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VRSSSRTPSD10KPVAHVVANP20QAEGQLQWLN30RRA

NALLANG40VELRDNQLVV50PSEGLYLIYS60QVLFKGQGCP70STHVLL

THTI80SRIAVSYQTK90VNLLSAIKSP100CQRETREGAE110AKPWYEPI

YL120GGVFQLEKGD130RLSAEINRPD140YLDFAESGQV150YFGIIAL157

(57) Abstract

The present invention provides peptides which have the ability to abrogate TNF toxicity and/or LPS toxicity. The present invention further relates to compositions including these peptides as the active ingredient and methods of anti-inflammatory treatment involving the administration of this composition. The peptides of the present invention are based primarily on residue 1 to 26 of human TNF.

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PEPTIDE WHICH ABROGATES TNF AND/OR LPS TOXICITY

Field of the Invention

The present invention relates to a group of peptides which have the ability to abrogate TNF toxicity and/or LPS toxicity. The present invention further relates to compositions including this peptide as the active ingredient and methods of anti-inflammatory treatment involving the administration of this composition.

Background of the Invention

Many of the clinical features of septicemic shock induced by Gram-negative bacteria which have lipopolysaccharide (LPS) in their cell walls may be reproduced in animals by the administration of LPS. This induces prompt severe metabolic and physiological changes which can lead to death. Associated with the injection of LPS is the extensive production of tumour necrosis factor alpha (TNF). Many of the effects of LPS injection or indeed of Gram-negative bacteria can be reproduced by TNF. Thus, mice injected with recombinant human TNF develop piloerection of the hair (ruffling), diarrhoea, a withdrawn, unkempt appearance and die if sufficient amounts are given. Rats treated with TNF become hypotensive, tachypneic and die of sudden respiratory arrest (Tracey et al., 1986 Science 234, 470). Severe acidosis, marked haemoconcentration and biphasic changes in blood glucose concentration were also observed. Histopathology revealed severe leukostatsis in the lungs, haemorraghic necrosis in the adrenals, pancreas and other organs and tubular necrosis of the kidneys. All these changes were prevented if the animals were pretreated with a neutralizing monoclonal antibody against TNF.

The massive accumulation of neutrophils in the lungs of TNF-treated animals reflects the activation of neutrophils by TNF. TNF causes neutrophil degranulation, respiratory burst, enhanced antimicrobiocidal and

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anti-tumour activity (Klebanoff et al., 1986 J. Immunol. 136, 4220; Tsujimoto et al., 1986 Biochem Biophys Res Commun 137, 1094). Endothelial cells are also an important target for the expression of TNF toxicity. TNF diminishes the anticoagulant potential of the endothelium, inducing procoagulant activity and down regulation of the expression of thrombomodulin (Stern and Nawroth, 1986 J Exp Med 163, 740).

TNF, a product of activated macrophages produced in response to infection and malignancy, was first identified as a serum factor in LPS treated mice which caused the haemorraghic necrosis of transplantable tumours in murine models and was cytoxoic for tumour cells in culture (Carswell et al., 1975 PNAS 72, 3666; Helson et al., 1975 Nature 258, 731). Cachexia is a common symptom of advanced malignancy and severe infection. It is characterised by abnormal lipid metabolism with hypertriglyceridemia, abnormal protein and glucose metabolism and body wasting. Chronic administration of TNF (also known as cachectin in the early literature) to mice causes anorexia, weight loss and depletion of body lipid and protein within 7 to 10 days (Cerami et al., 1985 Immunol Lett 11, 173, Fong et al., 1989 J Exp Med 170, 1627). These effects were reduced by concurrent administration of antibodies against TNF. Although TNF has been measured in the serum of patients with cancer and chronic disease associated with cachexia the results are inconclusive since large differences in TNF levels have been reported. These may be due to the short half-life of TNF (6 minutes), differences in TNF serum binding protein, or true differences in TNF levels in chronic disease states.

TNFα, as a mediator of inflammation, has been implicated in the pathology of other diseases apart from toxic shock and cancer-related cachexia. TNF has been

measured in synovial fluid in patients with both rheumatoid and reactive arthritis and in the serum of patients with rheumatoid arthritis (Saxne et al., 1988 Arthrit. Rheumat. 31, 1041). Raised levels of TNF have been detected in renal transplant patients during acute rejection episodes (Maury and Teppo 1987 J. Exp Med 166, 1132). In animals TNF has been shown to be involved in the pathogenesis of graft versus host disease in skin and gut following allogeneic marrow transplantation. Administration of a rabbit anti-murine TNF was demonstrated to prevent the histological changes associated with graft versus host disease and reduced mortality (Piquet et al., 1987 J Exp Med 166, 1280).

