues that lie within the ß sandwich and are effectively concealed from the antibody (79).

A surprise finding in my study was the effectiveness of the antibody (30.10.E) against human lymphokine activated killer (LAK) cells. This antibody was used as an unrelated control to exclude the possibility of the differences in mice survival being due to presence of IgG. However, the results indicated that as compared to control mice the

animals receiving 30.10.E or MRH.3 had a better possibility of surviving a lethal LPS dose. Other experiments comparing effectiveness of mouse IgG (WI-MN-1) (204) with MRH.3 and LAK cell antibody against LPS induced endotoxin shock revealed that non-specific mouse IgG was not effective in increasing survival of mice injected with a lethal dose of LPS.

The 30.10.E mAb prevents TNF-α induced L929 cytotoxicity as well. The possibility that this may be an antibody against TNF receptor can not be excluded. The antibody binds mostly natural killer cell population and these cells are considered to play an important role in immune surveillance against viral diseases and cancer (119, 205).

With the development of immunotherapy the treatment of endotoxin shock is rapidly changing. In addition to the supportive fluid replacement and antibiotics the physicians now have available antibodies against TNF-α and endotoxin. Besides, antibodies against IL-1 receptors are also in the development stages (190, 206-208).

Strong evidence from animal studies suggests that mAb against TNF-α or endotoxin can suppress progress of shock caused by LPS and these studies have now been extended into clinical trials for evaluation of treatment protocols for patients in septicemic shock.

Studies have shown that mAb against TNF-α seem to offer protection against both Gram-positive and Gram-negative infections, and maybe preferred over antibodies against endotoxin (190). This fact has to be borne in mind while treating patients with septicemic shock as mAb against endotoxin have no effect on the outcome of Gram-positive septicemia.

Most of the clinical research has focussed on mAb against endotoxin. Clinical trials with these mAb are currently underway and FDA approval has been granted to Centocor Incorporated for marketing a human anti-endotoxin monoclonal IgM (Centoxin/HA-1A) for treatment of Gram-negative bacteremia and sepsis syndrome. In randomized, double-blind, placebo-controlled studies, 32/261 patients receiving the mAb died as compared to 45/281 recieving the placebo and 33% of the patients suffering from shock died after infusion of the mAb in comparison to 57% of the controls. The study also showed that the reduction in patient mortality was clinically significant only in those patients who suffered from Gram-negative septicemia (207). Another murine anti-endotoxin monoclonal antibody (E5) is also being tested in phase I clinical trials. It has shown to improve outcome of Gram-negative sepsis in patients who are not refractory to treatment (206). Some pharmaceutical companies (Cetus Corp., Chiron, Cutter Biologicals) have

initiated Phase I trials using anti-TNF antisera or mAb in treatment of clinical sepsis and septic shock. The results of these trials have not been published but based on the data accumulated in the preclinical studies the results should be beneficial.

The time of administration of immunotherapy is the subject of much debate. In survival studies on BALB/c mice it was found that the antibody was effective in neutralizing the effect of intraperitoneal LPS *in vivo* if injected at the same time. Injection of the antibody 24 hours before or 3 hours after introduction of LPS did not offer protection, contrary to earlier results (161). The antibody therapy seems to be effective in most cases if given just before or immediately after exposure to endotoxin.

To predict the time and duration of administration of mAb in the clinical setting would be even more difficult. Blood cultures for diagnosis of Gram-negative take a few days to become positive and a delay in initiating therapy could be the difference between life and death in septicemic patients. The counter argument is that early intervention with immunotherapy, without awaiting culture reports, may give the patient and the physician a false sense of security. This could prove fatal if mAb against LPS is being used in a patient who is later found to have Gram-positive shock.

The mAb MRH.3 could also be employed in ELISA or RIA detection kits for diagnosis of increased serum TNF-α levels. Serum TNF-α is elevated in a variety of diseases and there is a positive correlation between the serum TNF-α levels and the outcome of endotoxic shock (140).

Measurements of TNF-α levels in septicemic patients could be a useful early marker and help the physician in deciding when to institute anti-TNF-α immunotherapy. This would also aid in optimizing the dose of the mAb. Studies with anti-endotoxin mAb employed either a single dose (HA-1A) or two doses (E5) given 12 to 24 hours apart (206, 207).

The route of administration for the mAb is usually intravenous. The half life of heterologous antibodies injected intravenously in mice has been demonstrated to be 1-3 days (209, 210). Some animal studies utilized i.p. injections which may prolong the half-life of the antibody. A single injection of MRH.3 (500 μg/mouse) was enough to combat the TNF-α produced by a single lethal injection of LPS. More controlled studies to choose the most therapeutic and the least toxic route of administration are needed.

The reuse of anti-TNF-α mAb in patients who have received prior immunotherapy is also controversial at present. No studies are available indicating the effectiveness or safety of this approach. The reuse of the mAb in surviving mice challanged with another lethal dose

of LPS was not examined in my study. Theoretically, the reactivity towards the Fc region or epitopes distinguishing immunoglobulin classes can lead to development of human antibodies. These antibodies potentially vitiate the future use of any mAb produced in rabbits and is a contraindication for the repetition of mAb therapy. In the case of anti-TNF-α antibody this is not considered to be a major hurdle as the therapy is given over a short time, but it may jeopardize any further therapy utilizing the antibodies from the same species, even if it was for a different purpose (211).

The efficacy of TNF-α as a non-specific anti-neoplastic agent alone, or in combination with IL-2 or IFN-τ, is being tested in clinical trials (120, 212). Its clinical use has been limited because of serious side effects experienced by the patients at therapeutic doses (10-100 $\mu g/m^2$). These include headaches, edema, hypotension and wasting. The adverse reactions prevent dose escalation to levels that might be useful for tumor lysis and eradication. Antibodies against TNF-α that minimize the side effects without altering its tumor killing properties may prove useful. This may enable the investigator to increase the TNF-α dose to an optimal biologic dose (OBD) or a maximally tolerated dose (MTD) (212).

The use of anti-TNF-α antibodies in diseases associated with increased levels of TNF-α may prove beneficial. These include diseases such as cerebral malaria, Reye's syndrome and autoimmune diseases such as insulin-dependent diabetes mellitus, rheumatoid arthritis and autoimmune thyroid disease (213, 214). The antibody may also be of possible use in preventing cachexia and wasting associated with malignancy and chronic diseases.

The drawbacks of using rabbit mAb for treatment purposes in humans is the production of human anti-murine or anti-rabbit antibodies directed against various epitopes on the mAb, similar to human anti-mouse antibodies. The human antibodies may be directed against $F(ab')_2$ structures that determine the antigenic specificity of the xenogeneic antibody molecule (the idiotypic epitopes) and hence inactivate the antibody (211).

One approach to this problem is the use of human antibodies. The problems with human anti-serum include the risk of developing AIDS and other diseases from transfusions and the difficulty in obtaining a constant, standardized source of antibody (190).

To circumvent the problems arising with interspecies antibody chimeric antibodies are being presented (211). One approach is to insert the murine idiotype on a human Ig

backbone. This approach has the prospect of attracting Fc receptor positive cells more avidly than a murine Fc piece and resulting in greater efficacy of these antibodies in antibody dependent cell mediated cytotoxicity and a greater propensity for thrombosis.

