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(54) Title: METHOD OF TREATING VIRAL INFECTION

(57) Abstract

The present invention provides a method of treating viral infection in a mammal. The method comprises administering an anti-TNF ligand either alone or in combination with TNF to the mammal. The anti-TNF ligand is characterised in that when it binds to TNF the biological activity of the TNF is modified. The present invention further provides a composition for use in treating viral infections in a mammal.

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METHOD OF TREATING VIRAL INFECTION

Field of the Invention

The present invention relates to a method of treating viral infection in a mammal comprising administering to the mammal an anti-TNF ligand either alone or in combination with tumour necrosis factor alpha (TNF). The ligand is characterised in that the binding of the ligand to the TNF is such that the biological activity of TNF is modified. The invention also relates to a composition for treating viral infection.

Background of the Invention

Tumor necrosis factor alpha (TNF) is a product of activated macrophages first observed in the serum of experimental animals presensitized with Bacillus Calmette-Guerin or Corynebacterium parvum and challenged with endotoxin (LPS). Following the systematic administration of TNF haemorrhagic necrosis was observed in some transplantable tumours of mice while in vitro TNF caused cytolytic or cytostatic effects on tumour cell lines.

In addition to its host-protective effect, TNF has been implicated as the causative agent of pathological changes in septicemia, cachexia and cerebral malaria. Passive immunization of mice with a polyclonal rabbit serum against TNF has been shown to protect mice against the lethal effects of LPS endotoxin, the initiating agent of toxic shock, when administered prior to infection.

The gene encoding TNF has been cloned allowing the usefulness of this monokine as a potential cancer therapy agent to be assessed. While TNF infusion into cancer patients in stage 1 clinical trials has resulted in tumour regression, side-effects such as thrombocytopaenia, lymphocytopaenia, hepatotoxicity, renal impairment and hypertension have also been reported. These quite significant side-effects associated with the clinical use of TNF are predictable in view of the many known effects of TNF, some of which are listed in Table 1.

TABLE 1

BIOLOGICAL ACTIVITIES OF TNF

-ANTI-TUMOUR
-ANTI-VIRAL
-ANTI-PARASITE

FUNCTION
cytotoxic action on tumour cells
pyrogenic activity
angiogenic activity
inhibition of lipoprotein lipase
activation of neutrophils
osteoclast activation
induction of endothelial, monocyte and tumour cell procoagulant activity
induction of surface antigens on endothelial cells
induction of IL-6
induction of c-myc and c-fos
induction of EGF receptor
induction of IL-1
induction of TNF synthesis
induction of GM-CSF synthesis
increased prostaglandin and collagenase synthesis
induction of acute phase protein C3

Of particular importance is the activation of coagulation which occurs as a consequence of TNF activation of endothelium and also peripheral blood monocytes. Disseminated intravascular coagulation is associated with toxic shock and many cancers including gastro-intestinal cancer, cancer of the pancreas, prostate, lung, breast and ovary, melanoma, acute leukaemia, myeloma, myeloproliferative syndrome and myeloblastic leukaemia. Clearly modifications of TNF activity such that tumour regression activity remains

intact but other undesirable effects such as activation of coagulation are removed or masked would lead to a more advantageous cancer therapy. Complete abrogation of TNF activity is sought for successful treatment of toxic shock.

Segregation of hormonal activity through the use of site-specific antibodies (both polyclonal and monoclonal) can result in enhanced hormonal activity (Aston et al, 1989, Mol. Immunol. 26, 435). To date few attempts have been made to assign antigenicity or function to particular regions of the TNF molecule for which the three-dimensional structure is now known. Assignment of function to such regions would permit the development of MAbs and other ligands of therapeutic use. Polyclonal antibodies to amino acids 1 to 15 have been reported to block Hela R19 cell receptor binding by TNF (Socher et al, 1987, PNAS 84, 8829) whilst monoclonal antibodies recognising undefined conformational epitopes on TNF have been shown to inhibit TNF cytotoxicity in vitro (Bringman and Aggarwal, 1987, Hybridoma 6, 489). However, the effects of these antibodies on other TNF activities is unknown.

Description of the Present Invention

The present inventors have produced panels of monoclonal antibodies active against human TNF and have characterised them with respect to their effects on the anti-tumour effect of TNF (both in vitro and in vivo), TNF receptor binding, activation of coagulation (both in vitro and in vivo) and defined their topographic specificities. This approach has led the inventors to show that different topographic regions of TNF alpha are associated with different activities. This work is described in detail in a co-pending patent application filed under the Patent Cooperation Treaty in the Australian Receiving Office on 7 August 1990 and disclosure of this application is incorporated herein by reference.

The present inventors have made the surprising

finding that the administration of TNF in combination with a specific anti-TNF ligand provides an effective anti-viral treatment. In addition, it is believed that the administration of the specific anti-TNF ligand alone will provide an effective anti-viral therapy as the ligand will bind to endogenous TNF, thereby providing the same effect as if the anti-TNF ligand was administered in combination with TNF. This administration of the anti-TNF ligand alone may be the preferred method of therapy in disease states in which the endogenous levels of TNF are elevated.

Summary of the Invention

Accordingly, in a first aspect the present invention consists in a method of treating viral infection in a mammal comprising administering to the mammal an anti-TNF ligand either alone or in combination with TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited and the anti-viral activity of the TNF is unaffected or enhanced.

In a preferred embodiment of the present invention the ligand is further characterised in that when it binds to TNF the binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities for the TNF are enhanced; the cytotoxicity is unaffected and the tumour receptor binding activities of the TNF are unaffected or enhanced.

In a further preferred embodiment of the present invention the ligand is characterised in that the epitope of the TNF defined by the topographic region of residues 1 to 18 is substantially prevented from binding to naturally occurring biologically active ligands.

In yet a further preferred embodiment of the present invention the ligand binds to TNF such that the epitope of the TNF defined by the topographic regions of residues 1 -

30, 117 - 128 and 141 - 153 and more preferably in the topographic regions of residues 1 - 26, 117 - 128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands. Such sequence regions are topographically represented in Figure 26.

In a second aspect the present invention consists in a method of treating viral infection in a mammal comprising administering to the animal an anti-TNF ligand either alone or in combination with TNF, the ligand being characterised in that it binds to residues 1 to 18 of human TNF.

In a third aspect the present invention consists in a method of treating viral infection in a mammal comprising administering to the mammal an anti-TNF ligand either alone or in combination with TNF, the ligand being characterised in that it binds to human TNF in the topographic regions of residues 1 - 30, 117 - 128 and 141-153.

In a preferred embodiment of this aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 1 - 26, 117 - 128 and 141-153. Such sequence regions are topographically represented in Figure 26.

In a preferred embodiment of the present invention the ligand is an antibody raised against a peptide having an amino acid sequence substantially corresponding to amino acids 1 to 18 of human TNF (Peptide 301).

In a preferred embodiment of the present invention the ligand is monoclonal antibody designated MAb 32. A sample of the hybridoma producing MAb 32 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury,

Wiltshire SP4 OJG, United Kingdom on 3 August 1989 and was accorded accession number 89080302.

In a preferred embodiment of the present invention the method of treatment includes the co-administration of another anti-viral agent, such as, IL-2, AZT or acyclovir.

The present inventors have also found that there is a synergistic effect in treatment of viral infection between gamma interferon and TNF to which the ligand of the present is bound.

This synergistic effect can be obtained either by the administration of the anti-TNF ligand alone by making use of endogenous TNF and endogenous interferon. As would be clear to a person skilled in the art this same effect may be obtained in the following ways:-

1. Administration of anti-TNF ligand bound to TNF (making use of endogenous interferon);
2. Anti-TNF ligand together with gamma interferon (making use of endogenous TNF); and
3. Administration of anti-TNF ligand bound to TNF together with gamma interferon.

Accordingly, in a preferred embodiment of the present invention the method of treatment includes the co-administration of gamma interferon with the anti-TNF ligand either alone or in combination with TNF.

In a fourth aspect the present invention consists in a composition for use in treating viral infection the composition comprising gamma interferon and an anti-TNF ligand either alone or bound to TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited and the anti-viral activity of the TNF is unaffected or enhanced.

In a preferred embodiment of the present invention the ligand is further characterised in that when it binds to TNF the binding of TNF to receptors on endothelial

cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities for the TNF are enhanced; the cytotoxicity is unaffected and the tumour receptor binding activities of the TNF are unaffected or enhanced.

In a further preferred embodiment of the present invention the ligand is characterised in that the epitope of the TNF defined by the topographic region of residues 1 to 18 is substantially prevented from binding to naturally occurring biologically active ligands.

In yet a further preferred embodiment of the present invention the ligand binds to TNF such that the epitope of the TNF defined by the topographic regions of residues 1 - 30, 117 - 128 and 141 - 153 and more preferably in the topographic regions of residues 1 - 26, 117 - 128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands. Such sequence regions are topographically represented in Figure 26.

In a fifth aspect the present invention consists in a composition for use in treating viral infection in a mammal the composition comprising gamma interferon and an anti-TNF ligand either alone or bound to TNF, the ligand being characterised in that it binds to residues 1 - 18 of human TNF.

In a sixth aspect the present invention consists in a composition for use in treating viral infection in a mammal comprising gamma interferon and an anti-TNF ligand either alone or bound to TNF, the ligand being characterised in that it binds to human TNF in the topographic regions of residues 1 - 30, 117 - 128 and 141-153.

In a preferred embodiment of this aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 1 - 26, 117 - 128 and

141-153. Such sequence regions are topographically represented in Figure 26.

In a preferred embodiment of the present invention the ligand is an antibody raised against a peptide having an amino acid sequence substantially corresponding to amino acids 1 to 18 of human TNF (Peptide 301).

In a preferred embodiment of the present invention the ligand is monoclonal antibody designated MAb 32.

In a seventh aspect the present invention consists in the use of an anti-TNF ligand either alone or in combination with TNF in the production of a medicament for the treatment of viral infection in a mammal, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited and the anti-viral activity of the TNF is unaffected or enhanced.

In a preferred embodiment of the present invention the ligand is further characterised in that when it binds to TNF the binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities for the TNF are enhanced; the cytotoxicity is unaffected and the tumour receptor binding activities of the TNF are unaffected or enhanced.

In a further preferred embodiment of the present invention the ligand is characterised in that the epitope of the TNF defined by the topographic region of residues 1 to 18 is substantially prevented from binding to naturally occurring biologically active ligands.

In yet a further preferred embodiment of the present invention the ligand binds to TNF such that the epitope of the TNF defined by the topographic regions of residues 1 - 30, 117 - 128 and 141 - 153 and more preferably in the topographic regions of residues 1 - 26, 117 - 128 and 141-153 is substantially prevented from binding to

naturally occurring biologically active ligands. Such sequence regions are topographically represented in Figure 26.

In an eighth aspect the present invention consists in the use of an anti-TNF ligand either alone or in combination with TNF in the production of a medicament for the treatment of viral infection in a mammal, the anti-TNF ligand being characterised in that it binds to residues 1 - 18 of human TNF.

In a ninth aspect the present invention consists in the use of an anti-TNF ligand either alone or in combination with TNF in the production of a medicament for the treatment of viral infection in a mammal, the ligand being characterised in that it binds to human TNF in the topographic regions of residues 1 - 30, 117 - 128 and 141 - 153.

In a preferred embodiment of this aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 1 - 26, 117 - 128 and 141-153. Such sequence regions are topographically represented in Figure 26.

In a preferred embodiment of the present invention the ligand is an antibody raised against a peptide having an amino acid sequence substantially corresponding to amino acids 1 to 18 of human TNF (Peptide 301).

In a preferred embodiment of the present invention the ligand is monoclonal antibody designated MAb 32.

In a preferred embodiment of all aspects of the present invention the ligand is selected from the group consisting of antibodies, F(ab) fragments, restructured antibodies (CDR grafted humanised antibodies), single domain antibodies (dABs), single chain antibodies, anti-idiotypic antibodies, serum binding proteins, receptors and natural inhibitors. The ligand may also be a protein or peptide which has been synthesised and which

is analogous to one of the foregoing fragments. However, it is presently preferred that the ligand is a monoclonal or polyclonal antibody or F(ab) fragment thereof.

The biological activities of TNF referred to herein by the terms "Tumour Regression", "Induction of Endothelial Procoagulant", "Induction of Tumour Fibrin Deposition", "Cytotoxicity" and "Receptor Binding" are to be determined by the methods described below.

The term "single domain antibodies" as used herein is used to denote those antibody fragments such as described in Ward et al (Nature, Vol. 341, 1989, 544 - 546) as suggested by these authors.

Further information regarding anti-idiotypic antibodies can be found in Gaulton, G.N. and Greane, M.I. 1986. Idiotypic mimicry of biological receptors, Ann. Rev. Immunol. 4, 253-280; Sege, K and Peterson, P.A., 1978. Use of anti-idiotypic antibodies as cell surface receptor probes. Proc. Natl. Acad. Sci. U.S.A. 75, 2443-2447).