TNF has also been shown to contribute significantly to the pathology of malaria (Clark et al., 1987; Am. J. Pathol. 129: 192-199). Further, elevated serum levels of TNF have been reported in malaria patients (Scuderi et al., 1986; Lancet 2: 1364-1365). TNF may also contribute to the brain pathology and consequent dementia observed in late stage HIV infections (Grimaldi et al Ann Nevrol 29 : 21)

The peptides encompassed in the present invention do not necessarily interfere directly with the bio-synthetic mechanisms of the disease-causing component. As will be described below in the experimental data the mechanism behind the alleviating effect of the peptides is to be found in the modulation of the different cytokines produced by activated cells belonging to the cell-lines encompassing the immune defence. This modulation of cytokines is not limited to TNF but may also be valid for the whole range of interleukins, from interleukin-1 to interleukin 10. LPS, a known component of bacteria important in inducing major inflammatory response was used as a model. LPS binds to receptors on neutrophils, monocytes, endothelial cells and machrophages, which

consequently become activated and start production of IL-1 and TNF and other cytokines, thus starting the inflammatory cascade. One parameter used to measure the effect of LPS is the concentration of blood glucose, which will normally decrease on exposure to TNF or LPS.

LPS normally combines with LPS-Binding-Protein (LBP) and exerts its dramatic effect through the CD14 receptor. The activation of the CD14 molecule by LPS results in TNF production by leucocytes. It is believed that the peptides of the present invention which abrogate LPS toxicity may exert their effect by interacting with the CD14 molecule and thus inhibit LPS binding.

The peptides identified by the present inventors which have the ability to abrogate TNF and/or LPS toxicity resemble peptide sequences found in the amino terminal of TNFα . Other investigators have also considered this area of the TNFα molecule but with little success in obtaining biologically active peptides.

In this regard attention is drawn to Canadian patent application Nos 2005052 and 2005056 in the name of BASF AG. Both these applications claim a wide range of peptide sequences and, by selecting appropriate alternatives it can be seen that application No 2005052 is directed toward the peptide sequence 7-42 of TNFα whilst application No 2005056 is directed toward amino acid sequence 1 to 24 of TNFα . Whilst each of these applications claim a broad range of peptide sequences it is noted that there is no indication as to what, if any, biological activity the claimed peptides may possess. Indeed there is no demonstration that any of the produced peptide have any biological activity. In contrast, the present inventors have produced a range of peptides which have specific activities in that they abrogate TNF and/or LPS toxicity.

Summary of the Invention

In a first aspect the present invention consists in a linear or cyclic peptide of the general formula:-

X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9

in which

X_1 is null, Cys or R_1

X_2 is null, Cys, R_1 or A_1-A_2-A_3-A_4-A_5

in which A_1 is Val or Ile or Leu or Met or His

A_2 is Arg or Cys or His

A_3 is Ser or Thr or Ala

A_4 is Ser or Thr or Ala

A_5 is Ser or Thr or Ala

X_3 is Cys, R_1 or A_6-A_7

in which A_6 is Arg or Cys or His or Absent

A_7 is Thr or Ser or Ala

X_4 is Cys, R_1 or A_8-A_9

in which A_8 is Pro or an Nα-alkylamino acid

A_9 is Ser or Thr or Ala

X_5 is Cys, R_1 or A_{10}

in which A_{10} is Asp or Ala or Cys or Glu or Gly or Arg or His

X_6 is Cys, R_2 or A_{11}-A_{12}-A_{13}

in which A_{11} is absent or Cys or Arg or His or Asp or Glu

A_{12} is Pro or an Nα-alkylamino acid

A_{13} is Val or Ile or Phe or Tyr or Trp or His or Leu or His or Met

X_7 is null, Cys, R_2 or A_{14}-A_{15}

in which A_{14} is Ala or Val or Gly or Ile or Phe or Trp or Tyr or Leu or His or Met

A_{15} is absent or His or Arg or Glu or Asa or Ala or Lys or Asp or Phe or Tyr or Trp or Glu or Gln or Ser or Thr or Gly

X_8 is null, Cys, R_2, A_{16}, A_{16}-A_{17}, A_{16}-A_{17}-A_{18} or A_{16}-A_{17}-A_{18}-A_{19}-A_{20}-A_{21}-A_{22}-A_{23}-A_{24}-A_{25}-A_{26}

in which A_{16} is Val or Ile or Leu or Met or His

A_{17} is Val or Ile or Leu or Met or His

A_{18} is Ala or Gly

A_{19} is Asp or Glu
A_{20} is Pro or an Nα-alkylamino acid
A_{21} is Gln or Asn
A_{22} is Ala or Gly
A_{23} is Glu or Asp
A_{24} is Gly or Ala
A_{25} is Gln or Asn
A_{26} is Leu or Ile or Val or Met or His