In light of all the theoretical benefits the cost of the immunotherapy can not be ignored. In times of rising medical costs the proposed use of expensive mAb against septic shock may not be the answer. The very people who most need the antibody, especially in the developing world, may not be able to afford the cost of treatment.

The final verdict on the physiological role of TNF-α has yet to be declared but all evidence points towards a multipotential role for this factor in the regulation of the inflammatory immune system to an antigen, be it a tumor associated protein or an invading organism. Why mammalian cells produce a potentially lethal cytokine in response to an immune affront remains to be answered.

With the expansion of our knowledge about the mechanisms of endotoxic shock new treatments will become available. Antibodies against IL-1, IL-6 , platelet activating factor could be instituted in the near future. Chemical blockers of endotoxin receptors such as monophosphorylated lipid A and lipid X may be useful prophylactically as well as therapeutically.

Pharmacological or immunological agents that bind or neutralize lipopolysaccharide binding protein (LBP) or CD 14 antigen may hold future promise. LBP is a plasma protein that binds the lipid A portion of bacterial LPS and CD 14 is a differentiation antigen of monocyte that binds LPS and LBP complexes (215, 216). Blockage of either LBP or CD 14 by mAb prevents synthesis of TNF-α *in vitro* and offer additional avenues for expansion in our arsenal against septicemia. In all probability the best results with immunotherapy may involve a combination of antibodies that neutralize cytokines at multiple points of the inflammatory cascade that leads to sepsis.

REFERENCES

1. **Robbins, S. L., R. S. Cotran, and V. Kumar.** 1984. Fluid and hemodynamic derangements. In *Pathologic Basis of Disease*. W. B. Saunders Company, Tokyo, Japan, p. 112.
2. **Sultzer, B. M.** 1968. Genetic control of leucocyte responses to endotoxin. *Nature 219:1253*.
3. **Bradley, S. G.** 1979. Cellular and molecular mechanisms of action of bacterial endotoxins. *Annu. Rev. Micro. 33:67*.
4. **Rietschel, E. T., C. Galanos, O. Lüderitz, and O. Westphal.** 1982. The chemistry and biology of lipopolysaccharides and their lipid A component. In *Immunopharmacology and the Regulation of Leucocyte Function*. D. R. Webb ed. Marcel Dekker Inc., New York, NY, p. 183.
5. **Wanebo H. J.** 1989. Tumor necrosis factors. *Semin. Surg. Oncol. 5:402*.
6. **Elin, R. J., and S. M. Wolff.** 1976. Biology of endotoxin. *Annu. Rev. Med. 27:127*.
7. **Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey III, A. Zentella, J. D.**

Albert, G. T. Shires, and A. Cerami. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science 234:470.*

8. **Beutler, B., and A. Cerami.** 1988. Cachectin, cachexia and shock. *Annu. Rev. Med. 39:75.*

9. **Beutler, B., and A. Cerami.** 1988. The common mediator of shock, cachexia and tumor necrosis. *Adv. Immunol. 42:213.*

10. **Carswell, E. A., L. J. Old, R. I. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA 72:3666.*

11. **Rothstein, J. L., and H. Schreiber.** 1987. Relationship of tumour necrosis factor and endotoxin to macrophage cytotoxicity, haemorrhagic necrosis and lethal shock. In *Tumour Necrosis Factor and Related Cytotoxins: Ciba Foundation Symposium 131.* J. Wiley and Sons, Sussex, U.K. p. 124.

12. **Mathison, J. C., E. Wolfson, and R. J. Ulevitch.** 1988. Participation of tumor necrosis factor in the mediation of Gram negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest. 81:1925.*

13. **Beutler, B., and A. Cerami.** 1987. Cachectin: more than a tumor necrosis factor. *N. Engl. J. Med. 316:379.*

14. **Ziegler, E. J.** 1988. Tumor necrosis factor in humans. *N. Engl. J. Med. 318:1533.*

15. **Dinarello, C. A., and J. W. Mier.** 1987. Current concepts: lymphokines. *N. Engl. J. Med. 317:940.*

16. **Beutler, B., and A. Cerami.** 1988. Cachectin: a monokine implicated as a mediator of cachexia and shock. In *Lymphokines, vol. 14.* E. Pick, and M. Landy eds. Academic Press Inc., San Diego, CA, p. 203.

17. **Cerami, A.** 1990. Cachectin/TNF and the immune response. *Adv. Oncol. 6:5.*

18. **Rothstein, J. L., and H. Schreiber.** 1988. Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. *Proc. Natl. Acad. Sci. USA 85:607.*

19. **Cerami, A., and B. Beutler.** 1988. The role of cachectin/TNF in endotoxic shock and cachexia. *Immunol. Today 9:28.*

20. **Old, L. J.** 1985. Tumor necrosis factor (TNF). *Science 230:630.*

21. **Flick, D. A., and G. E. Gifford.** 1985. Tumor necrosis factor. In *Biological Response Modifiers: New Approaches to Disease Intervention*. P. F. Torrence ed. Academic Press Inc., Orlando, FL, p. 171.

22. **Pennica, D., M. R. Shalaby, and M. A. Palladino, Jr.** 1987. Tumor necrosis factors alpha and beta. In *Recombinant Lymphokines and Their Receptors*. S. Gills, ed. Marcel Dekker Inc., New York, NY, p. 301.

23. **Torti, F. M., B. Dieckmann, B. Beutler, A. Cerami, and G. M. Ringold.** 1985. A macrophage factor inhibits adipocyte gene expression: an *in vitro* model of cachexia. *Science 229:867*.

24. **Averbook, B. J., T. R. Ulich, S. Orr, R. S. Yamamoto, I. Masunaka, and G. A. Granger.** 1988. Human lymphotoxin, macrophage toxin and tumor necrosis factor. In *Biological Response Modifiers*. D. J. W. Chiao ed. Marcel Dekker, New York, NY, p. 181.

25. **Matthews, N.** 1978. Tumour-necrosis factor from the rabbit. II. Production by monocytes. *Br. J. Cancer 38:310*.

26. **Fisch, H., and G. E. Gifford.** *In vitro* production of rabbit macrophage tumor cell cytotoxin. 1983. *Int. J. Cancer 32:105*.

27. **Beutler, B., N. Krochin, I. W. Milsark, C. Luedke, and A. Cerami.** 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science 232:977.*

28. **Beutler, B. A., I. W. Milsark, and A. Cerami.** 1985. Cachectin/tumor necrosis factor: production, distribution and metabolic fate *in vivo*. *J. Immunol. 135:3972.*

29. **Kornbluth, R. S., and T. S. Edgington.** 1986. Tumor necrosis factor production by human monocytes is a regulated event: induction of TNF-α-mediated cellular cytotoxicity by endotoxin. *J. Immunol. 137:2585.*

30. **Old, L. J.** 1988. Tumor necrosis factor. *Scientific American 258(5):59.*

31. **Grunfeld, C., and M. A. Palladino, Jr.** 1990. Tumor necrosis factor: immunologic, antitumor, metabolic and cardiovascular activities. *Adv. Intern. Med. 35:45.*

32. **Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, Jr., and J. V. O'Connor.** 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J. Exp. Med. 163:1433.*

33. **Kutsky, P.** 1989. Tumor necrosis factor$_{\alpha}$ decreases vascular contraction in vitro. *Circ. Shock 27:296.*

34. **Larsen, C., C. Zachariae, N. Mukaida, A. Anderson, M. Yamada, J. Oppenheim, and K. Matsushima.** 1990. Proinflammatory cytokines interleukin 1 and tumor necrosis factor induce cytokines that are chemotactic for neutrophils, T cells and monocytes. In *Progress in Clinical and Biological Research, vol.349: Cytokines and Lipocortins in Inflammation and Differentiation*. M. Melli, and L. Parente, eds. Wiley-Liss Inc., NY, p. 419.