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following example and accompanying figures in which:-

Fig. 1 shows the results of a titration assay with MAb 32 against TNF;

Fig. 2 shows the effect of anti-TNF monoclonal antibodies 1 and 32 on TNF cytotoxicity in WEHI-164 cells;

Fig. 3 shows the effect of anti-TNF MAbs on induction of endothelial cell procoagulant activity by TNF;

Fig. 4 is a schematic representation of epitopes on TNF;

Fig. 5 shows binding of radio labelled TNF to receptors on bovine aortic endothelial cells;

Fig. 6 shows receptor binding studies of TNF complexed with MAb 32 (—◆—), control antibody (—□—)

and MAb 47 (—■—) on melanoma cell line MM418E;

Fig. 7 shows receptor binding studies of TNF complexed with MAb 32 (—◆—), control antibody (—⊡—) and MAb 47 (—■—) on melanoma cell line IGR3;

Fig. 8 shows receptor binding studies of TNF complexed with MAb 32 (—◆—), control antibody (—⊡—) and MAb 47 (—■—) on bladder carcinoma cell line 5637;

Fig. 9 shows receptor binding studies of TNF complexed with MAb 32 (—◆—), control antibody (—⊡—) and MAb 47 (—■—) on breast carcinoma cell line MCF7;

Fig. 10 shows receptor binding studies of TNF complexed with MAb 32 (—◆—), control antibody (—⊡—) and MAb 47 (—■—) on colon carcinoma cell line B10;

Fig. 11 shows the effect on TNF-mediated tumour regression <u>in vivo</u> by MAb 32 (■) control MAb (▰) and MAb 47 (*);

Fig. 12 shows the effect on TNF-mediated tumour regression <u>in vivo</u> by control MAb, MAb 32 and univalent FAb' fragments of MAb 32;

Fig. 13 shows the effect on TNF induced tumour regression by control MAb (■), MAb 32 (▨) and peptide 301 antiserum (⁘);

Fig. 14 shows MAb 32 reactivity with overlapping peptides of 10 AA length;

Fig. 15 shows a schematic three dimensional representation of the TNF molecule;

Fig. 16 shows topographically the region of residues 1 - 26, 117 - 128 and 141 - 153;

Fig. 17 shows the virus levels in ovaries following treatment with TNF alone and with 200 µl TNF-MAb 32;

□ 2 µg TNF; ■ 2 µg TNF + Ab; ▨ 4 µg TNF;

▩ 4 µg TNF + Ab;

Fig. 18 shows the virus levels in lungs following administration of TNF alone and 200 µl TNF-MAb 32;

□ 2 µg TNF; ■ 2 µg TNF + Ab; ▨ 4 µg TNF;

▩ 4 µg TNF + Ab;

Fig. 19 shows virus levels in spleens following administration of TNF alone and 200 µl TNF-MAb 32; ☐ 2 µg TNF; ■ 2 µg TNF + Ab; ▨ 4 µg TNF; ▩ 4 µg TNF + Ab;

Fig. 20 shows the titration of virus from the ovaries of TNF plus 1/500 Ab 301 treated CBA/H mice four days after infection; ☐ PBS (N=9); ▨ 2µg TNF plus 1/500 AB 301 (N=7); ▥ 4µg TNF plus 1/500 AB 301 (N=7); ▤ 6µg TNF plus 1/500 AB 301 (N=4); ▧ 8µg TNF plus 1/500 AB 301 (N=4);

Fig. 21 shows in vitro anti-HSV-1 induction in L929 cells treated with TNF and MAb 32;

Fig. 22 shows HSV-1 titration in the ovaries of mice treated twenty-four hours before infection with various TNF concentrations with or without Ab301; ☐ TNF alone; ▨ TNF plus 1/50 Ab 301; and

Fig. 23 shows the binding of ^{125}I-TNF to L929 cells either alone or in the presence of MAb 32 or Ab 301; —— TNF alone; —○— TNF plus MAb 32; —●— TNF plus AB 301.

Animals and Tumour Cell Lines

In all experiments BALB/C female mice aged 10-12 weeks obtained from the CSIRO animal facility were used. Meth A solid tumour and Meth A ascites tumour cell lines were obtained from the laboratory of Dr. Lloyd J. Old (Sloan Kettering Cancer Centre) and the WEHI-164 fibrosarcoma line was obtained from Dr. Geeta Chauhdri (John Curtin School of Medical Research, Australian National University).

Fusions and Production of Hybridomas

Mice were immunised with 10 ug human recombinant TNF intra-peritoneally in Freund's complete adjuvant. One month later 10 ug TNF in Freund's incomplete adjuvant was administered. Six weeks later and four days prior to fusion selected mice were boosted with 10 ug TNF in PBS. Spleen cells from immune mice were fused with the myeloma Sp2/0 according to the procedure of Rathjen and Underwood

(1986, Mol. Immunol. 23, 441). Cell lines found to secrete anti-TNF antibodies by radioimmunoassay were subcloned by limiting dilution on a feeder layer of mouse peritoneal macrophages. Antibody subclasses were determined by ELISA (Misotest, Commonwealth Serum Laboratories).

Radioimmunoassay

TNF was iodinated using lactoperoxidase according to standard procedures. Culture supernatants from hybridomas (50 ul) were incubated with 125I TNF (20,000 cpm in 50 ul) overnight at 4°C before the addition of 100 ul Sac-Cel (donkey anti-mouse/rat immunoglobulins coated cellulose, Wellcome Diagnostics) and incubated for a further 20 minutes at room temperature (20°C). Following this incubation 1 ml of PBS was added and the tubes centrifuged at 2,500 rpm for 5 minutes. The supernatant was decanted and the pellet counted for bound radioactivity.

Antibody-Antibody Competition Assays

The comparative specificites of the monoclonal antibodies were determined in competition assays using either immobilized antigen (LACT) or antibody (PACT) (Aston and Ivanyi, 1985, Pharmac. Therapeut. 27, 403)

PACT

Flexible microtitre trays were coated with monoclonal antibody (sodium sulphate precipitated globulins from mouse ascites fluid, 100 micrograms per ml in sodium bicarbonate buffer, 0.05M, pH 9.6) overnight at 4°C prior to blocking non-specific binding sites with 1% bovine serum albumin in PBS (BSA/PBS). The binding of 125I TNF to immobilised antibody was determined in the presence of varying concentrations of a second anti-TNF monoclonal antibody. Antibody and TNF were added simultaneously and incubated for 24 hours prior to washing with PBS (4 times) and counting wells for bound radioactivity. 100% binding was determined in the absence of heterologous monoclonal antibody while 100% competition was determined in the

presence of excess homologous monoclonal antibody. All dilutions were prepared in BSA/PBS.

LACT

The binding of protein A purified, radiolabelled monoclonal antibodies to TNF coated microtitre wells was determined in the presence of varying concentrations of a second monoclonal antibody. Microtitre plates were coated with TNF (50 micrograms per ml) as described above. Quantities of competing antibodies (50 microlitres) were pre-incubated on plates for 4 hour at $4^{o}C$ prior to addition of 125I monoclonal antibody (30,000 cpm) for a further 24 hours. Binding of counts to wells was determined after four washes with PBS. 100% binding was determined in the absence of competing antibody while 100% competition was determined in the presence of excess unlabelled monoclonal antibody.

WEHI-164 Cytotoxicity Assay

Bioassay of recombinant TNF activity was performed according to Espevik and Nissen-Meyer (1986, J. Immunol. Methods 95, 99). The effect of the monoclonal antibody on TNF activity was determined by the addition of the monoclonal antibody to cell cultures at ABT90.

Tumour Regression Experiments

Modulation of TNF-induced tumour regression activity by monoclonal antibodies was assessed in three tumour models: the subcutaneous tumours WEHI-164 and Meth A sarcoma and the ascitic Meth A tumour. Subcutaneous tumours were induced by the injection of approximately 5 x 10^5 cells. This produced tumours of between 10 - 15 mm approximately 14 days later. Mice were injected intra-peritoneally with human recombinant TNF (10 micrograms) plus monoclonal antibody (200 microlitres ascites globulin) for four consecutive days. Control groups received injections of PBS alone or TNF plus monoclonal antibody against bovine growth hormone. At the

commencement of each experiment tumour size was measured with calipers in the case of solid tumours or tumour-bearing animals weighed in the case of ascites mice. These measurements were taken daily throughout the course of the experiment.

Radio-Receptor Assays

WEHI-164 cells grown to confluency were scrape harvested and washed once with 1% BSA in Hank's balanced salt solution (HBSS, Gibco). 100 ul of unlabelled TNF (1-10,000 ng/tube) or monoclonal antibody (10 fold dilutions commencing 1 in 10 to 1 in 100,000 of ascitic globulin) was added to 50ul 125I TNF (50,000 cpm). WEHI cells were then added (200 microlitres containing 2 x 10^6 cells). This mixture was incubated in a shaking water bath at 37°C for 3 hours. At the completion of this incubation 1 ml of HBSS was added and the cells spun at 16,000 rpm for 30 seconds. The supernatant was discarded and bound 125I TNF in the cell pellet counted. All dilutions were prepared in HBSS containing 1% BSA.

Procoagulant Induction by TNF on Endothelial Cells

Bovine aortic endothelial cells (passage 10) were grown in RPMI-1640 containing 10% foetal calf serum (FCS), penicillin, streptomycin, and 2-mercaptoethanol at 37°C in 5% CO_2. For induction of procoagulant activity by TNF the cells were trypsinised and plated into 24-well Costar trays according to the protocol of Bevilacqua et al., 1986 (PNAS 83, 4533). TNF (0-500 units/culture) and monoclonal antibody (1 in 250 dilution of ascitic globulin) was added after washing of the confluent cell monolayer with HBSS. After 4 hours the cells were scrape harvested, frozen and sonicated. Total cellular procoagulant activity was determined by the recalcification time of normal donor platelet-poor plasma performed at 37°C, 100 microlitres of citrated platelet-poor plasma was added to 100 ul of cell lysate and 100 ul of calcium chloride (30mM) and the

time taken for clot formation recorded. In some experiments tumour cell culture supernatant was added to endothelial cells treated with TNF and/or monoclonal antibody (final concentration of 1 in 2).

Incorporation of 125I Fibrinogen into Tumours of Mice Treated with TNF and Monoclonal Antibody

In order to examine the effect of TNF and monoclonal antibodies on fibrin formation in vivo, BALB/c mice were injected subcutaneously with WEHI-164 cells (10^5 cells/animal). After 7 - 14 days, when tumours reached a size of approximately 1 cm in diameter, animals were injected intra-peritoneally with TNF (10 ug/animal) and 125I human fibrinogen (7.5ug/animal, 122uCi/mg Amersham) either alone or in the presence of monoclonal antibody to human TNF (200ul/animal ascitic globulin). Monoclonal antibody against bovine growth hormone was used as control monoclonal antibody. Two hours after TNF infusion incorporation of 125I fibrinogen into mouse tissue was determined by removing a piece of tissue, weighing it and counting the sample in a gamma counter.

In all 13 monoclonal antibodies reacting with human TNF were isolated. These monoclonal antibodies were designated MAb 1, MAb 11, MAb 12, MAb 20, MAb 21, MAb 25, MAb 31, MAb 32, MAb 37, MAb 42, MAb 47, MAb 53 and MAb 54. The effect of these monoclonal antibodies on the bioactivity of human TNF is set out in Table 2.

As can be seen from Table 2, whilst some monoclonal antibodies inhibit both anti-tumour activity and activation of coagulation by human TNF (MAb 1, 47 and 54) not all antibodies which inhibit the anti-tumour activity inhibit activation of coagulation either in vitro or in vivo (MAb 11, 12, 25 and 53). Indeed MAb 21 which inhibited tumour regression enhanced the activation of coagulation in vivo.

TABLE 2

EFFECT OF MONOCLONAL ANTIBODIES ON TNF BIOACTIVITY

TNF BIOACTIVITY	MONOCLONAL ANTIBODY												
	1	11	12	20	21	25	31	32	37	42	47	53	54
Cytotoxicity	-	-	-	0	-	-	0	0	0	0	-	-	-
Tumour Regression	-	-	-	0	-	-	0	+	0	0	-	-	-
Induction of Procoagulant (Endothelial)	-	0	0	-	-	0	0	-	0	-	-	-	-
Fibrin Deposition (tumour)	-	-	-	+	+	+	+	+	0	-	-	0	-
Receptor Binding (WEHI-164)	-	-	-	0	-	-	0	+/0*	0	0	-	-	-

\+ Enhancement

0 No effect

\- Inhibition

* Depending on MAb concentration in the case of WEHI-164 tumour cells and tumour type (see Figs. 3, 13 - 17).

MAbs 1, 47 and 54, which have been shown in competition binding studies to share an epitope on TNF, can be seen to have highly desirable characteristics in treatment of toxic shock and other conditions of bacterial, viral and parasitic infection where TNF levels are high requiring complete neutralisation of TNF. Other monoclonal antibodies such as MAb 32 are more appropriate as agents for coadministration with TNF during cancer therapy since they do not inhibit tumour regression but do inhibit activation of coagulation. This form of therapy is particularly indicated in conjunction with cytotoxic drugs used in cancer therapy which may potentiate

activation of coagulation by TNF (e.g. vinblastin, acyclovir, IFN alpha, IL-2, actinomycin D, AZT, radiotherapy, adriamycin, mytomycin C, cytosine arabinoside, dounorubicin, cis-platin, vincristine, 5-flurouracil, bleomycin, (Watanabe N et al 1988 Immunopharmacol. Immunotoxicol. 10 117-127) or in diseases where at certain stages TNF levels are low (e.g. AIDS) and where individuals may have AIDS associated cancer e.g. Kaposi sarcoma, non-Hodgkins lymphoma and squamous cell carcinoma.

MAb 32 (Fig. 1) is an IgG2b,K antibody with an affinity for human TNF alpha of 8.77×10^{-9} moles/litre as determined by Scatchard analysis. This monoclonal antibody does not react with either human TNF beta (lymphotoxin) or mouse TNF alpha.

As shown in Figure 2 MAb 32 does not inhibit TNF cytotoxicity in vitro as determined in the WEHI-164 assay.

Monoclonal antibody 32 variably enhances TNF-induced tumour regression activity against WEHI-164 fibrosarcoma tumours implanted subcutaneously into BALB/c mice at a TNF dose of 10ug/day (see Fig. 11). This feature is not common to all monoclonal antibodies directed against TNF but resides within the binding site specificity of MAb 32 (Fig. 4) which may allow greater receptor mediated uptake of TNF into tumour cells (see Table 3).

TABLE 3

BINDING OF TNF TO RECEPTORS ON WEHI-164 CELLS IN THE PRESENCE OF MAb 32

	% BINDING ^{125} I-TNF	
MAB DILUTION	CONTROL MAB	MAB 32
1/10	36	141
1/100	74	88
1/1000	101	83
1/10,000	92	82
1/100,000	97	93

Enhancement of TNF activity by MAb 32 at lower doses of TNF is such that at least tenfold less TNF is required to achieve the same degree of tumour regression (see Fig. 11. The results for day 1, 2.5ug and 1ug TNF and day 2, 5ug, 2.5ug and 1ug are statistically significant in a t-test at $p < .01$ level. This level of enhancement also increases the survival rate of recipients since the lower dose of TNF used is not toxic. Fig. 12 shows that univalent Fab fragments of MAb 32 also cause enhancement of TNF-induced tumour regression in the same manner as whole MAb 32 (see below).

MAb 32 inhibits the expression of clotting factors on endothelial cells normally induced by incubation of the cultured cells with TNF (see Fig. 3). This response may be mediated by a previously unidentified TNF receptor which is distinct to the receptor found on other cells.

Conversely, MAb 32 enhances the <u>in vivo</u> activation of coagulation within the tumour bed as shown by the incorporation of radiolabelled fibrinogen. This may be due to activation of monocytes/macrophage procoagulant and may provide further insight into the mechanism of TNF-induced tumour regression.

The results obtained with MAb 32 are shown in comparison to other anti-TNF MAbs in Table 2.

The ability of MAb 32 and MAb 47 to inhibit the binding of TNF to endothelial cells was also assessed. Bovine aortic endothelial (BAE) cells (passage 11) were plated in 24-well culture dishes (Corning) which had been pre-coated with gelatin (0.2%) and grown to confluence in McCoys 5A (modified) medium supplemented with 20% foetal calf serum. For the radio-receptor assay all dilutions (of cold TNF and MAbs) were made in this medium. The BAE cells were incubated for one hour in the presence of either cold TNF (0 to 100ng) or MAb (ascites globulins diluted 1/100 to 1/100,000) and iodinated TNF (50,000

cpm). At the end of this time the medium was withdrawn and the cells washed before being lysed with 1M sodium hydroxide. The cell lysate was then counted for bound radioactive TNF. Specific binding of labelled TNF to the cells was then determined.