X_9 is null, Cys or R_2

R_1 is R-CO, where R is H, straight, branched or cyclic alkyl up to C20, optionally containing double bonds and/or substituted with halogen, nitro, amino, hydroxy, sulfo, phospho or carboxyl groups (which may be substituted themselves), or aralkyl or aryl optionally substituted as listed for the alkyl and further including alkyl, or R_1 is glycosyl, nucleosyl, lipoyl or R_1 is an L- or D-α amino acid or an oligomer thereof consisting of up to 5 residues

R_1 is absent when the amino acid adjacent is a desamino-derivative.

R_2 is
$-NR_{12}R_{13}$, wherein R_{12} and R_{13} are independently H, straight, branched or cyclic alkyl, aralkyl or aryl optionally substituted as defined for R_1 or N-glycosyl or N-lipoyl
$-OR_{14}$, where R_{14} is H, straight, branched or cyclic alkyl, aralkyl or aryl, optionally substituted as defined for R_1
-O-glycosyl, -O-lipoyl or
- an L- or D-α-amino acid or an oligomer thereof consisting of up to 5 residues

or R_2 is absent, when the adjacent amino acid is a decarboxy derivative of cysteine or a homologue thereof or the peptide is in a N-C cyclic form.

with the proviso that:

when X_6 is Cys or R_2 then X_5 is A_{10}, X_4 is A_8-A_9, X_3 is A_6-A_7 and X_2 is A_1-A_2-A_3-A_4-A_5

when X_5 is Cys or R_1 then X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15}, X_8 is A_{16}-A_{17}-A_{18} and A_{11} is absent

when X_4 is Cys or R_1 then X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15} and X_8 is A_{16}-A_{17}-A_{18}

when X_2 is A_1-A_2-A_3-A_4-A_5 then X_8 is not A_{16}

when X_1 is null, X_2 is Cys or R_1, X_3 is A_6-A_7, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15} and X_8 is A_{16} then A_{16} is not D-His.

X_1 is always and only null when X_2 is R_1, Lys or Null

X_2 is always and only null when X_3 is Cys or R_1

X_3 is always and only null when X_6 is Cys or R_2

X_7 is always and only null when X_7 is Cys, R_2 or Null

X_8 is always and only null when X_8 is Cys, R_2 or Null

X_9 is always and only null when X_8 is Cys, R_2 or Null

when X_1 and R_2 are null, X_3 is R_1, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15}, X_8 is R_2 and A_{14} is Ala and A_{15} is absent then R_1 is acetyl and R_2 is NH_2.

The amino acids may be D or L isomers, however generally the peptide will primarily consist of L-amino acids.

In a second aspect the present invention consists in a pharmaceutical composition for use in treating subjects suffering from toxic effects of TNF and/or LPS, the composition comprising a therapeutically effective amount of a peptide of the first aspect of the present invention and a pharmaceutically acceptable sterile carrier.

In a third aspect the present invention consists in a method of treating a subject suffering from the toxic effects of TNF and/or LPS, the method comprising administering to the subject a therapeutically effective amount of the composition of the second aspect of the present invention.

In a preferred embodiment of the present invention X_1 is H, X_2 is A_1-A_2-A_3-A_4-A_5, X_3 is A_6-A_7, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15}, X_8 is A_{16}-A_{17}-A_{18} and X_9 is OH.

In a further preferred embodiment of the present invention X_1 is null, X_2 is H or Ac, X_3 is A_6-A_7, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15}, X_8 is A_{16}-A_{17}-A_{18} and X_9 is OH or NH_2.

In a further preferred embodiment of the present invention X_1 is H, X_2 is A_1-A_2-A_3-A_4-A_5, X_3 is A_6-A_7, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is OH and X_6, X_7 and X_8 are null.

In a further preferred embodiment of the present invention the peptide is selected from the group consisting of:-

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Ala-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Lys-Asp-Pro-Val-Ala-His-Val-Val-Ala;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Arg-Val-Val-Ala;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Gln-Val-Val-Ala;

Ac-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-NH2;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Ala-Val;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Lys-Val;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val;

Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val;

Pro-Ser-Asp-Lys-Pro-Val-Ala-His;

Pro-Ser-Asp-Lys-Pro-Val;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Val-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-Asn-Pro-Gln-Ala-Glu-Gly-Gln-Leu;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp;

Ac-Pro-Ser-Asp-Lys-Pro-Val-Ala-NH2;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Asp-Val;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-Asn-Pro-Gln-Ala-Glu-Gly-Gln-Leu;

Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val;

Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Pro-Sir-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Pro-Val-Ala-His-Val-Val-Ala; and

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Val-His-Val.