35. **Kunkel, S. L., R. M. Strieter, S. W. Chensue, M. Basha, T. Standiford, J. Ham, and D. G. Remick.** 1990. Tumor necrosis factor-alpha, interleukin-8 and chemotactic cytokines. In *Progress in Clinical and Biological Research, vol.349: Cytokines and Lipocortins in Inflammation and Differentiation*. M. Melli, and L. Parente, eds. Wiley-Liss Inc., NY, p. 433.

36. **Dinarello, C. A.** 1987. Interleukins, tumor necrosis factors (cachectin), and interferons as endogenous pyrogens and mediators of fever. In *Lymphokines, vol. 14*. E. Pick, and M. Landy eds. Academic Press Inc., San Diego, CA, p. 1.

37. **Vlassara, H., M. Brownlee, K. R. Manogue, C. A. Dinarello, and A. Pasagian.** 1988. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science 240:1546.*

38. **Leibovich, S. J., P. J. Polverini, H. M. Shepard, D. M. Wiseman, V. Shively, and N. Nuseir.** 1987. Macrophage-induced angiogenesis is mediated by tumour necrosis factor-α. *Nature 329:630.*

39. **Folkman, J., and M. Klagsbrun.** 1987. A family of angiogenic peptides. *Nature 329:671.*

40. **Scuderi, P., K. S. Lam, K. J. Ryan, E. Petersen, K. E. Sterling, P. R. Finley, C. G. Ray, D. J. Slymen, and S. E. Salmon.** 1986. Raised serum levels of tumour necrosis factor in parasitic infections. *Lancet 2:1364.*

41. **Clark, I. A., J.-L. Virelizier, E. A. Carswell, and P. R. Wood.** 1981. Possible importance of macrophage-derived mediators in acute malaria. *Infect. Immun. 32:1058.*

42. **Taverne, J., N. Matthews, P. Depledge, and J. H. L. Playfair.** 1984. Malarial parasites and tumor cells are killed by the same component of tumor necrosis serum. *Clin. Exp. Immunol. 57:293.*

43. **Wozencraft, A. O., H. M. Dockrell, J. Taverne, G. A. T. Targett, and J. H. L. Playfair.** 1984. Killing of human malaria parasites by macrophage secretory products. *Infect. Immun. 43:664.*

44. **Grau, G. E., L. F. Fajardo, P.-F. Piguet, B. Allet, P.-H. Lambert, and P. Vassalli.** 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science 237:1210.*

45. **Taverne, J., H. M. Dockrell, and J. H. L. Playfair.** 1981. Endotoxin-induced serum factor kills malarial parasites *in vitro*. *Infect. Immun. 33:83.*

46. **Taverne, J., P. Depledge, and J. H. L. Playfair.** 1982. Differential sensitivity *in vivo* of lethal and nonlethal malarial parasites to endotoxin-induced serum factor. *Infect. Immun. 37:927.*

47. **Havell, E. A.** 1989. Evidence that tumor necrosis factor has an important role in antibacterial resistance. *J. Immunol. 143:2894.*

48. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. *Infect. Immun. 57:291.*

49. **Waage, A., A. Halstensen, and T. Espevik.** 1987. Association between tumour necrosis factor in serum

and fatal outcome in patients with meningococcal disease. *Lancet 1:355*.

50. **Girardin, E., G. E. Grau, J.-M. Dayer, P. Roux-Lombard, and P.-H. Lambert** *et al*. 1988. Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N. Engl. J. Med. 319:397*.

51. **Ammann, A. J., M. A. Palladino, P. Volberding, D. Abrams, N. L. Martin, and M. Conant.** 1987. Tumor necrosis factors alpha and beta in acquired immunodeficiency syndrome (AIDS) and Aids-related complex. *J. Clin. Immunol. 7:481*.

52. **Porat, O.** 1989. The effect of tumor necrosis factor α on the activity of lipoprotein lipase in adipose tissue. *Lymphokine Res. 8:459*.

53. **Beutler, B., J. Mahoney, N. Le Trang, P. Pekala, and A. Cerami.** 1985. Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin induced RAW 264.7 cells. *J. Exp. Med. 161:984*.

54. **Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bringman, G. E. Nedwin, D. V. Goeddel, and R. N. Harkins.** 1985. Human tumor necrosis factor: production purification and characterization. *J. Biol. Chem. 260:2345*.

55. **Pennica, D., J. S. Hayflick, T. S. Bringman, M. A. Palladino, and D. V. Goeddel.** 1985. Cloning and expression in *Escherichia coli* of the cDNA for murine tumor necrosis factor. *Proc. Natl. Acad. Sci. USA 82:6060.*

56. **Shirai, T., H. Yamaguchi, H. Ito, C. W. Todd, and R. B. Wallace.** 1985. Cloning and expression in *Escherichia coli* of the gene for human tumour necrosis factor. *Nature 313:803.*

57. **Beutler. B., D. Greenwald, J. D. Hulmes, M. Chang, Y.-C. E. Pan, J. Mathison, R. Ulevitch, and A. Cerami.** 1985. Identity of tumor necrosis factor and the macrophage-secreted factor cachectin. *Nature 316:552.*

58. **De Maeyer, E., and J. De Maeyer-Guignard.** 1988. Interaction of interferons, tumor necrosis factor, interleukin-1, and interleukin-2 as part of the cytokine network. In *Interferons and Other Regulatory Cytokines*. John Wiley and Sons Inc., New York, NY, p. 288.

59. **Müller, U., C. V. Jongeneel, S. A. Nedospasov, K. F. Lindahl, and M. Steinmetz.** 1987. Tumour necrosis factor and lymphotoxin genes map close to *H-2D* in the mouse major histocompatibility complex. *Nature 325:265.*

60. **Gray, P. W., B. B. Aggarwal, C. V. Benton, T. S. Bringman, W. J. Henzel, J. A. Jarrett, D. W. Leung, B. Moffat, P. Ng, L. P. Svedersky, M. A. Palladino, and G. E. Nedwin.** 1984. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. *Nature 312:721.*

61. **Pennica, D., G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal, and D. V. Goeddel.** 1984. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature 312:724.*

62. **Aggarwal, B. B.** 1990. Lymphotoxin and tumor necrosis factor: qualitative and quantitative differences in their receptors and signal transduction in various cell types. In *Progress in Clinical and Biological Research, vol.349: Cytokines and Lipocortins in Inflammation and Differentiation*. M. Melli, and L. Parente, eds. Wiley-Liss Inc., NY, p. 375.