The results obtained in this assay with MAb 32, MAb 47 and a control MAb are set out in Figure 5.

The results obtained in the clotting assay using BAE cells cultured in the presence of TNF and anti-TNF MAb correlate with the results obtained in the BAE radioreceptor assay i.e. MAbs which inhibit the induction of clotting factors on the surface of endothelial cells (as shown by the increase in clotting time compared to TNF alone) also inhibit the binding of TNF to its receptor. This is exemplified by MAbs 32 and 47.

MAb 32, which does not inhibit TNF binding to WEHI-164 cells, does inhibit binding of TNF to endothelial cells. This result provides support for the hypothesis that distinct functional sites exist on the TNF molecule and that these sites interact with distinct receptor subpopulations on different cell types. Thus ligands which bind to defined regions of TNF are able to modify the biological effects of TNF by limiting its binding to particular receptor subtypes.

As shown in Figure 5 MAb 47 is a particularly potent inhibitor of TNF interaction with endothelial cells, the percentage specific binding at a dilution of 1/100 to 1/10,000 being effectively zero.

RECEPTOR BINDING STUDIES OF HUMAN TNF COMPLEXED WITH MAB 32 ON HUMAN CARCINOMA CELL LINES IN VITRO

MAb 32 has been shown to enhance the anti-tumour activity of human TNF. The mechanisms behind the enhancement may include restriction of TNF binding to particular (tumour) receptor subtypes but not others (endothelial) with subsequent decrease in TNF toxicity to

non-tumour cells. This mechanism does not require enhanced uptake of TNF by tumour cells in in vitro assays. In addition, MAb 32 also potentiates the binding of human TNF directly to TNF receptors on certain human carcinoma cell lines.

MATERIALS AND METHODS

The following human carcinoma cell lines have been assayed for enhanced receptor-mediated uptake of TNF in the presence of MAb 32: B10, CaCo, HT 29, SKC01 (all colon carcinomas), 5637 (Bladder carcinoma), MM418E (melanoma), IGR3 (melanoma), MCF 7 (breast carcinoma). The cells were propogated in either RPMI-1640 (MM418E) DMEM (CaCo and IGR 3) or Iscoves modified DMEM (B10, HT 29, SK01, S637, MCF 7) supplemented with 10% foetal calf serum, penecillin/streptomycin and L-glutamine. Receptor assays were performed as previously described for endothelial cells except that the incubation time with iodinated TNF was extended to 3 hours for all but the B10 cells for which the radiolabel was incubated for 1 hour.

RESULTS

Enhanced TNF uptake was observed in the presence of MAb32 by the melanoma cell lines tested MM418E and IGR 3 (Figs. 13 and 14), the bladder carcinoma 5637 (Fig. 8), and the breast carcinoma MCF 7 (Fig. 9). MAb 32 did not affect TNF-receptor interaction in any of the other cell lines as shown by B 10 (Fig. 10) MAb 47, which has been shown to inhibit TNF binding to WEHI-164 cells and endothelial cells, and which also inhibits TNF-mediated tumour regression was found to markedly inhibit TNF binding to all the cell lines tested (Figs. 6-10).

CONCLUSIONS

Receptor binding analyses have indicated a second mechanism whereby MAb 32 may potentiate the anti-tumour activity of TNF. This second pathway for enhancement of TNF results from increased uptake of TNF by tumour all

receptors in the presence of MAb 32.

ENHANCEMENT OF TNF-MEDIATED TUMOUR REGRESSION IN VIVO BY MAB 32 OR UNIVALENT FAB' FRAGMENTS OF MAB 32

Tumour regression studies were carried out as described above in mice carrying WEHI-164 subcutaneous tumours (N = 5 animals/group). Tumour size was determined daily during the course of the experiment. The results obtained using MAb 32 are set out in Fig. 11 and show the mean +/- SD% change in tumour area at the completion of treatment (day 2) (MAb 32: control MAb: *MAb 47). Differences observed between control MAb-TNF and MAb 32-TNF treated groups are statistically significant in a T-test at the $p < .01$ level.

The results using the univalent FAb' fragments of MAb 32 are shown in Fig. 12. Tumour size was determined daily during the course of the experiment. The results show the mean +/- SD% change in tumour area at the completion of treatment (day 2). Differences between the control, TNF and MAb 32-TNF treated groups are statistically significant in a T-test at the $p < .01$ level.

TNF INDUCED TUMOUR REGRESSION : EFFECT OF ANTI-PEPTIDE 301 SERA

Fig. 13 shows the percent change in tumour area in tumour-bearing mice treated for three days with TNF plus control MAb (antibody against bovine growth hormone), TNF plus MAb 32 or TNF plus antiserum (globulin fraction) against peptide 301. In an unpaired T-test the control group is significantly different from both of the test groups (MAb 32, antiserum 301) while the MAb 32 and peptide antiserum 301 groups are not significantly different from each other. (control vs MAb 32, $p < .002$; control vs antipeptide 301, $p < .025$). Thus antisera raised using a peptide which comprises part of the MAb 32 specificity, also causes TNF enhancement of tumour regression.

As shown in Fig. 4 competition binding studies has shown that the thirteen monoclonal antibodies can be sub-divided into two main groups, namely MAbs 1, 21, 47, 54, 37, 32 and 25 and MAbs 11, 12, 53 and 42. Experiments were then conducted to identify the regions on human TNF recognised by these monoclonal antibodies.

IDENTIFICATION OF REGIONS ON HUMAN TNF RECOGNISED BY MONOCLONAL ANTIBODIES

Methods

1. Overlapping peptides of 7 and 10 amino acid residues long were synthesized on polypropylene pins according to the method of Geysen et al., 1984, PNAS 81, 3998-4002. The overlap was of 6 and 9 residues respectively and collectively the peptides covered the entire TNF amino acid sequence. The peptides were tested for reactivity with the MAbs by ELISA. MAbs which had TNF reactivity absorbed from them by prior incubation with whole TNF were also tested for reactivity with the peptides and acted as a negative control.

2. Longer peptides of TNF were synthesized as described below. These peptides were used to raise antisera in sheep using the following protocol. Merino sheep were primed with TNF peptide conjugated to ovalbumin and emulsified in Freunds Complete adjuvant and boosted at 4 weekly intervals with peptide-ovalbumin and sera assayed for the presence of anti-TNF antibody by radioimmunoassay. Of the peptides shown only peptides 275, 301, 305, 306 and 307 elicited sera reacting with whole TNF. The positive sera were then used in competitive binding assays (PACT assays) with the MAbs.

The following peptides were synthesised and are described using the conventional three letter code for each amino acid with the TNF sequence region indicated in brackets.

Peptide 275

H-Ala-Lys-Pro-Trp-Tyr-Glu-Pro-Ile-Tyr-Leu-OH (111-120)

Peptide 301

H-Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-OH (1-18)

Peptide 302

H-Leu-Arg-Asp-Asn-Gln-Leu-Val-Val-Pro-Ser-Glu-Gly-Leu-Tyr-Leu-Ile-OH (43-58)

Peptide 304

H-Leu-Phe-Lys-Gly-Gln-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile-Ser-Arg-Ile-OH (63-83)

Peptide 305

H-Leu-Ser-Ala-Glu-Ile-Asn-Arg-Pro-Asp-Tyr-Leu-Asp-Phe-Ala-Glu-Ser-Gly-Gln-Val-OH (132-150)

Peptide 306

H-Val-Ala-His-Val-Val-Ala-Asn-Pro-Gln-Ala-Glu-Gly-Gln-Leu-OH (13-26)

Peptide 307

H-Ala-Glu-Gly-Gln-Leu-Gln-Trp-Leu-Asn-Arg-Arg-Ala-Asn-Ala-Leu-Leu-Ala-Asn-Gly-OH (22-40)

Peptide 308

H-Gly-Leu-Tyr-Leu-Ile-Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-OH (54-68)

Peptide 309

H-His-Val-Leu-Leu-Thr-His-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Thr-Gln-Thr-Lys-Val-Asn-Leu-Leu-COOH (73-94)

Peptide 323

H-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Thr-Gln-Thr-OH (79-89)

These peptides were synthesised using the following general protocol.

All peptide were synthesised using the Fmoc-polyamide method of solid phase peptide synthesis (Atherton et al, 1978, J.Chem.Soc.Chem.Commun., 13, 537-539). The solid resin used was PepSyn KA which is a polydimethylacrylamide gel on Kieselguhr support with 4-hydroxymethylphenoxy-

acetic acid as the functionalised linker (Atherton et al., 1975, J.Am.Chem. Soc. 97, 6584-6585).

The carboxy terminal amino acid was attached to the solid support by a DCC/DMAP-mediated symmetrical-anhydride esterification.

All Fmoc-groups were removed by piperidine/DMF wash and peptide bonds were formed either via pentafluorophenyl active esters or directly by BOP/NMM/HOBt (Castro's reagent) (Fournier et al, 1989, Int.J.Peptide Protein Res., 33, 133-139) except for certain amino acids as specified in Table 4.

Side chain protection chosen for the amino acids was removed concomittantly during cleavage with the exception of Acm on cysteine which was left on after synthesis.

TABLE 4

Amino Acid	Protecting Group	Coupling Method
Arg	Mtr or Pmc	Either
Asp	OBut	Either
Cys	Acm (permanent)	Either
Glu	OBut	Either
His	Boc	OPfp only
Lys	Boc	Either
Ser	But	BOP only
Thr	But	BOP only
Tyr	But	Either
Trp	none	Either
Asn	none	OPfp only
Gln	none	OPfp only

Cleavage and Purification

Peptide 301, 302, 305 are cleaved form the resin with 95% TFA and 5% thioanisole (1.5 h) and purified on reverse phase C4 column, (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

Peptide 303, 304 are cleaved from the resin with 95% TFA and 5% phenol (5-6 h) and purified on reverse phase C4 column. (Buffers as above).

Peptide 306, 308 are cleaved from the resin with 95%

TFA and 5% water (1.5 h) and purified on reverse phase C4 column. (Buffers as above).

Peptide 309 Peptide was cleaved from the resin with 95% TFA and 5% thioanisole and purified on reverse phase C4 column. (Buffers as above).

Peptide 307 Peptide was cleaved from the resin with a mixture of 93% TFA, 3.1% Anisole, 2.97% Ethylmethylsulfide and 0.95% Ethanedithiol (3 h) and purified on reverse phase C4 column. (Buffers as above).

RESULTS

Typical results of MAb ELISA using the 7 and 10 mers are shown in Fig. 21. Together with the results of PACT assays using the sheep anti-peptide sera (shown in Table 6) the following regions of TNF contain the binding sites of the anti-TNF MAbs.

MAb 1 : residues 1-18, 58-65, 115-125, 138-149
MAb 11: residues 49-98
MAb 12: residues 22-40, 70-87
MAb 21: residues 1-18, 76-90
MAb 25: residues 12-22, 36-45, 96-105, 132-157
MAb 32: residues 1-26, 117-128, 141-153
MAb 37: residues 22-31, 146-157
MAb 42: residues 22-40, 49-96, 110-127, 136-153
MAb 47: residues 1-18, 108-128
MAb 53: residues 22-40, 69-97, 105-128, 135-155
MAb 54: residues 56-79, 110-127, 136-155

TABLE 5

COMPETITIVE BINDING OF TNF BY ANTI-TNF MONOCLONES IN THE PRESENCE OF ANTI PEPTIDE SERA

MAB/PEPTIDE SERA	275	301	305	306	307
1	-	+	-	-	-
11	-	+/-	-	-	-
12	-	+	-	-	++
21	-	++	-	-	-
25	-	+	-	-	-
32	-	++++	+	+	-
37	-	+	+/-	-	+
47	-	+	-	-	-
53	-	+	-	-	+
54	-	+	-	-	-
42	-	+	+	-	+

Note 1: - indicates no competition, + indicates slight competition at high concentration of anti-peptide antisera (1/50), ++++ indicates strong competition by anti-peptide sera equal to that of the homologous MAb. Note 2: Only peptides which elicited sera recognising whole TNF were used in this assay.

Antibody 301

Sheep were immunised with Peptide 301 conjugated with ovalbumin in Freunds adjuvant. Antiserum reactive with Peptide 301 was obtained from immunised sheep.

CONCLUSIONS

Mapping of the regions recognised by each of the MAbs has indicated that MAbs in group I (MAbs 1, 21, 47, 54, 37, 32 and 25) as shown on the schematic diagram bind TNF in the region of residues 1-18 with the exception of MAbs 37 and 54, while MAbs in group II of the schematic diagram (MAbs 11, 12, 53 and 42) bind TNF in the region of residues 70-96 which encompasses a so-called pallendromic loop on the TNF 3-D structure. MAbs which inhibit the induction of endothelial cell procoagulant activity (MAbs 1, 32, 42, 47, 54 and 53) all bind in the region of residues 108-128 which again contains a loop structure in

the 3-D model and may indicate that this region interacts with TNF receptors which are found on endothelial cells but not tumour cells. MAb 32 which potentiates the in vivo tumour regression and anti-viral activity of TNF is the only antibody which binds all the loop regions associated with residues 1-26, 117-128, and 141-153 and hence binding of these regions is crucial for enhanced TNF bioactivity with concommittant reduction of toxicity for normal cells.

As is apparent from Table 2 MAb 1, 47 and 54 have the same effect on the bioactivity of TNF. From the results presented above it is noted that these three monoclonals bind to similar regions of the TNF molecule. Accordingly, it is believed that a ligand which binds to TNF in at least two regions selected from the group consisting predominately of the region of residues 1-20, the region of residues 56-77, the region of residues 108-128 and the region of residues 138-149 will effect the bioactivity of TNF in a manner similar to that of MAbs 1, 47 and 54. Similarly, it is believed that a ligand which binds to TNF predominately in the regions of residues 1-20 and 76-90 will have the same effect on the bioactivity of TNF as MAb 21. A ligand which binds to TNF predominately in the regions of residues 22-40 and 69-97 will have the same effect on bioactivity of TNF as MAb 12. A ligand which binds to TNF predominately in the regions of residues 1-30, 117-128, and 141-153 would be expected to have the same effect on the bioactivity of TNF as MAb 32 and a ligand which binds to TNF predominately in the regions of residues 22-40, 49-97, 110-127 and 136-153 would be expected to have the same effect on the bioactivity of TNF as MAb 42. A ligand which binds to TNF predominately in the regions of residues 22-31 and 146-157 would be expected to have the same effect on the bioactivity of TNF as MAb 37 and a ligand which binds to TNF predominately in

the regions of residues 22-40, 69-97, 105-128 and 135-155 would be expected to have the same effect on the bioactivity of TNF as MAb 53.