The composition and method of the present invention would be expected to be useful as an anti-inflammatory agent in a wide range of disease states including toxic shock, adult respiratory distress syndrome, hypersensitivity pneumonitis, systemic lupus erythromatosis, cystic fibrosis, asthma, bronchitis, drug withdrawal, schistosomiasis, sepsis, rheumatoid arthritis, acquired immuno-deficiency syndrome, multiple sclerosis, leperosy, malaria, systemic vasculitis, bacterial meningitis, cachexia, dermatitis, psoriasis, diabetes, neuropathy associated with infection or autoimmune disease, ischemia/reperfusion injury, encephalitis, Guillame Barre Syndrome, atherosclerosis, chronic fatigue syndrome, TB, other viral and parasitic diseases, OKT3 therapy, and would be expected to be useful in conjunction with radiation therapy, chemotherapy and transplantation, to ameliorate the toxic effects of such treatments or procedures.

As the peptide of the present invention suppresses activation of neutrophils the composition and method of the present invention may also be useful in the treatment of diseases with an underlying element of local, systemic, acute or chronic inflammation. In general, it is believed

the composition and method of the present invention will be useful in treatment of any systemic or local infection leading to inflammation.

The peptides of the present invention may also be administered in cancer therapy in conjunction with cytotoxic drugs which may potentiate the toxic effects of TNFα (Watanabe et al., 1988; Immunopharmacol. Immunotoxicol. 10: 117-127) such as vinblastin, acyclovir, interferon alpha, cyclosporin A, IL-2, actinomycin D, adriamycin, mitomycin C, AZT, cytosine arabinoside, daunororubin, cis-platin, vincristine, 5-flurouracil and bleomycin; in cancer patients undergoing radiation therapy; and in AIDS patients (or others suffering from viral infection such as viral meningitis, hepatitis, herpes, green monkey virus etc.) and in patients receiving immunostimulants such as thymopentin and muramyl peptides or cytokines such as IL-2 and GM-CSF. In this use peptides of the present invention will serve to abrogate the deleterious effects of TNFα

It will be appreciated by those skilled in the art that a number of modifications may be made to the peptide of the present invention without deleteriously effecting the biological activity of the peptide. This may be achieved by various changes, such as insertions, deletions and substitutions (e.g., sulfation, phosphorylation, nitration, halogenation), either conservative or non-conservative (e.g., W-amino acids, desamino acids) in the peptide sequence where such changes do not substantially altering the overall biological activity of the peptide. By conservative substitutions the intended combinations are:-

G, A; V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkylamino acids.

It may also be possible to add various groups to the peptide of the present invention to confer advantages such as increased potency or extended half-life in vivo,

without substantially altering the overall biological activity of the peptide.

The term peptide is to be understood to embrace peptide bond replacements and/or peptide mimetics, i.e. pseudopeptides, as recognised in the art (see for example: Proceedings of the 20th European Peptide Symposium, edt. G. Jung. E. Bayer, pp. 289-336, and references therein), as well as salts and pharmaceutical preparations and/or formulations which render the bioactive peptide(s) particularly suitable for oral, topical, nasal spray, ocular pulmonary, I.V., subcutaneous, as the case may be, delivery. Such salts, formulations, amino acid replacements and pseudopeptide structures may be necessary and desirable to enhance the stability, formulation, deliverability (e.g., slow release, prodrugs), or to improve the economy of production, and they are acceptable, provided they do not negatively affect the required biological activity of the peptide.

Apart from substitutions, three particular forms of peptide mimetic and/or analogue structures of particular relevance when designating bioactive peptides, which have to bind to a receptor while risking the degradation by proteinases and peptidases in the blood, tissues and elsewhere, may be mentioned specifically, illustrated by the following examples: Firstly, the inversion of backbone chiral centres leading to D-amino acid residue structures may, particularly at the N-terminus, lead to enhanced stability for proteolytical degradation while not impairing activity. An example is given in the paper "Tritriated D-ala^1-Peptide T Binding", Smith, C.S. et al, Drug Development Res. 15, pp. 371-379 (1988). Secondly, cyclic structure for stability, such as N to C interchain imides and lactames (Ede et al in Smith and Rivier (Eds) "Peptides: Chemistry and Biology", Escom, Leiden (1991), p268-270), and sometimes also receptor binding may be enhanced by forming cyclic analogues. An