63. **Beutler, B.** 1990. Regulation of cachectin biosynthesis occurs at multiple levels. In *Progress in Clinical and Biological Research, vol.349: Cytokines and Lipocortins in Inflammation and Differentiation*. M. Melli, and L. Parente, eds. Wiley-Liss Inc., NY, p. 433.

64. **Cuturi, M. C., M. Murphy, M. P. Costa-Giomi, R. Weinmann, B. Perussia, and G. Trinchieri.** 1987. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. *J. Exp. Med. 165:1581.*

65. **Kasid, A., E. P. Director, M. C. Stovroff, M. T. Lotze, and S. A. Rosenberg.** 1990. Cytokine regulation of tumor necrosis factor-α and -ß (lymphotoxin)-messenger RNA expression in human peripheral blood mononuclear cells. *Cancer Res. 50:5072.*

66. **Gifford, G. E., D. A. Flick, N. A. AbdAllah, and H. Fisch.** 1984. Production of a cytotoxin from phorbol myristate acetate-treated human promyelocytes. *J. Natl. Cancer Inst. 73:69.*

67. **Chen, A. R., K. P. McKinnon, and H. S. Koren.** 1985. Lipopolysaccharide (LPS) stimulates fresh human monocytes to lyse actinomycin D-treated WEHI-164 target cells via increased secretion of a monokine similar to tumor necrosis factor. *J. Immunol. 135:3978.*

68. **Scuderi, P.** 1989. Suppression of human leukocyte tumor necrosis factor secretion by the serine protease inhibitor *p*-toluenesulfonyl-L-arginine methyl ester (TAME). *J. Immunol. 143:168.*

69. **Spofford, B. T., R. A. Daynes, and G. A. Granger.** 1974. Cell-mediated immunity *in vitro*: a highly sensitive assay for human lymphotoxin. *J. Immunol. 112:2111.*

70. **Kramer, S. M., and M. E. Carver.** 1986. Serum-free *in vitro* bioassay for the detection of tumor necrosis factor. *J. Immunol. Methods 93:201.*

71. **Matthews, N., and M. L. Neale.** 1987. Cytotoxicity assays for tumour necrosis factor and lymphotoxin. In *Lymphokines and Interferons: A Practical Approach*. M. J. Clemens, A. J. Morris, and A. J. H. Gearing, eds. IRL Press Ltd., Oxford, U.K., p.221.

72. **Dealtry, G. B., and F. R. Balkwill.** 1987. Cell growth inhibition by interferons and tumour necrosis factor. In *Lymphokines and Interferons: A Practical Approach*. M. J. Clemens, A. J. Morris, and A. J. H. Gearing, eds. IRL Press Ltd., Oxford, U.K., p.195.

73. **Khan. A., D. Weldon, J. Duvall, S. Pitchyangkul, and N. O. Hill.** 1982. A standardized automated computer assisted micro-assay for lymphotoxin. In *Human Lymphokines, The Biological Immune Response Modifiers*. A. Khan, and N. O. Hill, eds. Academic Press Inc., New York, NY, p. 23.

74. **Decker, T., and M.-L. Lohmann-Matthes.** 1988. A quick and simple method for quantitation of lactate

dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods 15:61.*

75. **Flick, D. A., and G. E. Gifford.** 1984. Comparison of *in vitro* cell cytotoxic assays for tumor necrosis factor. *J. Immunol. Methods. 68:167.*

76. **Teppo, A.-M., and C. P. J. Maury.** 1987. Radioimmunoassay of tumor necrosis factor in serum. *Clin. Chem. 33:2024.*

77. **Smith, R. A., and C. Baglioni.** 1989. Multimeric structure of the tumor necrosis factor receptor of HeLa cells. *J. Biol. Chem. 264:14646.*

78. **Lam, K. S., P. Scuderi, and S. E. Salmon.** 1988. Analysis of the molecular organization of recombinant human tumor necrosis factor (rTNF) in solution using ethylene glycolbis (succinimidyl-succinate) as the cross-linking agent. *J. Biol. Response Mod. 7:267.*

79. **Jones, E. Y., D. I. Stuart, and N. P. C. Walker.** 1990. The three-dimensional structure of tumour necrosis factor. In *Progress in Clinical and Biological Research, vol.349: Cytokines and Lipocortins in Inflammation and Differentiation* M. Melli, and L. Parente, eds. Wiley-Liss Inc., NY, p. 321.

80. **Matthews, N., and M. L. Neale.** 1987. Studies on the mode of action of tumor necrosis factor on tumor cells *in vitro*. In *Lymphokines, vol. 14*. E. Pick, and M. Landy eds. Academic Press Inc., San Diego, CA, p. 223.

81. **Nawroth, P. P., I. Bank, D. Handley, J. Cassimeris, L. Chess, and D. Stern.** 1986. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. *J. Exp. Med. 163:1363*.

82. **Engelmann, H., H. Holtmann, C. Brakebusch, Y. S. Avni, I. Sarov, Y. Nophar, E. Hadas, O. Leitner, and D. Wallach.** 1990. Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J. Biol. Chem. 265:14497*.

83. **Hohmann, H.-P., R. Remy, B. Pöschl, and A. P. G. M. van Loon.** 1990. Tumor necrosis factors-α and -ß bind to the same two types of tumor necrosis factor receptors and maximally activate the transcription factor NF-*k*B at low receptor occupancy and within minutes after receptor binding. *J. Biol. Chem. 265:15183*.

84. **Nakamura, S., T. Masegi, M. Fukuoka, K. Kitai, A. Kato, Y. Ichikawa, N. Watanabe, and Y. Niitsu.** 1991. Interactions between novel tumor necrosis factor-α

mutants and receptors on tumor and normal cells. *Agric. Biol. Chem. 55:53.*

85. **Nakamura, S., H. Moriya, J. Kaneko, T. Masegi, Y. Yamanaka, K. Kozai, Y. Ichikawa, and M. Osumi.** 1991. The properties of a tumor necrosis factor mutant with the deletion of alanine-14. *Agric. Biol. Chem. 55:257.*

86. **Oppenheim, J. J., C. M. Dubois, F. W. Ruscetti, and J. R. Keller.** 1990. Regulation of the production and receptors for interleukin 1, and tumor necrosis factor. In *Progress in Clinical and Biological Research, vol.349: Cytokines and Lipocortins in Inflammation and Differentiation*. M. Melli, and L. Parente, eds. Wiley-Liss Inc., NY, p. 183.