The present inventors have quite clearly shown that the bioactivity of TNF can be altered by the binding of a ligand to the TNF, and that the effect on the bioactivity is a function of the specificity of the ligand. For example, the binding of MAb 32 to TNF in the regions of residues 1-26, 117-128 and 141-153 results in the induction of endothelial procoagulant activity of the TNF and binding of TNF to receptors on endothelial cells being inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF being enhanced; the cytotoxicity being unaffected and the tumour receptor binding activities of the TNF being unaffected or enhanced. It is believed that this effect on the bioactivity of the TNF may be due to the prevention of the binding of the epitope of the TNF recognised by MAb 32 to naturally occurring biologically active ligands. Accordingly, it is believed that a similar effect to that produced by MAb 32 could also be produced by a ligand which binds to a region of TNF in a manner such that the epitope recognised by MAb 32 is prevented from binding to naturally occurring biologically active ligands. This prevention of binding may be due to steric hindrance or other mechanisms.

Accordingly, it is intended that the prevention of the binding of epitopes recognised by MAb 32 and Ab 301 described herein to naturally occurring biologically active ligands is within the scope of the present invention.

Effect of Anti-TNF MAb 32 on Anti-Viral Activity of Human TNF Alpha

TNF has been shown to exert an anti-viral effect both in vitro (Mestan et al Nature 323, 816-819, 1986; Wong

and Goeddel Nature 323, 819-822, 1986) and in vivo (Doherty et al J.Immunol. 142, 376-380, 1989). The present inventors investigated the effect of TNF-MAb 32 complexes on the anti-viral effect of TNF in vaccinia infected mice.

Twenty-four hours prior to infection of CBA-H mice with vaccinia (10^7 PFU VV-HA-TK, Ramshaw et al Nature 329, 44-46, 1987) the mice were treated with either TNF alone (recombinant human TNF) or TNF and MAb 32 (200 ul ascites globulin) which had been mixed twenty minutes prior to inoculation. Virus titres in ovaries, lung and spleen samples which had been homogenised and treated with trypsin (1 mg/ml) were determined four days later using the 143B indicator cell line.

Mice which were treated with TNF-MAb 32 showed reduced virus levels in ovaries (Fig. 17), lungs (Fig. 35), and spleen (Fig. 19) compared to mice treated with TNF alone.

Similar results were obtained with mice treated with TNF and antibody 301 (Fig. 20).

HSV-1 In Vivo Protocol

CBA/H mice were treated 24 hours before infection with 107 pfu Herpes Simplex Virus 1 (HSV-1) (ip) with the relevant TNF +/- Ab 301 administrations. The antibody was diluted 1/50 before mixing with 6.0 micrograms of TNF, and the mixture then left for an hour at room temperature. From this stock TNF+Ab 301 the various concentration sof TNF in complex with the Ab was removed and diluted in PBS (i.e., 0.5-2.0 micrograms). The mice were left for three days post infection after which the animals were sacrificed and the ovaries aseptically removed. These organs were then homogenised in PBS and 100 μl treated with 0.1% trypsin for 30 minutes. Trypsinisation was stopped by the addition of FCS. This sample was then serially diluted by a factor of 1/10. These dilutions

were then absorbed onto Vero cells for 1 hour at 370C, overlaid with F15 + 5% FCS and left for 2 days at 370C. The plaques were then counted and the virus concentration calculated. The results are shown in Fig. 22.

HSV-1 In Vitro Protocol

L929 cells were seeded at a concentration of 500 000 cells/well (Linbro 24 well plate) in the presence of TNF alone (10-400 ng) or in complex with Mab 32 (complex procedure described above). These cultures were then left for 24 hours after which they were infected with HSV-1 (0.1-2.0 MOI). 100 µl of virus-containing PBS was left to absorb on the cells for 1 hour at 370C, then the excess virus was removed and the cells overlaid with F15 +5% FCS. These cultures were then left for 48 hours. After this period the cells were frozen and thawed x2 and the supernatents serially diluted (trypsin treatment not necessary) and absorbed and grown on Vero cells as described above. The results are shown in Fig. 21.

The Binding of ^{125}I-TNF to L929 Cells Either Alone or in the Presence of Mab 32 or Ab 301

To different dilutions of Mab 32 or Ab 301 in RPMI plus 10% FCS iodinated TNF (50 000 cpm/50 µl) was added followed by 2.0 x 10^6 L929 cells, again in RPMI. These treatments were then incubated for 3 hours after which the cells were washed once and the pellets counted. All treatments were done in triplicate. The results are shown in Fig. 23.

Effect of Co-Administration of Gamma Interferon on Antiviral Activity of Anti-TNF Ligand and TNF

Antibody 301 complexed to TNF was produced by diluting antibody 301 1:50 prior to mixing with 6 µg TNF. The TNF plus antibody was administered (i.p.) alone as well as 10^5 U/mouse murine gamma interferon alone. A TNF with Ab 301 plus mIFN-γ treatment was also administered. The mIFN-γ was not mixed with the TNF:

Ab 301 complex until after the 1 hour room temperature incubation of TNF with the antibody. 24 hours later the mice were injected with 10^6 pfu vaccinia intravenously (i.v.). The results of this experiment are shown in Table 6.

TABLE 6

Cytokine Treatment	Log^{10} Virus Titre (pfu/ml) ± SEM
PBS (n=5)	8.20 ± 0.29
6 μg TNF + 1/50 Ab 301 (n=4)	5.93 ± 0.33
10^5U mIFN-γ (n=5)	5.66 ± 0.35
6 μg TNF + 1/50 Ab 301 + 10^5U mIFN-γ	<2.0

As can be seen from the results set out in Table 6 there is a marked synergistic anti-viral effect between the mIFN-γ and the MAb 301: TNF complex.

As is readily apparent from the results shown in Figs. 17, 18 and 19 the administration of TNF in combination with the anti-TNF ligand MAb 32 results in a decrease in the number of virus particles recoverable from the infected animal. Without wishing to be bound by scientific theory, it is believed that the enhanced anti-viral effect provided by the administration of TNF in combination with an anti-TNF ligand is a result of the ligand increasing the amount of TNF available by either preventing the binding of the TNF to endothelial receptors or by directly increasing the binding of TNF to receptors on virus infected cells (Fig. 23). Accordingly, it is

believed that the method of the present invention would be particularly applicable to the treatment of viral infections which are not confined to the infection of endothelial surfaces. In particular, it is believed that the method of the present invention is applicable in the treatment of infection with the following viruses, hepatitis, AIDS, herpes, viral meningitis, green monkey virus and vaccinia.

It will be recognised by persons skilled in the art that numerous variations and modifications may be made to the invention as described above without departing from the spirit or scope of the invention as broadly described.

CLAIMS:-

1. A method of treating viral infection in a mammal comprising administering to the mammal an anti-TNF ligand either alone or in combination with TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited and the anti-viral activity of the TNF is unaffected or enhanced.

2. A method as claimed in claim 1 in which the ligand is further characterised in that when it binds to TNF the binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities for the TNF are enhanced; the cytotoxicity is unaffected and the tumour receptor binding activities of the TNF are unaffected or enhanced.

3. A method as claimed in claims 1 or 2 in which the ligand is characterised in that the epitope of the TNF defined by the topographic region of residues 1 to 18 is substantially prevented from binding to naturally occurring biologically active ligands.

4. A method as claimed in claims 1 or 2 in which the ligand binds to TNF such that the epitope of the TNF defined by the topographic regions of residues 1 - 30, 117 - 128 and 141 - 153 and more preferably in the topographic regions of residues 1 - 26, 117 - 128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands.

5. A method of treating viral infection in a mammal comprising administering to the animal an anti-TNF ligand either alone or in combination with TNF, the ligand being characterised in that it binds to residues 1 to 18 of human TNF.

6. A method of treating viral infection in a mammal comprising administering to the mammal an anti-TNF ligand either alone or in combination with TNF, the ligand being

characterised in that it binds to human TNF in the topographic regions of residues 1 - 30, 117 - 128 and 141-153.

7. A method as claimed in claim 6 in which the ligand binds to human TNF in the topographic regions of residues 1 - 26, 117 - 128 and 141-153.

8. A method as claimed in claims 3 or 5 in which the ligand is an antibody raised against a peptide having an amino acid sequence substantially corresponding to amino acids 1 to 18 of human TNF (Peptide 301).

9. A method as claimed in any one of claims 1-7 in which the ligand is monoclonal antibody designated MAb 32.

10. A method as claimed in any one of claims 1-9 in which the method of treatment includes the co-administration of another anti-viral agent, such as, IL-2, AZT or acyclovir.

11. A method as claimed in any one of claims 1-10 in which the method of treatment includes the co-administration of gamma interferon with the anti-TNF ligand either alone or in combination with TNF.

12. A composition for use in treating viral infection the composition comprising gamma interferon and an anti-TNF ligand either alone or bound to TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited and the anti-viral activity of the TNF is unaffected or enhanced.

13. A composition as claimed in claim 12 in which the ligand is further characterised in that when it binds to TNF the binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities for the TNF are enhanced; the cytotoxicity is unaffected and the tumour receptor binding activities of the TNF are unaffected or enhanced.

14. A composition as claimed in claims 12 or 13 in which

the ligand is characterised in that the epitope of the TNF defined by the topographic region of residues 1 to 18 is substantially prevented from binding to naturally occurring biologically active ligands.

15. A composition as claimed in claims 12 or 13 in which the ligand binds to TNF such that the epitope of the TNF defined by the topographic regions of residues 1 - 30, 117 - 128 and 141 - 153 and more preferably in the topographic regions of residues 1 - 26, 117 - 128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands.

16. A composition for use in treating viral infection in a mammal the composition comprising gamma interferon and an anti-TNF ligand either alone or bound to TNF, the ligand being characterised in that it binds to residues 1 - 18 of human TNF.

17. A composition for use in treating viral infection in a mammal comprising gamma interferon and an anti-TNF ligand either alone or bound to TNF, the ligand being characterised in that it binds to human TNF in the topographic regions of residues 1 - 30, 117 - 128 and 141-153.

18. A composition as claimed in claims 15 or 17 in which the ligand binds to human TNF in the topographic regions of residues 1 - 26, 117 - 128 and 141-153.

19. A composition as claimed in claims 14 or 16 in which the ligand is an antibody raised against a peptide having an amino acid sequence substantially corresponding to amino acids 1 to 18 of human TNF (Peptide 301).

20. A method as claimed in any one of claims 11-19 in which the ligand is monoclonal antibody designated MAb 32.

21. The use of an anti-TNF ligand either alone or in combination with TNF in the production of a medicament for the treatment of viral infection in a mammal, the ligand being characterised in that when it binds to TNF the

induction of endothelial procoagulant activity of the TNF is inhibited and the anti-viral activity of the TNF is unaffected or enhanced.

22. The use as claimed in claim 21 in which the ligand is further characterised in that when it binds to TNF the binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities for the TNF are enhanced; the cytotoxicity is unaffected and the tumour receptor binding activities of the TNF are unaffected or enhanced.

23. The use as claimed in claims 21 or 22 in which the ligand is characterised in that the epitope of the TNF defined by the topographic region of residues 1 to 18 is substantially prevented from binding to naturally occurring biologically active ligands.

24. The use as claimed in claims 21 or 22 in which the ligand binds to TNF such that the epitope of the TNF defined by the topographic regions of residues 1 - 30, 117 - 128 and 141 - 153 and more preferably in the topographic regions of residues 1 - 26, 117 - 128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands.

25. The use of an anti-TNF ligand either alone or in combination with TNF in the production of a medicament for the treatment of viral infection in a mammal, the anti-TNF ligand being characterised in that it binds to residues 1 - 18 of human TNF.

26. The use of an anti-TNF ligand either alone or in combination with TNF in the production of a medicament for the treatment of viral infection in a mammal, the ligand being characterised in that it binds to human TNF in the topographic regions of residues 1 - 30, 117 - 128 and 141 - 153.

27. The use as claimed in claim 26 in which the ligand binds to human TNF in the topographic regions of residues

1 - 26, 117 - 128 and 141-153.

28. The use as claimed in claim 25 in which the ligand is an antibody raised against a peptide having an amino acid sequence substantially corresponding to amino acids 1 to 18 of human TNF (Peptide 301).

29. The use as claimed in an one of claims 21-28 in which the ligand is monoclonal antibody designated MAb 32.

30. A method as claimed in any one of claims 1-9 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, restructured antibodies (CDR grafted humanised antibodies), single domain antibodies (dABs), single chain antibodies, anti-idiotypic antibodies, serum binding proteins, receptors and natural inhibitors.

31. A method as claimed in claim 30 in which the ligand is a monoclonal or polyclonal antibody or F(ab) fragment thereof.