example of this is given in "Confirmationally restricted thymopentin-like compounds", U.S. pat. 4,457,489 (1985), Goldstein, G. et al. Finally, the introduction of ketomethylene, methylsulfide or retroinverse bonds to replace peptide bonds, i.e. the interchange of the CO and NH moieties may both greatly enhance stability and potency. An example of the latter type is given in the paper "Biologically active retroinverso analogues of thymopentin", Sisto A. et al in Rivier, J.E. and Marshall, G.R. (eds.) "Peptides, Chemistry, Structure and Biology", Escom, Leiden (1990), p.722-773.

The peptides of the invention can be synthesized by various methods which are known in principle, namely by chemical coupling methods (cf. Wunsch, E.: "Methoden der organischen Chemie", Volume 15, Band 1 + 2, Synthese von Peptiden, Thieme Verlag, Stuttgart (1974), and Barrany, G.; Merrifield, R.B: "The Peptides", eds. E. Gross, J. Meienhofer., Volume 2, Chapter 1, pp. 1-284, Academic Press (1980)), or by enzymatic coupling methods (cf. Widmer, F., Johansen, J.T., Carlsberg Res. Commun., Volume 44, pp. 37-46 (1979), and Kullmann, W.: "Enzymatic Peptide Synthesis", CRC Press Inc., Boca Raton, Florida (1987), and Widmer, F., Johansen, J.T. in "Synthetic Peptides in Biology and Medicine:, eds., Alitalo, K., Partanen, P., Vatieri, A., pp. 79-86, Elsevier, Amsterdam (1985)), or by a combination of chemical and enzymatic methods if this is advantageous for the process design and economy.

It will be seen that one of the alternatives embraced in the general formula set out above is for a cysteine residue to be positioned at both the amino and carboxy terminals of the peptide. This will enable the cylisation of the peptide by the formation of di-sulphide bond.

It is intended that such modifications to the peptide of the present invention which do not result in a decrease in biological activity are within the scope of the present invention.

As would be recognized by those skilled in the art there are numerous examples to illustrate the ability of anti-idiotypic (anti-Ids) antibodies to an antigen to function like that antigen in its interaction with animal cells and components of cells. Thus, anti-Ids to a peptide hormone antigen can have hormone-like activity and interact specifically with the receptors to the hormone. Conversely, anti-Ids to a receptor can interact specifically with a mediator in the same way as the receptor does. (For a review of these properties see: 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-iodiotypic antibodies as cell surface receptor probes. Proc. Natl. Acad. Sci. U.S.A. 75, 2443-2447).

As might be expected from this functional similarity of anti-Id and antigen, anti-Ids bearing the internal image of an antigen can induce immunity to such an antigen. (This nexus is reviewed in Hiernaux, J.R. 1988. Idiotypic vaccines and infectious diseases. Infect. Immun. 56, 1407-1413.)

As will be appreciated by persons skilled in the art from the disclosure of this application it will be possible to produce anti-idiotypic antibodies to the peptide of the present invention which will have similar biological activity. It is intended that such anti-idiotypic antibodies are included within the scope of the present invention.

Accordingly, in a fourth aspect the present invention consists in an anti-idiotypic antibody to the peptide of the first aspect of the present invention, the anti-idiotypic antibody being capable of abrogating TNF and/or LPS toxicity.

The individual specificity of antibodies resides in the structures of the peptide loops making up the Complementary Determining Regions (CDRs) of the variable

domains of the antibodies. Since in general, the amino acid sequences of the CDR peptide loops of an anti-Id are not identical to or even similar to the amino acid sequence of the peptide antigen from which it was originally derived, it follows that peptides whose amino acid sequence is quite dissimilar, in certain contexts can take up a very similar three-dimensional structure. The concept of this type of peptide, termed a "functionally equivalent sequence" or mimotope by Geyson is familiar to those expert in the field. (Geyson, H.M. et al 1987. Strategies for epitope analysis using peptide synthesis. J. Immun. Methods. 102, 259-274).

Moreover, the three-dimensional structure and function of the biologically active peptides can be simulated by other compounds, some not even peptidic in nature, but which mimic the activity of such peptides. This field of science is summarised in a review by Goodman, M. (1990). (Synthesis, spectroscopy and computer simulations in peptide research. Proc. 11th American Peptide Symposium published in Peptides-Chemistry, Structure and Biology pp 3-29. Ed Rivier, J.E. and Marshall, G.R. Publisher ESCOM.)