87. **Aggarwal, B. B., T. E. Eessalu, and P. E. Hass.** 1985. Characterization of receptors for human tumor necrosis factor and their regulation by τ-interferon. *Nature 318:665.*

88. **Billard, C., F. Sigaux, and J. Wietzerbin.** 1990. IFN-α *in vivo* enhances tumor necrosis factor receptor levels on hairy cells. *J. Immunol. 145:1713.*

89. **Ruggiero, V., J. Tavernier, W. Fiers, and C. Baglioni.** 1986. Induction of the synthesis of tumor necrosis factor receptors by interferon-τ. *J. Immunol. 136:2445.*

90. **Scheurich, P., B. Thoma, U. Ücer, and K. Pfizenmaier.** 1987. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-α: induction of TNF receptors on human T cells and TNF -α-mediated enhancement of T cell responses. *J. Immunol. 138:1786.*

91. **Porteu, F., and C. Nathan.** 1990. Shedding of tumor necrosis factor receptors by activated human neutrophils. *J. Exp. Med. 172:599.*

92. **Sprang, S. R.** 1990. The divergent receptors for TNF. *Trends Biochem. Sci. 15:366.*

93. **Brockhaus, M., H.-J. Schoenfeld, E.-J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher.** 1990. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA 87:3127.*

94. **Luettig, B., T. Decker, and M.-L Lohmann-Matthes.** 1989. Evidence for the existence of two forms of membrane tumor necrosis factor: an integral protein and a molecule attached to its receptor. *J. Immunol. 143:4034.*

95. **Andrews, J. S., A. E. Berger, and C. F. Ware.** 1990. Characterization of the receptor for tumor necrosis factor (TNF) and lymphotoxin (LT) on human T lymphocytes. TNF and LT differ in their receptor

binding properties and the induction of MHC Class I proteins on a human $CD4^+$ T cell hybridoma. *J. Immunol. 144:2582.*

96. **Aiyer, R. A., and B. B. Aggarwal.** 1990. Characterization of receptors for recombinant human tumor necrosis factor-α from human placental membranes. *Lymphokine Res. 9:333.*

97. **Hass, P. E., A. Hotchkiss, M. Mohler, and B. B. Aggarwal.** 1985. Characterization of specific high affinity receptors for human tumor necrosis factor on mouse fibroblasts. *J. Biol. Chem. 260:12214.*

98. **Rubin, B. Y., S. L. Anderson, S. A. Sullivan, B. D. Williamson, E. A. Carswell, and L. J. Old.** 1985. High affinity binding of ^{125}I-labeled human tumor necrosis factor (LuKII) to specific cell surface receptors. *J. Exp. Med. 162:1099.*

99. **Pichyangkul, S., D. Schick, F. L. Jia, S. Berent, A. Bollon, and A. Kahn.** 1987. Binding of tumor necrosis factor alpha (TNF-α) to high-affinity receptors on polymorphonuclear cells. *Exp. Hematol. 15:1055.*

100. **Kull, Jr., F. C., S. Jacobs, and P. Cuatrecasas.** 1985. Cellular receptor for ^{125}I-labeled tumor necrosis factor: specific binding, affinity

labeling, and relationship to sensitivity. *Proc. Natl. Acad. Sci. USA 82:5756.*

101. **Baglioni, C., S. McCandless, J. Tavernier, and W. Fiers.** 1985. Binding of human tumor necrosis factor to high affinity receptors on HeLa and lymphoblastoid cells sensitive to growth inhibition. *J. Biol. Chem. 260:13395.*

102. **Niitsu, Y., N. Watanabe, H. Sone, H. Neda, N. Yamauchi, M. Maeda, and I. Urushizaki.** 1988. Analysis of the TNF receptor on KYM cells by binding assay and affinity cross-linking. *J. Biol. Response Mod. 7:276.*

103. **Hohmann, H.-P., R. Remy, M. Brockhaus, and A. P. G. M. van Loon.** 1989. Two different cell types have different major receptors for human tumor necrosis factor (TNFα). *J. Biol. Chem. 264:14927.*

104. **Smith, R. A., M. Kirstein, W. Fiers, and C. Baglioni.** 1986. Species specificity of human and murine tumor necrosis factor. A comparative study of tumor necrosis factor receptors. *J. Biol. Chem. 261:14871.*

105. **Meyer, R., E. N. Hatada, H.-P. Hohmann, M. Haiker, C. Bartsch, U. Röthlisberger, H.-W. Lahm, E. J. Schlaeger, A. P. G. M. van Loon, and C. Scheidereit.** 1991. Cloning of the DNA-binding

subunit of human nuclear factor$_k$B: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. *Proc. Natl. Acad. Sci. USA 88:966.*

106. **Warren, R. S., D. B. Donner, H. F. Starnes, Jr., and M. F. Brennan.** 1987. Modulation of endogenous hormone action by recombinant human tumor necrosis factor. *Proc. Natl. Acad. Sci. USA 84:8619.*

107. **Marino, M. W., L. J. Feld, E. A. Jaffe, L. M. Pfeffer, H.-M. Han, and D. B. Donner.** 1991. Phosphorylation of the proto-oncogene product eukaryotic initiation factor 4E is a common cellular response to tumor necrosis factor. *J. Biol. Chem. 266:2685.*

108. **Wietzerbin, J., C. Gaudelet, L. Catinot, J. Chebath, and R. Falcoff.** 1990. Synergistic effect of interferon-τ and tumor necrosis factor-α on antiviral activity and (2′-5′) oligo (A) synthetase induction in a myelomonocytic cell line. *J. Leukocyte Biol. 48:149.*

109. **Piacibello, W., F. Sanavio, A. Severino, S. Morelli, A. M. Vaira, A. Stacchini, and M. Aglietta.** 1990. Opposite effect of tumor necrosis factor α on granulocyte colony-stimulating factor and

granulocyte-macrophage colony-stimulating factor-dependent growth of normal and leukemic hemopoietic progenitors. *Cancer Res. 50:5065.*

110. **Elbaz, O., L. M. Budel, H. Hoogerbrugge, I. P. Touw, R. Delwel, L. A. Mahmoud, and B. Löwenberg.** 1991. Tumor necrosis factor regulates the expression of granulocyte-macrophage colony-stimulating factor and interleukin-3 receptors on human acute myeloid leukemia cells. *Blood 77:989.*

111. **Malik, S. T. A., D. B. Griffin, M. S. Naylor, W. Fiers, A. Oliff, and F. R. Balkwill.** 1990. The complex effects of recombinant tumour necrosis factor-alpha (rhTNF-α) in human ovarian cancer xenograft models. In *Progress in Clinical and Biological Research, vol.349: Cytokines and Lipocortins in Inflammation and Differentiation.* M. Melli, and L. Parente, eds. Wiley-Liss Inc., NY, p. 393.

112. **Helson, L., C. Helson, and S. Green.** 1979. Effects of murine tumor necrosis factor on heterotrans-planted human tumors. *Exp. Cell Biol. 47:53.*

113. **Krönke, M., G. Hensel, C. Schlüter, P. Scheurich, S. Schütze, and K. Pfizenmaier.** 1988. Tumor necrosis factor and lymphotoxin gene expression in human tumor cell lines. *Cancer Res. 48:5417.*

114. **Darzynkiewicz, Z., B. Williamson, E. A. Carswell, and L. J. Old.** 1984. Cell cycle-specific effects of tumor necrosis factor. *Cancer Res. 44:83.*

115. **Kull, Jr., F. C.** The TNF receptor in TNF-mediated cytotoxicity. 1988. *Nat. Immun. Cell Growth Regul. 7:254.*

116. **Tsujimoto, M., Y. K. Yip, and J. Vilcek.** 1985. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. *Proc. Natl. Acad. Sci. USA 82:7626.*