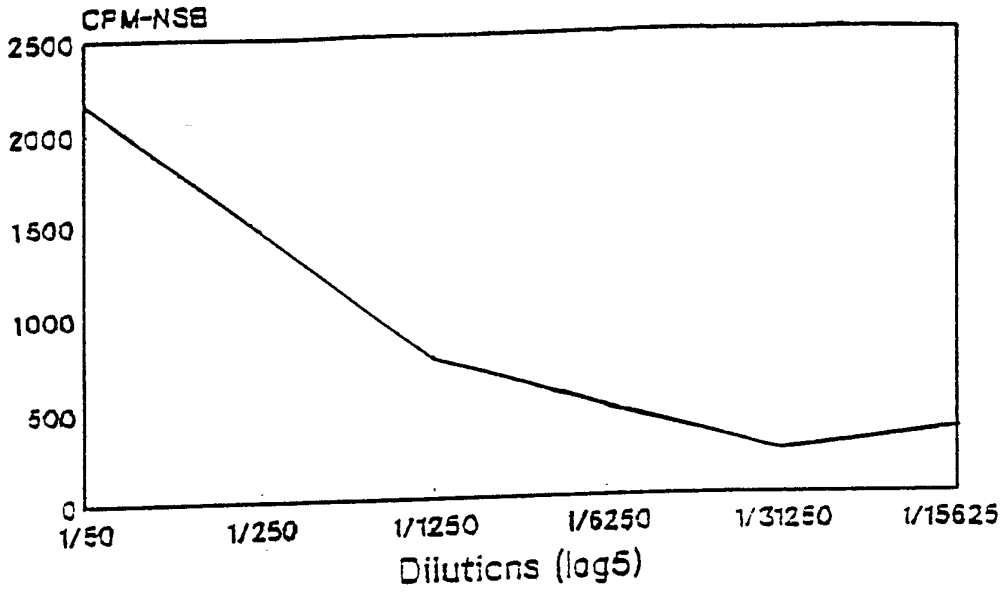

CPM-NSB
2500
2000
1500
1000
500
0
1/50
1/250
1/1250
1/6250
1/31250
1/15625
Dilutions (log5)

FIG. 1

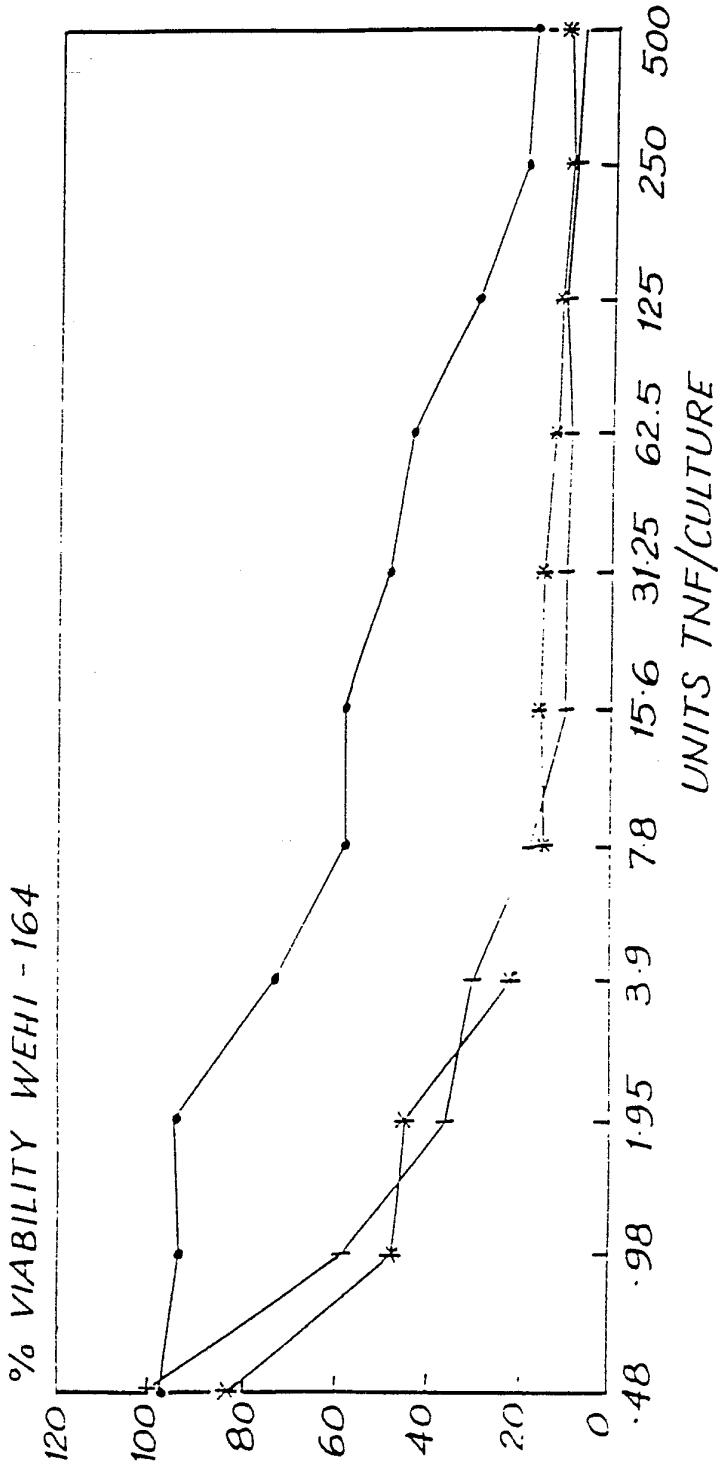

% VIABILITY WEHI - 164
120
100
80
60
40
20
0
·48
·98
1·95
3·9
7·8
15·6
31·25
62·5
125
250
500
UNITS TNF/CULTURE
LEGEND
TNF + MAb 1 ;
TNF ONLY ;
TNF + MAb 32
MEAN % VIABILITY of TRIPLICATE CULTURES

FIG. 2

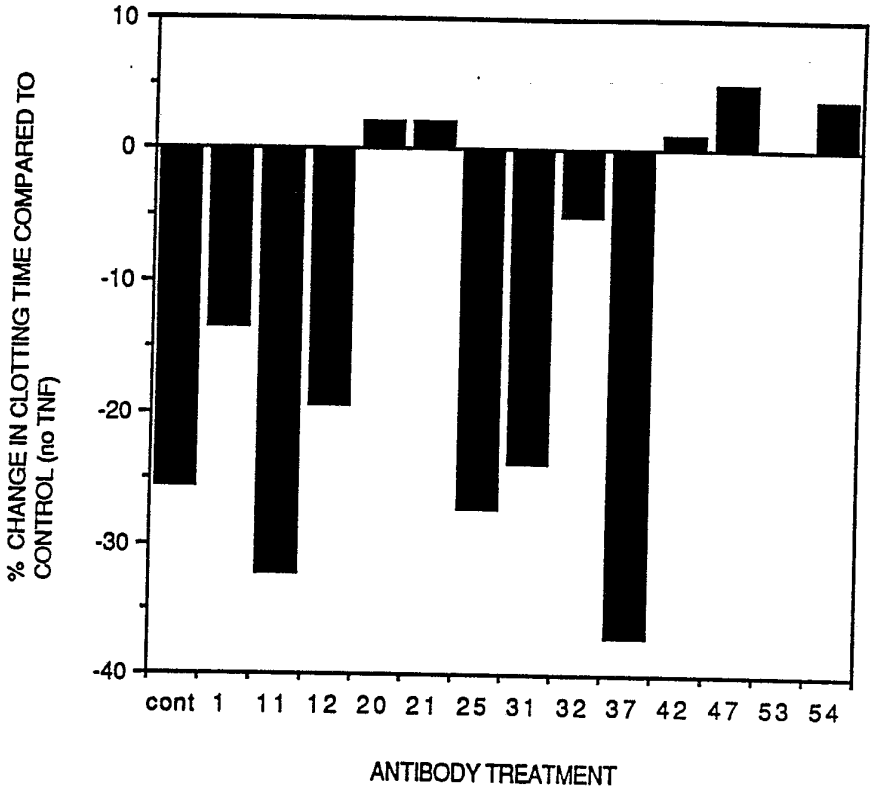

10
0
-10
-20
-30
-40
% CHANGE IN CLOTTING TIME COMPARED TO CONTROL (no TNF)
cont 1 11 12 20 21 25 31 32 37 42 47 53 54
ANTIBODY TREATMENT

FIG. 3

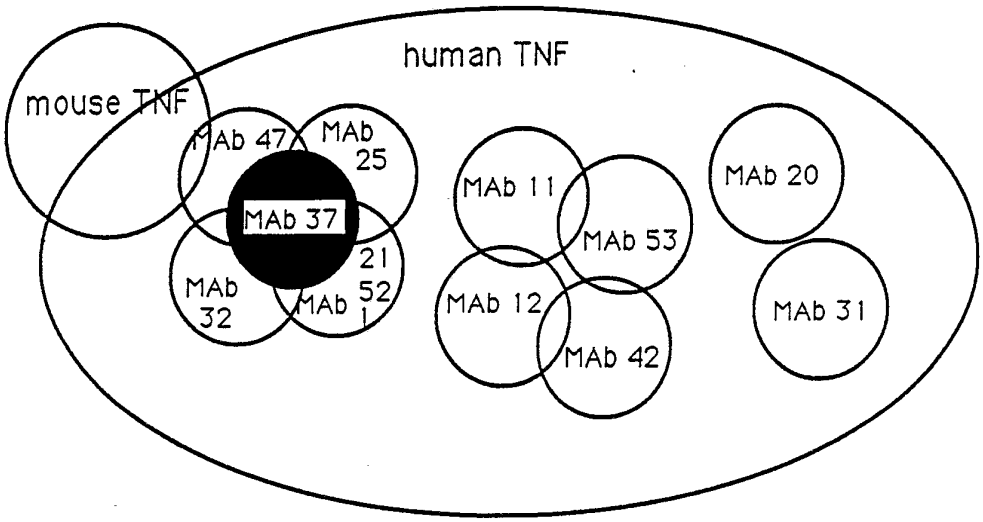

human TNF
mouse TNF
MAb 47
MAb 25
MAb 37
21
MAb 32
MAb 52
MAb 11
MAb 53
MAb 12
MAb 42
MAb 20
MAb 31

FIG. 4

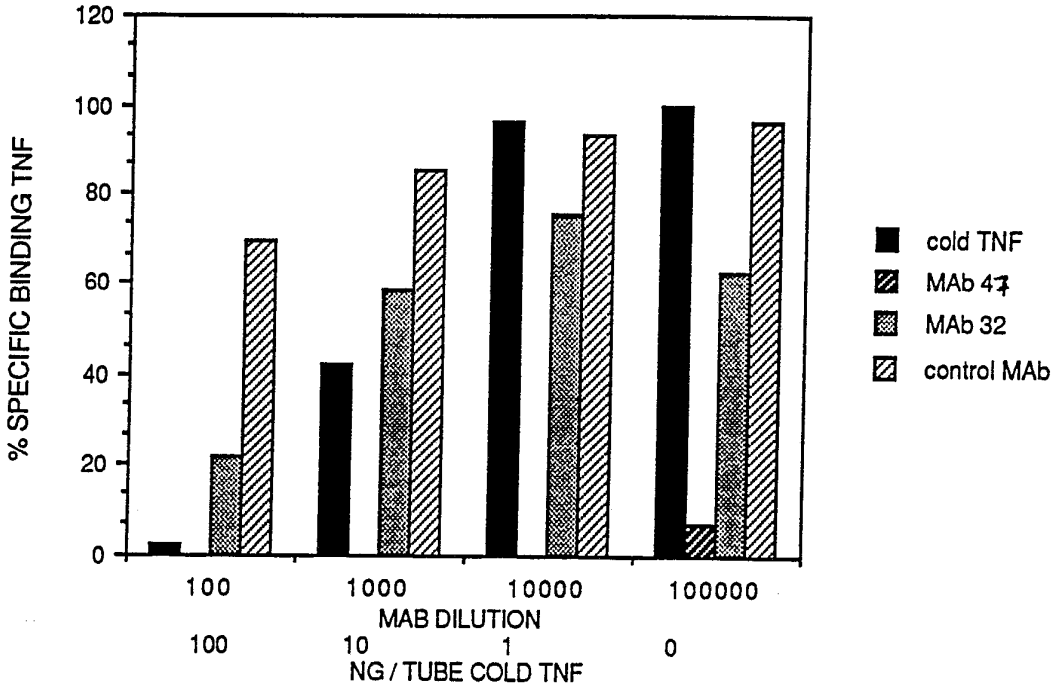

120
100
80
60
40
20
0
% SPECIFIC BINDING TNF
100
1000
10000
100000
MAB DILUTION
100
10
1
0
NG / TUBE COLD TNF
cold TNF
MAb 47
MAb 32
control MAb

FIG. 5

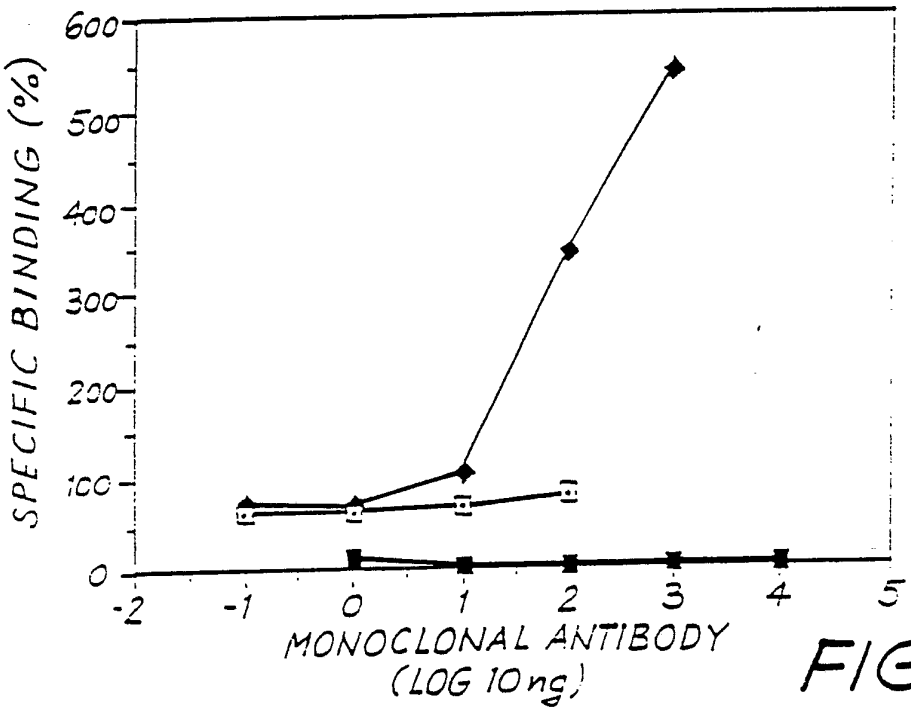

SPECIFIC BINDING (%)
600
500
400
300
200
100
0
-2
-1
0
1
2
3
4
5
MONOCLONAL ANTIBODY
(LOG 10 ng)

FIG. 6

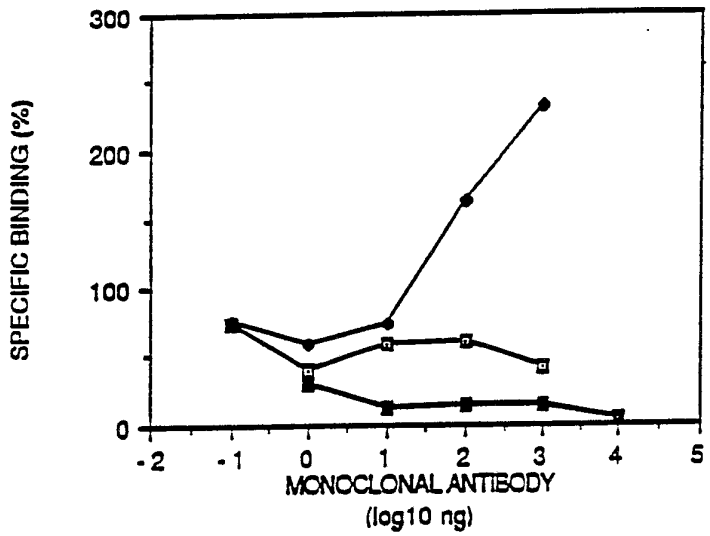


FIG. 7

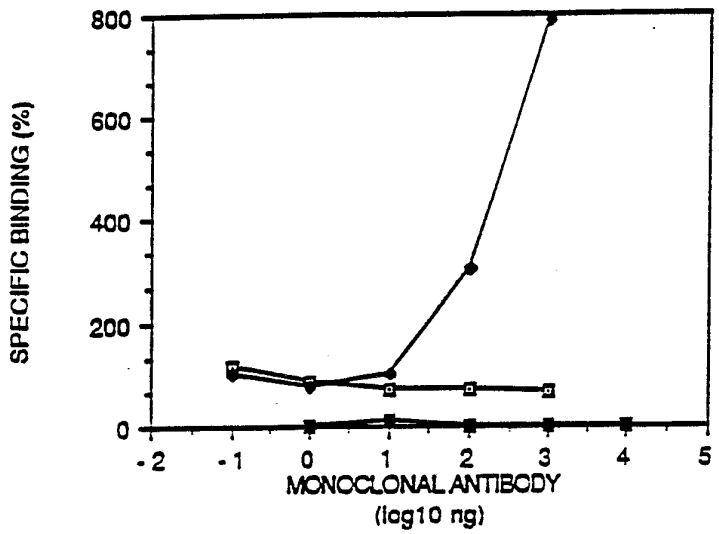

800
600
400
200
0
SPECIFIC BINDING (%)
-2
-1
0
1
2
3
4
5
MONOCLONAL ANTIBODY
(log10 ng)