As will be recognized by those skilled in the art, armed with the disclosure of this application, it will be possible to produce peptide and non-peptide compounds having the same three-dimensional structure as the peptide of the present invention. These "functionally equivalent structures" or "peptide mimics" will react with antibodies raised against the peptide of the present invention and may also be capable of abrogating TNF toxicity. It is intended that such "peptide mimics" are included within the scope of the present invention.

Accordingly, in a fifth aspect the present invention consists in a compound the three-dimensional structure of which is similar as a pharmacophore to the three-dimensional structure of the peptide of the first aspect

of the present invention, the compound being characterized in that it reacts with antibodies raised against the peptide of the first aspect of the present invention and that the compound is capable of abrogating TNF and/or LPS toxicity.

More detail regarding pharmacophores can be found in Bolin et al. p 150, Polinsky et al. p 287, and Smith et al. p 485 in Smith and Rivier (Eds) "Peptides: Chemistry and Biology", Escom, Leiden (1991).

Detailed Description of the Invention

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

Fig. 1 shows the amino acid sequence of human TNFα ;

Fig. 2: Effect of TNF (□) and TNF+ Peptide 1 (◆) on blood glucose levels in malaria primed mice-Peptide 1 abrogates TNF induced hypoglycaemia in malaria primed mice.

Fig. 3: Effect of Peptide 1 on TNF-induced tumour regression.

Fig. 4: Effect of Peptide 1 (●), peptide 308 (▼), peptide 309 (■), peptide 305 (⊠) and peptide 302 (○) on binding of radiolabelled TNF to TNF receptors on WEH1-164 tumour cells - Peptide 1 does not inhibit binding of TNF to tumour cells.

Fig. 5: Plasma reactive nitrogen intermediate levels in TNF± Peptide 1 treated malaria primed mice - this shows that induction of RNI by TNF is inhibited by treatment with Peptide 1.

Fig. 6 shows the effect on blood glucose levels in mice treated with PBS (□); TNF alone (◆); TNF + Peptide 1 (■) and TNF + Peptide 2 (○).

Fig. 7 shows the effect of Peptide 1 on TNF-induced decrease in blood glucose levels in mice administered with 200μg TNF.

Fig. 8 shows the effect of Peptide 1 on TNF-induced decrease in blood glucose levels in ascites tumour-bearing mice.

Fig. 9 shows the effect of Peptide 1 on TNF-induced weight loss in ascites tumour-bearing mice.

Fig. 10 shows the effect of peptides on LPS toxicity in Meth A ascites tumour-bearing mice (10 animals per group scored positive if 7 or more survive);

Fig. 11 shows the effect of peptides on LPS toxicity in Meth A ascites tumour-bearing mice (10 animals per group scored positive if 7 or more survive);

Fig. 12 shows the effect of peptides on TNF toxicity in Meth A ascites tumour-bearing mice (each group contains 20 animals: scored positive if 7 or more survived);

Fig. 13 shows the effect of peptides on TNF toxicity in Meth A ascites tumour-bearing mice (each group contains 20 animals: scored positive if 10 or more survived);

Fig. 14 shows effect of peptides on TNF toxicity in D-galactosamine sensitized mice (each group contains 10 animals: scored positive if 6 or more survive).

Fig. 15 shows the effect of peptides on direct induction of chemiluminescence by TNF on human neutrophils;

Fig. 16 shows inhibition of TNF priming of human neutrophils by Peptide 21;

Fig. 17 shows inhibition of TNF priming of human neutrophils by Peptide 19;

Fig. 18 shows inhibition of LPS stimulation of neutrophils by Peptide 19;

Fig. 19 shows dose-dependent effects of Peptide 9 on TNF-induced chemiluminescence;

Fig. 20 shows effect of peptide 2 on human TNF priming of human neutrophils;

Fig. 21 shows inhibition of LPS-induced chemiluminescence response of human neutrophils by Peptide 21; and

Fig. 22 shows inhibition of TNF priming of human neutrophils by Peptide 21.

Production of Peptides

Synthesis of Peptides Using the FMOC-Strategy

Peptides (1-6, 9-18, 22-25, 27-29, 35, 36, 39, 40 Table 3) were synthesized on the continuous flow system as provided by the Milligen synthesizer Model 9050 using the standard Fmoc-polyamide method of solid phase peptide synthesis (Atherton et al, 1978, J.Chem. Soc. Chem. Commun., 13, 537-539).

For peptides with free carboxyl at the C-terminus, the solid resin used was PepSyn KA which is a polydimethylacrylamide gel on Kieselguhr support with 4-hydroxymethylphenoxyacetic 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.