117. **Patek, P. Q., Y. Lin, and P. G. Case.** 1989. Cell lines cultured at high density are resistant to lysis by tumor necrosis factor and natural cytotoxic cells (42854). *Proc. Soc. Exp. Biol. Med. 190:234.*

118. **Larrick, J. W., and S. C. Wright.** 1990. Cytotoxic mechanisms of tumor necrosis factor-α. *FASEB J. 4:3215.*

119. **Patek, P. Q., Y. Lin, and J. L. Collins.** 1987. Natural cytotoxic cells and tumor necrosis factor activate similar lytic mechanisms. *J. Immunol. 138:1641.*

120. **Schiller, J. H., B. E. Storer, P. L. Witt, D. Alberti, M. B. Tombes, R. Arzoomanian, R. A. Proctor, D. McCarthy, R. R. Brown, S. D. Voss, S. C. Remick, J. L. Grem, E. C. Borden, and D. L. Trump.**

1991. Biological and clinical effects of intravenous tumor necrosis factor-α administered three times weekly. *Cancer Res. 51:1651.*

121. **Winkelhake, J. L., S. Stampfl, and R. J. Zimmerman.** 1987. Synergistic effects of combination therapy with human recombinant interleukin-2 and tumor necrosis factor in murine tumor models. *Cancer Res. 47:3948.*

122. **Rosenberg, S. A., P. Aebersold, K. Cornetta, A. Kasid, R. A. Morgan, R. Moen, E. M. Karson, M. T. Lotze, J. C. Yang, S. L. Topalian, M. J. Merino, K. Culver, A. D. Miller, R. M. Blaese, and W. F. Anderson.** 1990. Gene transfer into humans. Immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med. 323:570.*

123. **Ciancio, M. J., J. Hunt, S. B. Jones, and J. P. Filkins.** 1991. Comparative and interactive *in vivo* effects of tumor necrosis factor α and endotoxin. *Circ. Shock 33:108.*

124. **Ganfield, D. J., P. A. Rebers, and K. L. Heddleston.** 1976. Immunogenic and toxic properties of a purified lipopolysaccharide-protein complex from *Pasteurella multocida. Infect. Immun. 14:990-99.*

125. **Yelich, M. R.** 1990. Glucoregulatory, hormonal, and metabolic responses to endotoxicosis or cecal ligation and puncture sepsis in the rat: a direct comparison. *Circ. Shock 31:351.*

126. **Möller, G., O. Sjöberg, and J. Andersson.** 1973. Immunogenicity, tolerogenicity and mitogenicity of lipopolysaccharides. *J. Infect. Dis. 128S:S52.*

127. **Neter, E., H. Y. Whang, and H. Mayer.** 1973. Immunogenicity and antigenicity of endotoxic lipopolysaccharides: reversible effects of temperature on immunogenicity. *J. Infect. Dis. 128S:S56.*

128. **Reed. N. D., J. K. Manning, and J. A. Rudbach.** 1973. Immunologic responses of mice to lipopolysaccharide from *Escherichia coli*. *J. Infect. Dis. 128S:S70.*

129. **Hsueh, W., F. González-Crussi, and J. L. Arroyave.** 1987. Platelet-activating factor: an endogenous mediator for bowel necrosis in endotoxemia. *FASEB J. 2:403.*

130. **Brennan, F. M., D. Chantry, A. Jackson, R. Maini, and M. Feldmann.** 1989. Inhibitory effect of TNFα antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet 2:244.*

131. **Bona, C. A.** 1973. Fate of endotoxin in macrophages: biological and ultrastructural aspect. *J. Infect. Dis. 128S:S74.*

132. **Chang, C.-M., and A. Nowotny.** 1975. Relation of structure to function in bacterial O-antigens-VII. Endotoxicity of 'lipid A'. *Immunochemistry 12:19.*

133. **Kiener, P. A., F. Marek, G. Rodgers, P.-F. Lin, G. Warr, and J. Desiderio.** 1988. Induction of tumor necrosis factor, IFN-τ, and acute lethality in mice by toxic and non-toxic forms of lipid A. *J. Immunol. 141:870.*

134. **Kawakami, M.** 1973. Antigenic determinant involved in hypersensitivity of infected mice to lipopoly-saccharide of *Salmonella. J. Infect. Dis. 128S:S246.*

135. **Mäkelä, P. H., V. V. Valtonen, and M. Valtonen.** 1973. Role of O-antigen (lipopolysaccharide) factors in the virulence of *Salmonella. J. Infect. Dis. 128S:S81.*

136. **Vas, S. I., R. S. Roy, and H. G. Robson.** 1973. Endotoxin sensitivity of inbred mouse strains. *Can. J. Microbiol. 19:767.*

137. **Boggs, S. S., D. R. Boggs, and R. A. Joyce.** 1980. Response to endotoxin of endotoxin-"resistant"

C3H/HeJ mice: a model for study of hematopoietic control. *Blood 55:444.*

138. **Green, S., A. Dobrjansky, M. A. Chiasson, E. Carswell, M. K. Schwartz, and L. J. Old.** 1977. *Corynebacterium parvum* as the priming agent in the production of tumor necrosis factor in the mouse. *J. Natl. Cancer Inst. 59:1519.*

139. **Engelhardt, R., A. Mackensen, and C. Galanos.** 1991. Phase I trial of intravenously administered endotoxin (*Salmonella abortus equi*) in cancer patients. *Cancer Res. 51:2524.*

140. **Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, C. A. Dinarello, A. Cerami, S. M. Wolff, and D. W. Wilmore.** 1988. Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med. 318:1481.*

141. **Mózes, T., S. Ben-Efraim, C. J. A. M. Tak, J. P. C. Heiligers, P. R. Saxena, and I. L. Bonta.** 1991. Serum levels of tumor necrosis factor determine the fatal or non-fatal course of endotoxic shock. *Immunol. Lett. 27:157.*

142. **Ruff, M. R., and G. E. Gifford.** 1980. Purification and physico-chemical characterization of rabbit tumor necrosis factor. *J. Immunol. 125:1671.*

143. **Matthews, N., and J. F. Watkins.** 1978. Tumour-necrosis factor from the rabbit. I. Mode of action, specificity and physicochemical properties. *Br. J. Cancer 38:302.*

144. **Matthews, N.** 1979. Tumour-necrosis factor from the rabbit. III. Relationship to interferons. *Br. J. Cancer 38:534.*

145. **Lehmann, V., M. A. Freudenberg, and C. Galanos.** 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine -treated mice. *J. Exp. Med. 165:657.*

146. **Greisman, S. E., and B. DuBuy.** 1975. Mechanisms of endotoxin tolerance. IX. Effect of exchange transfusion (38608). *Proc. Soc. Exp. Biol. Med. 148:675.*

147. **Beutler, B., I. W. Milsark, and A. C. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science 229:869.*

148. **Nagai, M., T. Saigusa, Y. Shimada, H. Inagawa, H. Oshima, and M. Iriki.** 1988. Antibody to tumor necrosis factor (TNF) reduces endotoxin fever. *Experientia 44:606.*

149. **Shimozato T., M. Iwata, and N. Tamura.** 1990. Suppression of tumor necrosis factor alpha

production by a human immunoglobulin preparation for intravenous use. *Infect. Immun. 58:1384.*

150. **Wakabayashi, G., J. A. Gelfand, J. F. Burke, R. C. Thompson, and C. A. Dinarello.** 1991. A specific receptor antagonist for interleukin 1 prevents *Escherichia coli*-induced shock in rabbits. *FASEB J. 5:338.*

151. **Muchmore, A., J. Decker, A. Shaw, and P. Wingfield.** 1990. Evidence that high mannose glycopeptides are able to functionally interact with recombinant tumor necrosis factor and recombinant interleukin 1. *Cancer Res. 50:6285.*

152. **Dunbar, B. S. and E. D. Schwoebel.** 1990. Preparation of polyclonal antibodies. *Methods Enzymol. 182:663.*

153. **Köhler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature 256:495.*

154. **Milstein, C.** 1986. Overview: monoclonal antibodies. In *Handbook of Experimental Immunology, vol. 4: Applications of Immunological Methods in Biomedical Sciences*. D. M. Weir, L. A. Herzenberg, and C. Blackwell, eds. Blackwell Scientific Publications Inc., Palo Alto, CA, p. 107.1.