FIG. 8

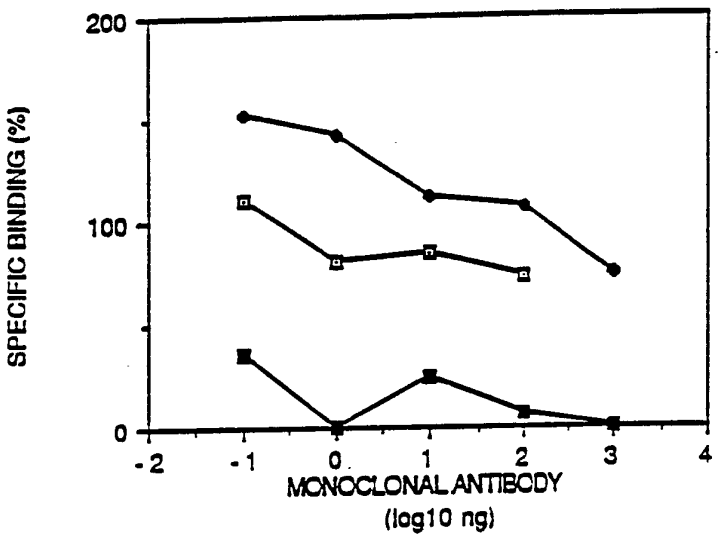

200
100
0
SPECIFIC BINDING (%)
-2
-1
0
1
2
3
4
MONOCLONAL ANTIBODY
(log10 ng)

FIG. 9

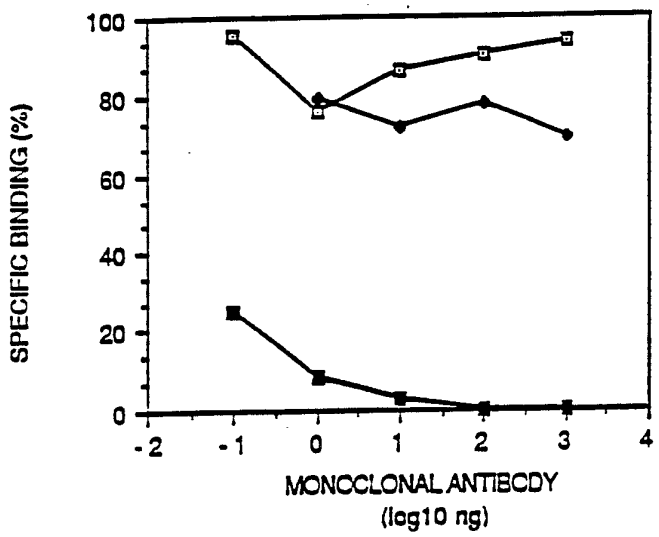

SPECIFIC BINDING (%)
100
80
60
40
20
0
-2
-1
0
1
2
3
4
MONOCLONAL ANTIBODY
(log10 ng)

FIG. 10

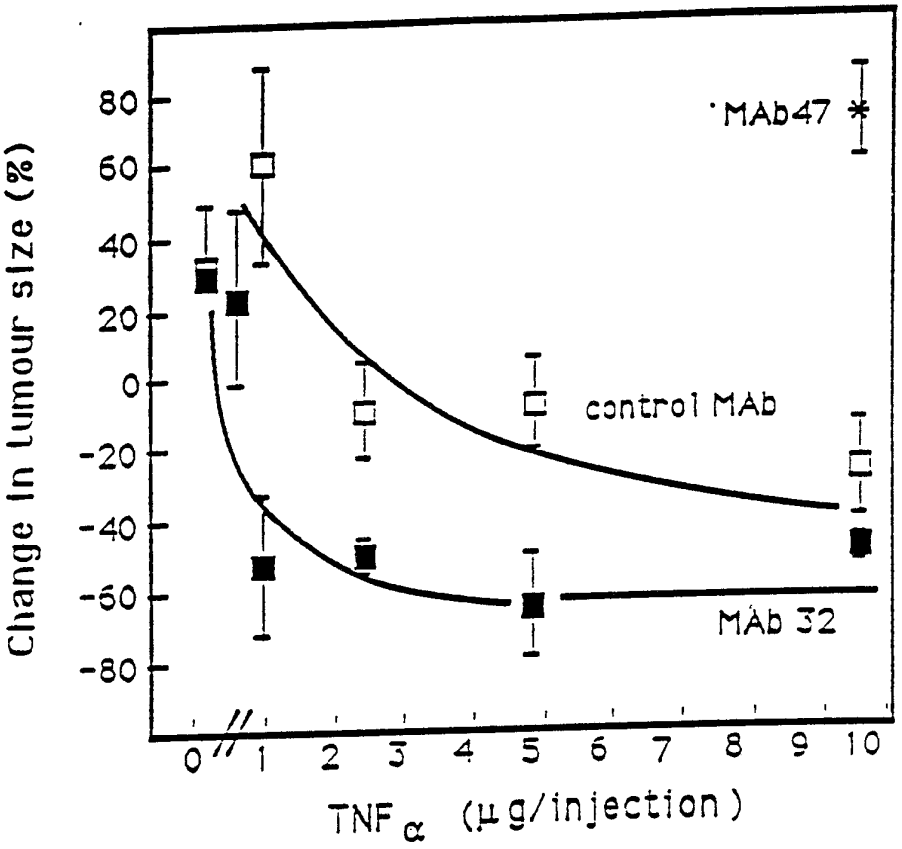

Change in tumour size (%)
80
60
40
20
0
-20
-40
-60
-80
MAb47
control MAb
MAb 32
0
1
2
3
4
5
6
7
8
9
10
TNF α (μg/injection)

FIG. 11

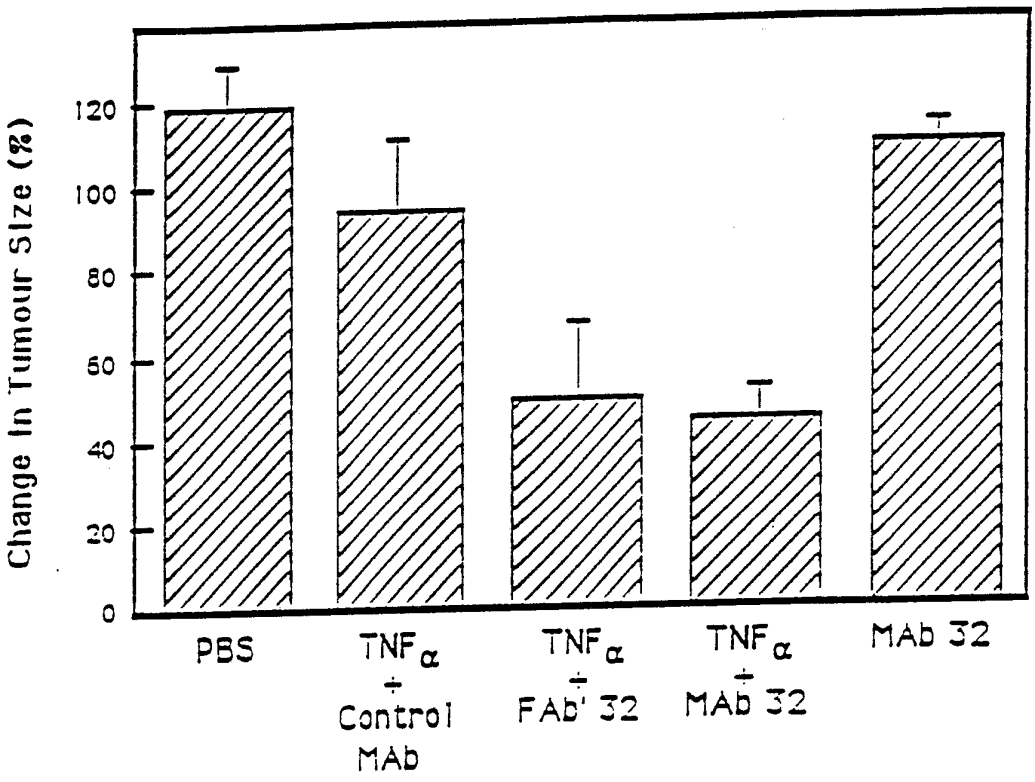
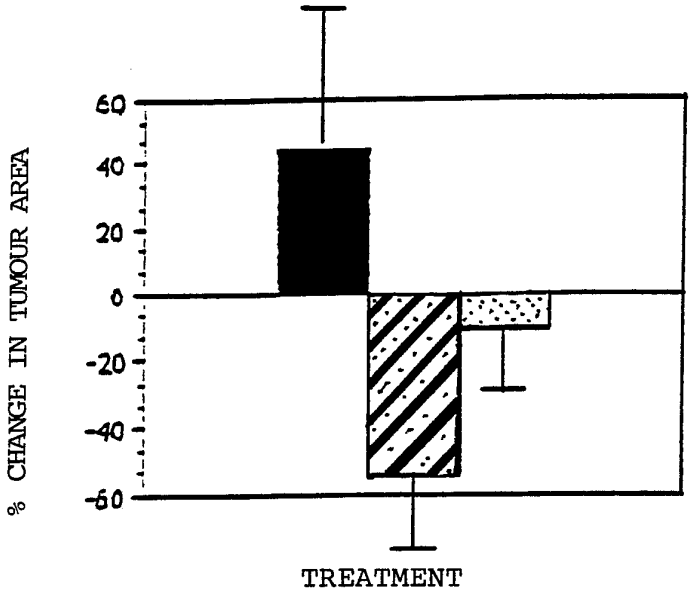

Change In Tumour Size (%)
120
100
80
60
40
20
0
PBS
TNFα + Control MAb
TNFα + FAb' 32
TNFα + MAb 32
MAb 32

FIG. 12



(200ul globulins + 10ug TNF injected/day/mouse)