For peptides with carboxamides at the C-terminus, the solid resin used was Fmoc-PepSyn L Am which is analogous polyamides resin with a Rink linker, p-[(R,S)-α[1-(9H-fluoren-9-yl)-methoxyformamido]-2, 4-dimethoxybenzyl]-phenoxyacetic acid (Bernatowicz et al, 1989, Tet.Lett. 30, 4645). The synthesis starts by removing the Fmoc-group with an initial piperidine wash and incorporation of the first amino acid is carried out by the usual peptide coupling procedure.

The Fmoc strategy was also carried out in the stirred cell system in synthesis of peptides (33,34,37,38) where the Wang resin replaced the Pepsyn KA.

All Fmoc-groups during synthesis were removed by 20% piperidine/DMF and peptide bonds were formed either of the following methods except as indicated in Table 1:

1. Pentafluorophenyl active esters. The starting materials are already in the active ester form.

2. Hydroxybenzotriazol esters. These are formed in situ either using Castro's reagent, BOP/NMM/HOBt (Fournier et al, 1989, Int.J.Peptide Protein Res., 33, 133-139) or

using Knorr's reagent, HBTU/NMM/HOBt (Knorr et al, 1989, Tet.Lett., 30, 1927).

Side chain protection chosen for the amino acids was removed concomitantly during cleavage with the exception of Acm on cysteine which was left on after synthesis. Intramolecular disulphide bridges where needed are then formed by treating the Acm protected peptide with iodine/methanol at high dilution.

TABLE 1

Amino Acid	Protecting Group	Coupling Method
Arg	Pmc	HOBt or OPfp
Asp	OBut	HOBt or OPfp
Cys	Acm	HOBt or OPfp
Glu	OBut	HOBt or OPfp
His	Boc or Trt	HOBt or OPfp
Lys	But	HOBt or OPfp
Ser	But	HOBt only
Thr	But	HOBt only
Tyr	But	HOBt or OPfp
Asn	none	OPfp only
Gln	none	OPfp only

Cleavage Conditions

Peptides were cleaved from the PepSyn KA and PepSyn K Am using 5% water and 95% TFA where Arg(Pmc) is not present. Where Arg(Pmc) is present a mixture of 5% thioanisole in TFA is used. The cleavage typically took 3 h at room temperature with stirring. Thioanisole was removed by washing with ether or ethyl acetate and the peptide was extracted into an aqueous fraction. Up to 30% acetonitrile was used in some cases to aid dissolution. Lyophilization of the aqueous/acetonitrile extract gave the crude peptide.

Peptides from the Wang resin were cleaved using 5% phenol, 5% ethanedithiol and 90% TFA for 16 h at ambient temperature with stirring. Thioanisole was removed by

washing with ether or ethyl acetate and the peptide was extracted into an aqueous fraction. Up to 30% acetonitrile was used in some cases to aid dissolution. Lyophilization of the aqueous/acetonitrile extract gave the crude peptide.

Peptides from the Wang resin were cleaved using 5% phenol, 5% ethanedithiol and 90% TFA for 16 h at ambient temperature with stirring.

Purification

Crude peptide is purified by reverse phase chromatography using either a C4 or C18 column and the Buffer system: Buffer A - 0.1% aqueous TFA, Buffer B - 80% Acetonitrile and 20% A.

N-Terminal Acetylation

The peptide resin obtained after the synthesis (with Fmoc removed in the usual manner was) placed in a 0.3 MDMF solution of 10 equivalents of Ac-OHSu for 60 minutes. The resin was filtered, washed with DMF, CH2C12, ether and used in the next step.

Cyclization

The purified and lyophilized bis-S-(acetamidomethyl) cysteine peptide (100-400 mg) was dissolved in 5 mls of methanol containing 1 ml of acetic acid. This was added dropwise to a 1 litre methanol solution containing 1 g of iodine.

After 2 h reaction, the excess iodine was removed by addition of a dilute sodium thiosulfate solution until the colour turns to a pale yellow, methanol was removed in vacuo at room temperature and the concentrated solution was finally completely decolourised with dropwise addition of sodium thiosulfate and applied immediately onto a preparatively reverse phase chromatography column.

Synthesis of Peptides using the Boc-Strategy

Syntheses of these peptides were carried out on the ABI 430A instrument using polystyrene based resins. For peptide with C-terminal acids, the appropriate Merrified

resin Boc-amino acid-O-resin or the 100-200 mesh PAM resin is used (7, 8, 19-21, 26, 31). Peptides with C-terminal amides are synthesized on MBHA resins (32, 33).