155. **Kipps, T. J., and L. A. Herzenberg.** 1986. Schemata for the production of monoclonal antibody-producing

hybridomas. In *Handbook of Experimental Immunology, vol.4: Applications of Immunological Methods in Biomedical Sciences*. D. M. Weir, L. A. Herzenberg, and C. Blackwell, eds. Blackwell Scientific Publications Inc., Palo Alto, CA, p. 108.1.

156. **Oi, V. T., and L. A. Herzenberg.** 1980. Immunoglobulin-producing hybrid cell lines. In *Selected Methods in Cellular Immunology*. B. B. Mishell, and S. M. Shiigi, eds. W. H. Freeman and Co., San Francisco, CA, p. 351.

157. **Littlefield, J. W.** 1964. Selection of hybrids from matings of fibroblasts *in vitro* and their presumed recombinants. *Science 145:709*.

158. **Mourik, P. V., and W. P. Zeijlemaker.** 1986. Improved hybridoma technology: spleen cell separation and soluble growth factors. *Methods Enzymol. 121:174*.

159. **Cianfriglia, M., M. Mariani, D. Armellini, A. Massone, M. Lafata, R. Presentini, and G. Antoni.** 1986. Methods for high frequency production of soluble antigen-specific hybridomas; specificities and affinities of the monoclonal antibodies obtained. *Methods Enzymol. 121:193*.

160. **Dunbar, B. S. and S. M. Skinner.** 1990. Preparation of monoclonal antibodies. *Methods Enzymol. 182:670*.

161. **Shimamoto, Y., R. L. Chen, A. Bollon, A. Chang, and A. Khan.** 1988. Monoclonal antibodies against human recombinant tumor necrosis factor: prevention of endotoxic shock. *Immunol. Lett. 17:311.*

162. **Kuo, M.-C., J. A. Sogn, E. E. Max, and T. J. Kindt.** 1985. Rabbit-mouse hybridomas secreting intact rabbit immunoglobulin. *Mol. Immunol. 22:351.*

163. **Dreher, K. L., J. A. Sogn, F. T. Gates III, M.-C. Kuo, and T. J. Kindt.** 1983. Allotype-defined mRNA for rabbit immunoglobulin H and L chains isolated from rabbit-mouse hybridomas. *J. Immunol. 130:442.*

164. **Raybould, T. J. G., and M. Takahashi.** 1988. Production of stable rabbit-mouse hybridomas that secrete rabbit mAb of defined specificity. *Science 240:1788.*

165. **Ostrove, S.** 1990. Affinity chromatography: general methods. *Methods Enzymol. 182:483.*

166. **Cuatrecasas, P., M. Wilchek, and C. B. Anfinsen.** 1968. Selective enzyme purification by affinity chromatography. *Proc. Natl. Acad. Sci. USA 61:636.*

167. **Engvall, E.** 1980. Enzyme immunoassay ELISA and EMIT. *Methods Enzymol. 70:419.*

168. **Hoogenraad, N. J., and C. J. Wraight.** 1986. The effect of pristane on ascites tumor formation and

monoclonal antibody production. *Methods Enzymol. 121:375.*

169. **Firestone, G. L., and S. D. Winguth.** 1990. Immunoprecipitation of proteins. *Methods Enzymol. 182:688.*

170. **Ouchterlony, Ö. and L.-Å. Nilsson.** 1986. Immunodiffusion and immunoelectrophoresis. In *Handbook of Experimental Immunology, vol. 1: Immunochemistry*. D. M. Weir, L. A. Herzenberg, and C. Blackwell, eds. Blackwell Scientific Publications Inc., Palo Alto, CA, p. 32.1.

171. **Kabat, E. A.** 1980. Basic principles of antigen-antibody reactions. *Methods Enzymol. 70:3.*

172. **Stoll, V. S. and J. S. Blanchard.** 1990. Buffers: principles and practice. *Methods Enzymol. 182:24.*

173. **Ham, R. G., and W. L. McKeehan.** 1979. Media and growth requirements. *Methods Enzymol. LVIII:44.*

174. **Stoscheck, C. M.** 1990. Quantitation of protein. *Methods Enzymol. 182:50.*

175. **Ames, G. F.-L.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs: membrane, soluble, and periplasmic fractions. *J. Biol. Chem. 249:634.*

176. **Mishell, B. B., S. M. Shiigi, C. Henry, E. L. Chan, J. North, R. Gallily, M. Slomich, K. Miller, J. Marbrook, D. Parks, and A. H. Good.** 1980.

Preparation of mouse cell suspensions. In *Selected Methods in Cellular Immunology*. B. B. Mishell, and S. M. Shiigi, eds. W. H. Freeman and Co., San Francisco, CA, p. 3.

177. **Roubinian, J.** 1980. Selected surgical procedures. In *Selected Methods in Cellular Immunology*. B. B. Mishell, and S. M. Shiigi, eds. W. H. Freeman and Co., San Francisco, CA, p. 325.

178. **Westerwoudt, R. J.** 1986. Factors affecting production of monoclonal antibodies. *Methods Enzymol. 121:3*.

179. **Herbert, W. J., F. Kirstensen, R. M. Aitken, M. B. Eslami, A. Ferguson, K. G. Gray, and W. J. Penhale.** Laboratory animal techniques for immunology. In *Handbook of Experimental Immunology, vol. 4: Applications of Immunological Methods in Biomedical Sciences*. D. M. Weir, L. A. Herzenberg, and C. Blackwell, eds. Blackwell Scientific Publications Inc., Palo Alto, CA, p. 133.1.

180. **Nakamura, R. M.,A. Voller and D. E. Bidwell.** 1986. Enzyme immunoassays: heterogeneous and homogeneous systems. In *Handbook of Experimental Immunology, vol. 1: Immunochemistry*. D. M. Weir, L. A. Herzenberg, and C. Blackwell, eds. Blackwell Scientific Publications Inc., Palo Alto, CA, p. 27.1

181. **Lewis, J. A.** 1987. Biological assays for interferons. In *Lymphokines and Interferons: A Practical Approach*. M. J. Clemens, A. J. Morris, and A. J. H. Gearing, eds. IRL Press Ltd., Oxford, U.K., p.73.