FIG. 13

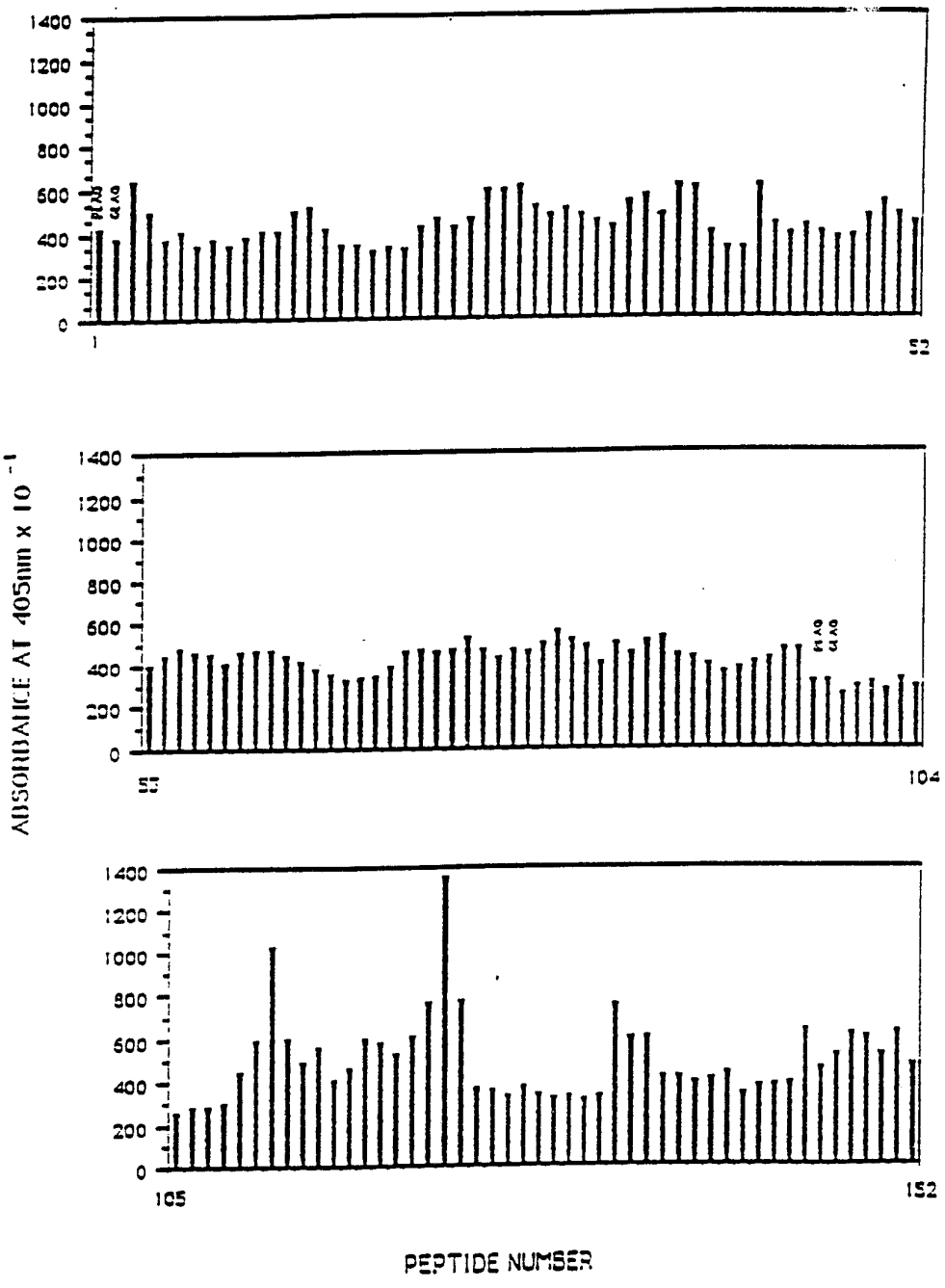

1400
1200
1000
800
600
400
200
0
1
52
53
104
105
152
ABSORBANCE AT 405nm x 10⁻¹
PEPTIDE NUMBER

FIG. 14

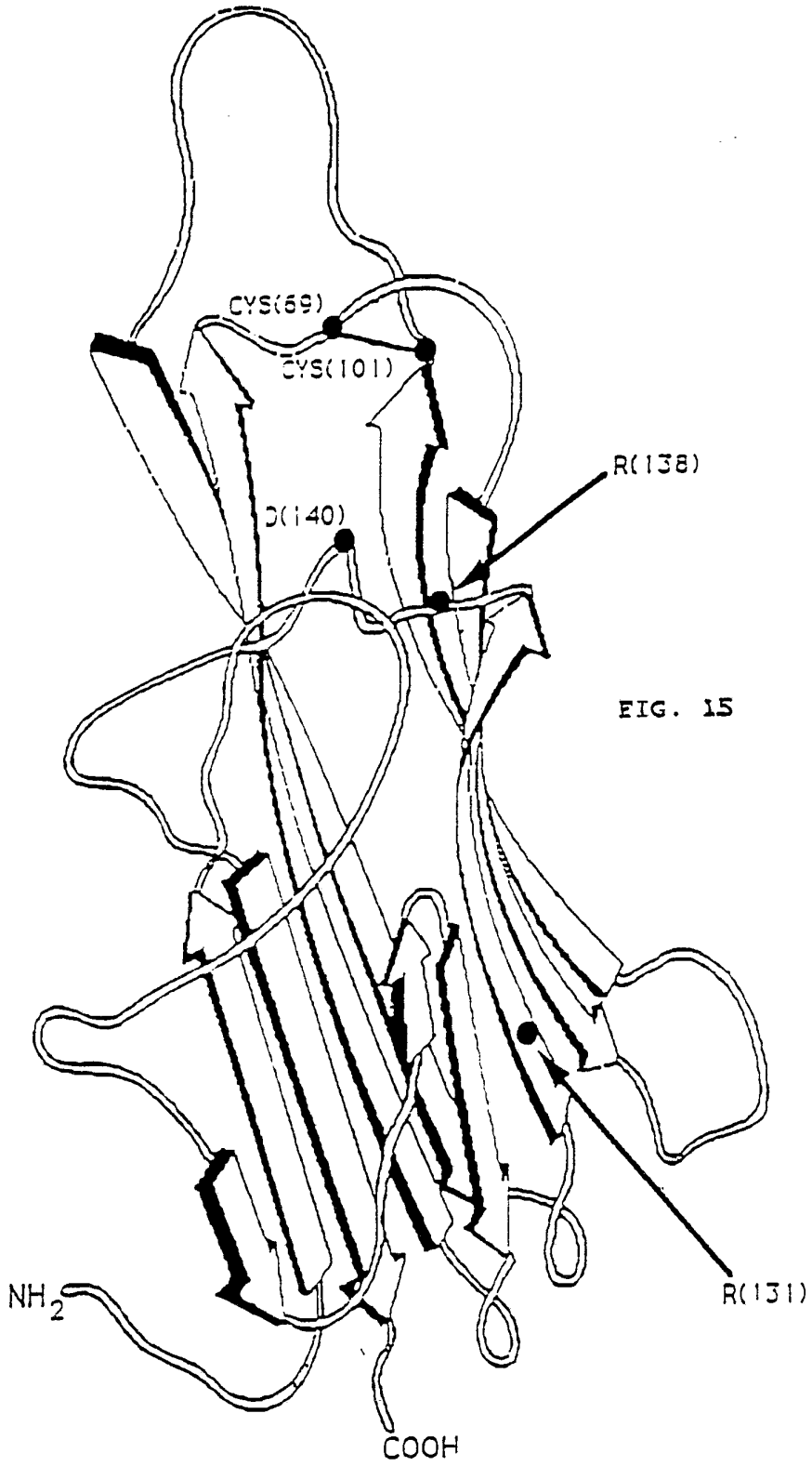


FIG. 15

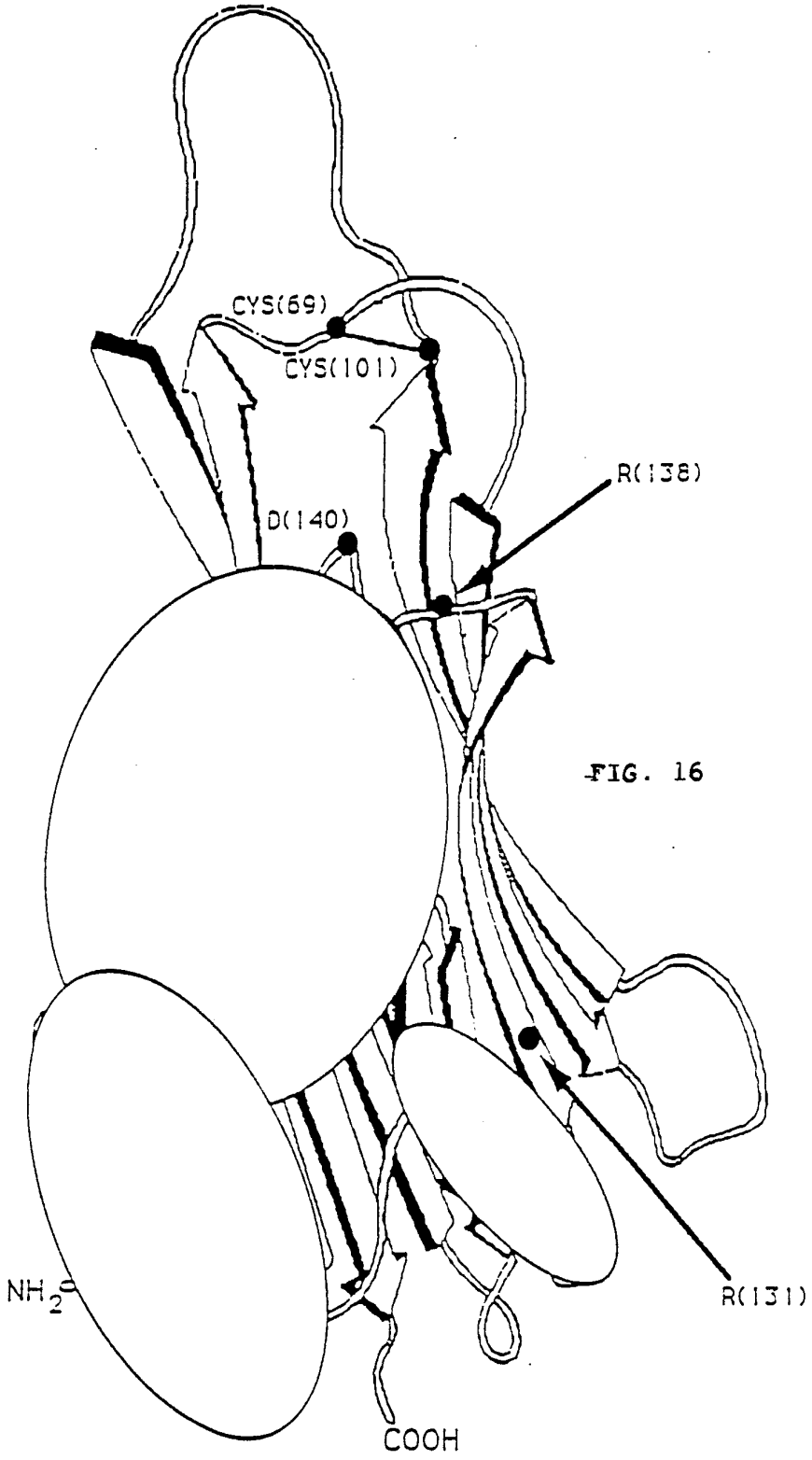


FIG. 16

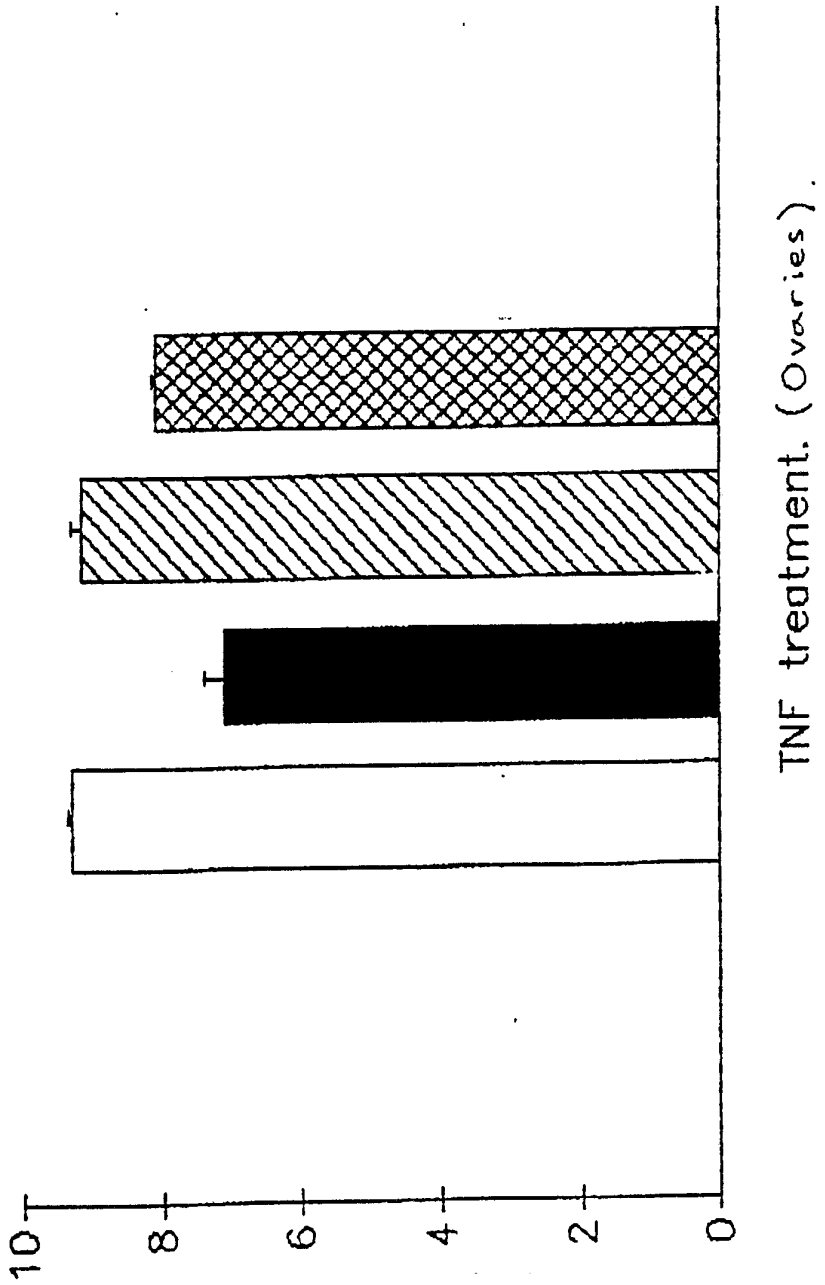


FIG. 17

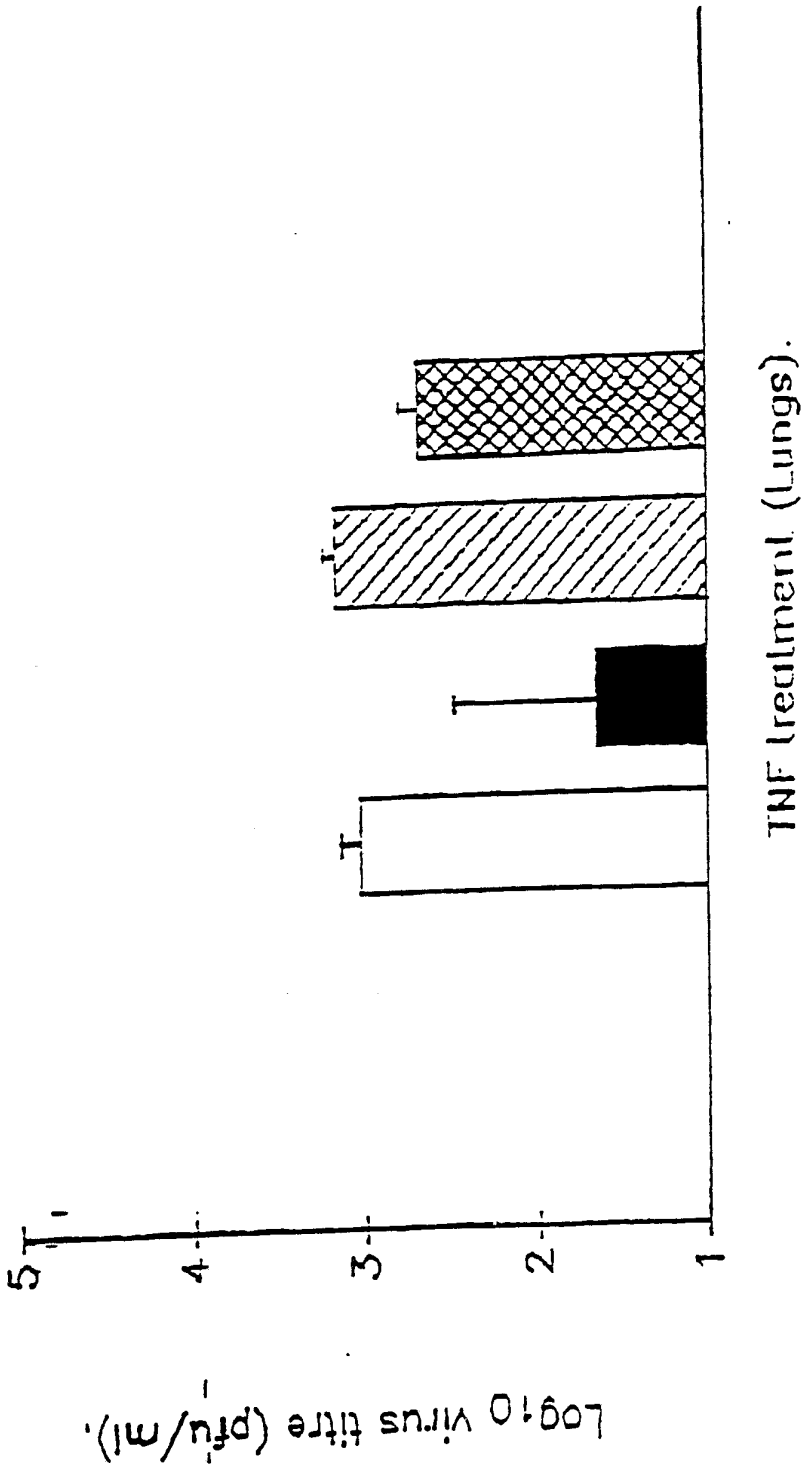


FIG. 18

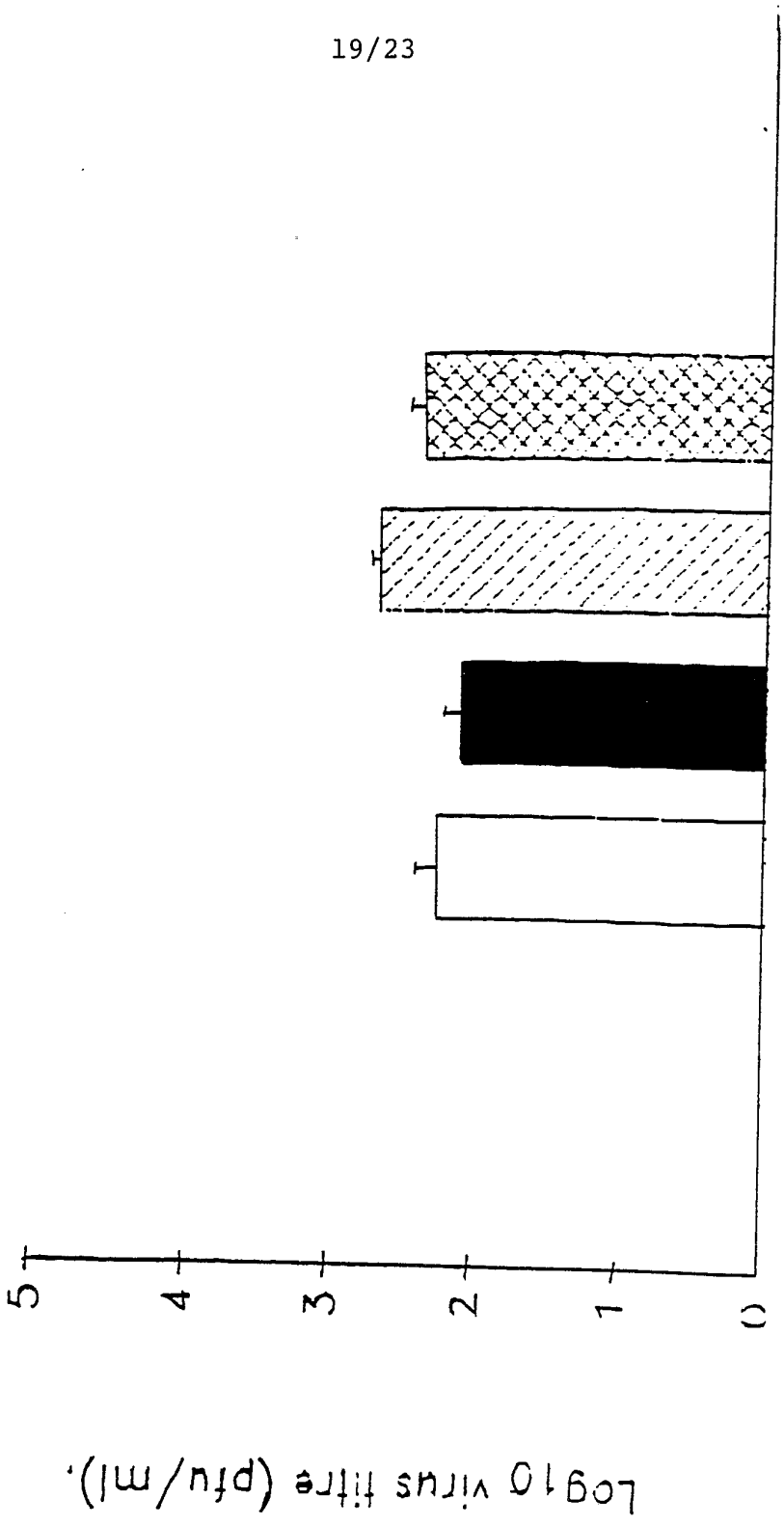
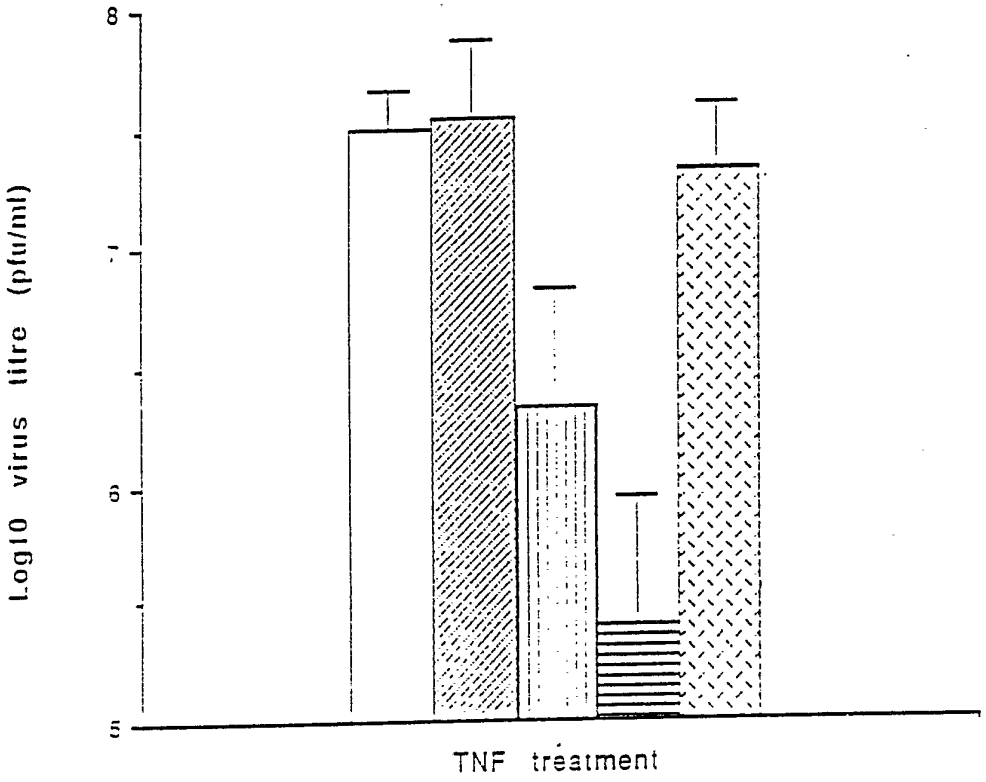


FIG. 19

FIG. 20



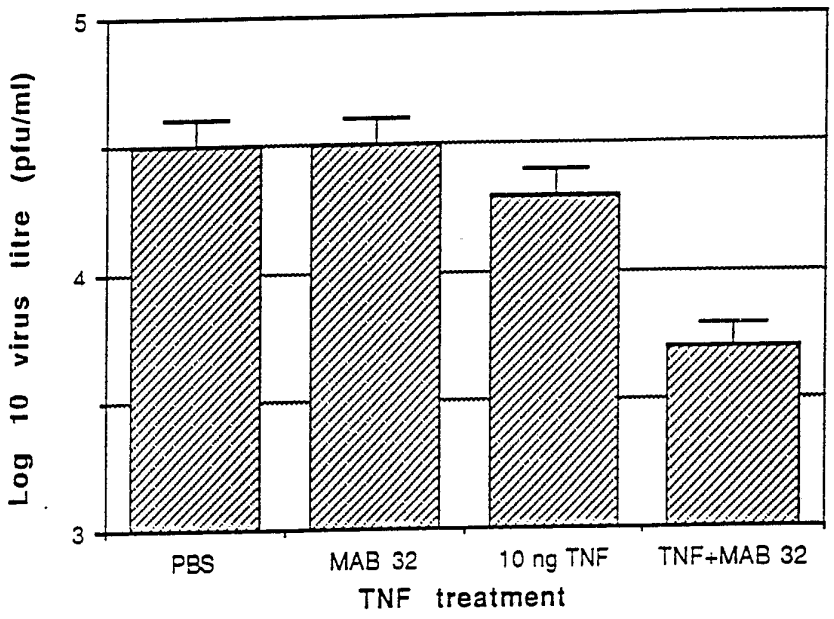

5
4
3
Log 10 virus titre (pfu/ml)
PBS
MAB 32
10 ng TNF
TNF+MAB 32
TNF treatment

FIG. 21

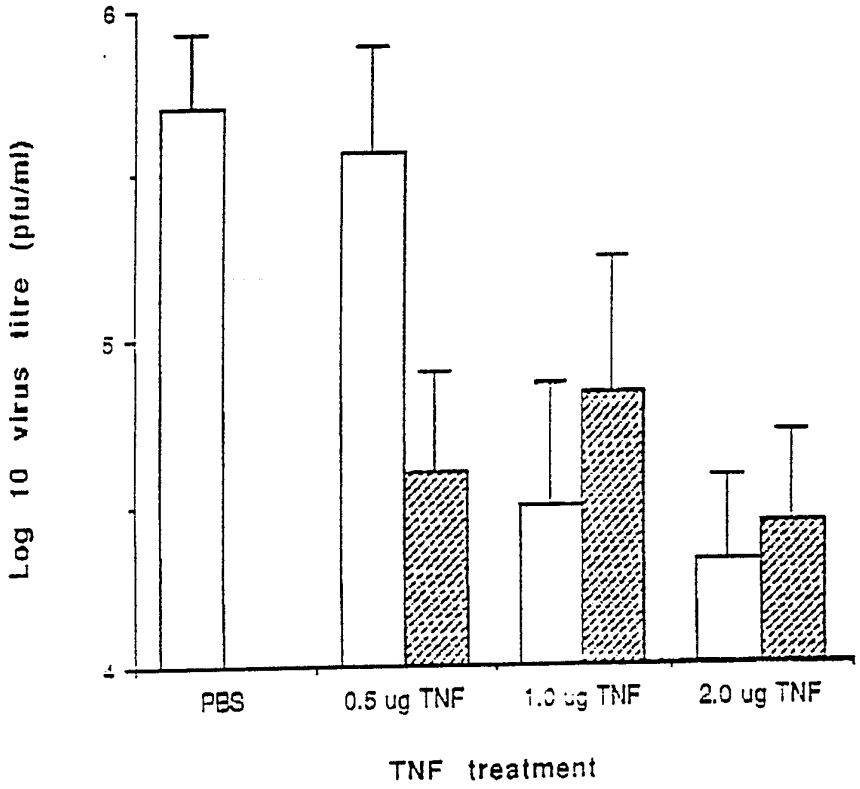

6
5
4
Log 10 virus titre (pfu/ml)
PBS
0.5 ug TNF
1.0 ug TNF
2.0 ug TNF
TNF treatment

FIG. 22

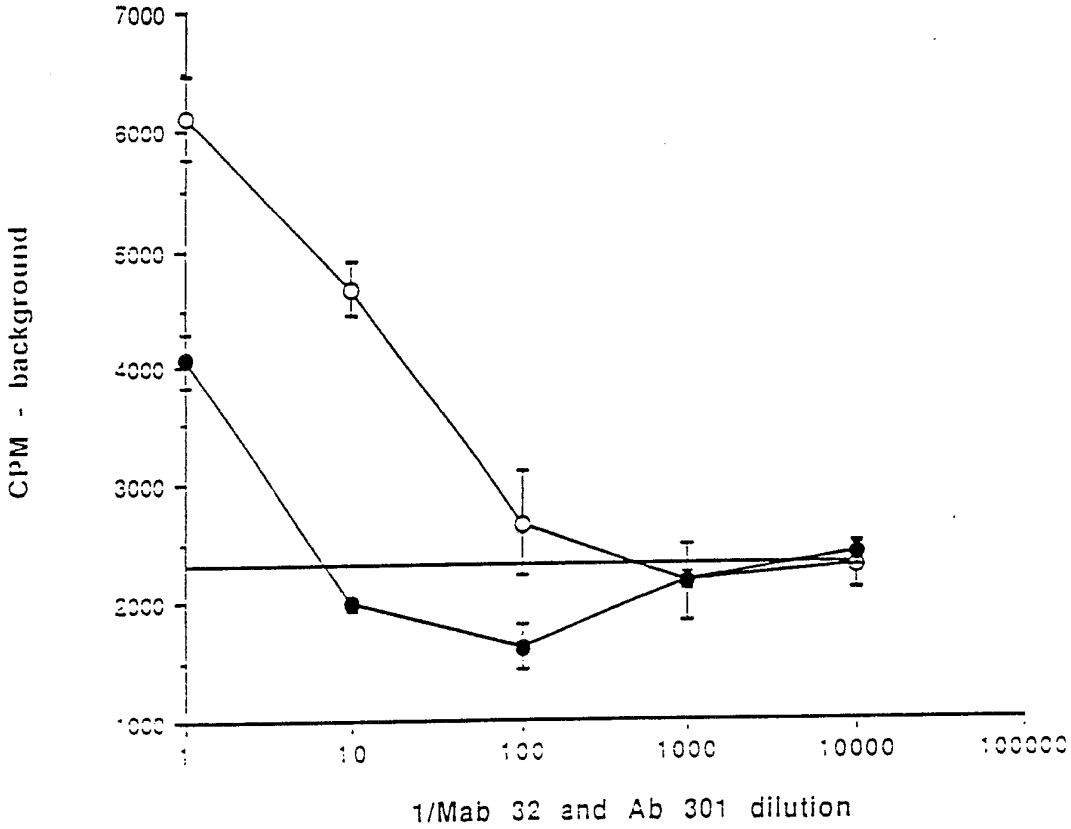

7000
6000
5000
4000
3000
2000
1000
CPM - background
1
10
100
1000
10000
100000
1/Mab 32 and Ab 301 dilution

FIG. 23

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent classification (IPC) or to both National Classification and IPC

Int. Cl.[5] A61K 37/02, 39/395, 37/66

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A61K 37/02, 39/395

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
P,X	WO 91/04054 (MILLAR, A.B.) 4 April 1991 (04.04.91)	(1-11,21-23)
P,X	WO 90/10707 (JONKER, M.) 20 September 1990 (20.09.90)	(12-18,25,29-31)
P,X	75th Annual Meeting Of The Federation Of American Societies For Experimental Biology, Atlanta, Georgia, U.S.A., April 21-25, 1991. (FED. AM. SOC. EXP. BIOL.) J. 5(6) 1991. A 1620, "Murine Anti-TNF Antibodies Protect Human Cells From The Cytotoxic Activity Of Human TNF". GALLOW, C J.; MADANAT, M.S.; MITRA, G.	(1-11,21-31)
A	WO 90/01950 (CELLTECH LIMITED) 8 March 1990 (08.03.90)	(12-18)
A	WO 89/06544 (CENTOCOR, INC.) 27 July 1989 (27.07.89)	(1-31)

* Special categories of cited documents : [10]

"A" Document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior t the international filing date but later than the priority date claimed

"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 November 1991 (26.11.91)	29 November 91
International Searching Authority	**Signature of Authorized Officer**
AUSTRALIAN PATENT OFFICE	J.P. PULVIRENTI J P Pulverenti

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers , because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4a

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.