182. **Meager, A.** 1987. Quantification of interferons by anti-viral assays and their standardization. In *Lymphokines and Interferons: A Practical Approach*. M. J. Clemens, A. J. Morris, and A. J. H. Gearing, eds. IRL Press Ltd., Oxford, U.K., p.129.

183. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA 76:4350*.

184. **Castora, F. J.** 1989. Western blotting of proteins. *Clin. Biotechnol. 1:43*.

185. **Morrison, M.** 1980. Lactoperoxidase-catalyzed iodination as a tool for investigation of proteins. *Methods Enzymol. 70:214*.

186. **Thorell, J. I., and B. G. Johansson.** 1971. Enzymatic iodination of polypeptides with ^{125}I to high specific activity. *Biochimic. Biophys. Acta 251:363*.

187. **Bradley, L. M.** 1989. Cell proliferation. In *Selected Methods in Cellular Immunology*. B. B. Mishell, and S. M. Shiigi, eds. W. H. Freeman and Co., San Francisco, CA, p. 153.

188. **Litchfield, Jr., J. T., and F. Wilcoxon.** 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther. 96:99.*

189. **Huang, C. Y.** 1979. Derivation of initial velocity and isotope exchange rate equations. *Methods Enzymol. 63:54.*

190. **Bone, R. C.** 1991. A critical evaluation of new agents for the treatment of sepsis. *JAMA 266:1686.*

191. **Bone, R. C.** 1991. The pathogenesis of sepsis. *Ann. Intern. Med. 115:457.*

192. **Chouaib, S., D. Branellec, and W. A. Buurman.** 1991. More insights into the complex physiology of TNF. *Immunol. Today 12:141.*

193. **Himeno, T., N. Watanabe, N. Yamauchi, M. Maeda, Y. Tsuji, T. Okamoto, H. Neda, and Y. Niitsu.** 1990. Expression of endogenous tumor necrosis factor as a protective protein against the cytotoxicity of exogenous tumor necrosis factor. *Cancer Res. 50:4941.*

194. **Plaetinck, G., M.-C. Combe, P. Corthésy, P. Sperisen, H. Kanamori, T. Honjo, and M. Nabholz.**

1990. Control of IL-2 receptor-α expression by IL-1, tumor necrosis factor, and IL-2: complex regulation via elements in the 5′ flanking region. *J. Immunol. 154:3340.*

195. **Pirofski, L., A. Casadevall, L. Rodriguez, L. S. Zuckier, and M. D. Scharff.** 1990. Current state of the hybridoma technology. *J. Clin. Immunol. 10:5S.*

196. **Bone, R. C., C. J. Fisher, T. P. Clemmer, G. J. Slotman, C. A. Metz, and R. A. Balk *et al.*** 1987. A controlled clinical trial of high-dose methyl-prednisolone in the treatment of severe sepsis and septic shock. *N. Engl. J. Med. 317:653.*

197. **Hinshaw, L., P. Peduzzi, E. Young, C. Sprung, C. Shatney, J. Sheagren, M. Wilson, and C. Haakenson.** 1987. Effect of high-dose glucocorticoid therapy on mortality in patients with clinical signs of systemic sepsis. *N. Engl. J. Med. 317:659.*

198. **Pichyangkul, S., D. Schick, W. Schober, G. Dixon, and A. Khan.** 1988. Increased expression of adhesive proteins on leukocytes by TNFα. *Exp. Hematol. 16:588.*

199. **Kettelhut, I. C., W. Fiers, and A. L. Goldberg.** 1987. The toxic effects of tumor necrosis factor *in vivo* and their prevention by cyclooxygenase inhibitors. *Proc. Natl. Acad. Sci. USA 84:4273.*

200. **Satomi, N., A. Sakurai, R. Haranaka, and K. Haranaka.** 1988. Preventive effects of several chemicals against lethality of recombinant human tumor necrosis factor. *J. Biol. Response Mod. 7:54.*

201. **Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami.** 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature 330:662.*

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

203. **Exley, A. R., J. Cohen, W. Buurman, R. Owen, G. Hanson, J. Lumley, J. M. Aulakh, M. Bodmer, A. Riddell, S. Stephens, and M. Perry.** 1990. Monoclonal antibody to TNF in severe septic shock. *Lancet 335:1275.*

204. **Khan, A., S. Grammer, D. Chang, N. O. Hill, and J. Miller.** 1983. Mouse monoclonal antibody (WI-MN-1) against malignant melanoma. *Cancer Res. 43:5868.*

205. **Wherry J. C., R. D. Schreiber, and E. R. Unanue.** 1991. Regulation of gamma interferon production by natural killer cells in *scid* mice: roles of tumor

necrosis factor and bacterial stimuli. *Infect. Immun. 59:1709.*

206. **Greenman, R. L., R. M. H. Schein, M. A. Martin, R. P. Wenzel, N. R. MacIntyre, G. Emmanuel, H. Chmel, R. B. Kohler, M. McCarthy, J. Plouffe, and J. A. Russell.** 1991. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of Gram-negative sepsis. *JAMA 266:1097.*

207. **Ziegler, E. J., C. J. Fisher, C. L. Sprung, R. C. Straube, J. C. Sadoff, G. E. Foulke, C. H. Wortel, M. P. Fink, R. P. Dellinger, N. N. H. Teng, I. E. Allen, H. J. Berger, G. L. Knatterud, A. F. Lo-Buglio, and C. R. Smith.** 1991. Treatment of Gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin: a randomized double-blind placebo-controlled trial. *N. Engl. J. Med. 324:429.*

208. **Bone, R. C.** 1991. Monoclonal antibodies to endotoxin: new allies against sepsis? *JAMA 266:1125.*

209. **Order, S. E. , J. L. Klein, D. Ettinger, P. Alderson, S. Siegelman, and P. Leichner.** 1980. Use of isotopic immunoglobulin in therapy. *Cancer Res. 40:3001.*

210. **Zalcberg, J. R., C. H. Thompson, M. Lichtenstein, and I. F. C. McKenzie.** 1984. Tumor immunotherapy in

the mouse with the use of ^{131}I-labeled monoclonal antibodies. *J. Natl. Cancer Inst. 72:697.*

211. **Winter, G., and C. Milstein.** 1991. Man-made antibodies. *Nature 349:293.*

212. **Urba, W. J., W. C. Kopp, J. W. Clark, J. W. Smith II, R. G. Steis, C. Huber, D. Coggin, and D. L. Longo.** 1991. The *in vivo* immunomodulatory effects of recombinant interferon gamma plus recombinant tumor necrosis factor-alfa. *J. Clin. Oncol. 9:1831.*

213. **Larrick J. W., and S. L. Kunkel.** 1986. Is Reye's syndrome caused by augmented release of tumour necrosis factor? *Lancet 2:132.*

214. **Bendtzen, K., A. K. Rasmussen, K. Bech, U. Feldt-Rasmussen, and J. Egeberg.** 1987. Cytokines in auto-immunity. *Immunol. Today 8:203.*

215. **Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch.** 1990. Structure and function of lipopolysaccharide binding protein. *Science 249:1429.*

216. **S. D. Wright, R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison.** 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science 249:1431.*