Couplings of Boc-amino acids (Table 2) were carried out either using symmetrical anhydride method or a HOBt ester method mediated by DCC or HTBU.

TABLE 2

Amino Acid	Protecting Group	Coupling Method
Arg	Tos	HOBt or S.A.
Asp	Cxl,OBzl	HOBt or S.A.
Cys	4-MeBzl	HOBt or S.A.
Glu	Cxl	HOBt or S.A.
His	Dnp, Bom	HOBt or S.A.
Lys	2-ClZ	HOBt or S.A.
Ser	Bzl	HOBt or S.A.
Thr	Bzl	HOBt or S.A.
Tyr	Br-Z	HOBt or S.A.
Asn	Xan	HOBt or S.A.
Gln	none	HOBt only

Cleavage

Peptides were cleaved in HF with p-cresol or anisole as scavenger for up to 90 min. For His with Dnp protection, the resin required pre-treatment with mercaptoethanol:DIPEA:DMF (2:1:7), for 30 min. After removal of scavengers by ether wash, the crude peptide is extracted into 30% acetonitrile in water.

N-Terminal Acetylation

Acetylation was achieved by treating the deblocked resin with acetic anhydride in DMF solution.

TABLE 3

No	hTNF	Sequence
1	1-18	VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS VAL VAL ALA
2	6-18	ARG THR PRO SER ASP LYS PRO VAL ALA HIS VAL VAL ALA

3	2-15	ART SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS
4	1-26	VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU
5	10-18	ASP LYS PRO VAL ALA HIS VAL VAL ALA
6	15-22	HIS VAL VAL ALA ASN PRO GLN ALA
7	6-16	ARG THR PRO SER ASP LYS PRO VAL ALA HIS VAL
8	6-17	ARG THR PRO SER ASP LYS PRO VAL ALA HIS VAL VAL
9	8-16	PRO SER ASP LYS PRO VAL ALA HIS VAL
10	8-15	PRO SER ASP LYS PRO VAL ALA HIS
11	8-15	PRO SER ASP LYS PRO VAL ALA
12	8-13	PRO SER ASP LYS PRO VAL
13	7-18	THR PRO SER ASP LYS PRO VAL ALA HIS VAL VAL ALA
14	8-18	PRO SER ASP LYS PRO VAL ALA HIS VAL VAL ALA
15	9-18	SER ASP LYS PRO VAL ALA HIS VAL VAL ALA
16	11-18	LYS PRO VAL ALA HIS VAL VAL ALA
17	12-18	PRO VAL ALA HIS VAL VAL ALA
18	12-18	Ac PRO VAL ALA HIS VAL VAL ALA NH2
19	6-18	ARG THR PRO SER ALA LYS PRO VAL ALA HIS VAL VAL ALA
	Ala(10)	
20	6-18	ARG THR PRO SER ASP ALA PRO VAL ALA HIS VAL VAL ALA
	Ala(11)	
21	6-18	ARG THR PRO SER LYS ASP PRO VAL ALA HIS VAL VAL ALA
	Lys(10)	
	Asp(11)	
22	1-18	VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA ARG VAL VAL ALA
	Arg(15)	

	23	1-18	VAL ARG SER SER SER ARG THR PRO SER ASP
		GLN(15)	LYS PRO VAL ALA GLN VAL VAL ALA
	24	1-18	VAL ARG SER SER SER ARG THR PRO SER ASP
		Leu(14)	LYS PRO VAL LEU HIS VAL VAL ALA
	25	1-18	VAL ARG SER SER SER ARG THR PRO SER ASP
			LYS PRO VAL VAL HIS VAL VAL ALA
		Val(14)	
	26	6-26	ARG THR PRO SER ASP LYS PRO VAL ALA HIS
			VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN
			LEU
	27	1-16	VAL ARG SER SER SER ARG THR PRO SER ASP
			LYS PRO VAL ALA HIS VAL
	28	1-10	VAL ARG SER SER SER ARG THR PRO SER ASP
	29	8-14	Ac PRO SER ASP LYS PRO VAL ALA NH2
	30	6-16	Ac ARG THR PRO SER ASP LYS PRO VAL ALA
			HIS VAL NH2
	31	6-16	ARG THR PRO SER ASP LYS PRO VAL VAL HIS
			VAL
		Val(14)	
	32	6-16	ARG THR PRO SER ASP LYS PRO VAL ALA HIS
			ALA
		ALA(16)	
	33	6-16	ARG THR PRO SER ASP LYS PRO VAL ALA ALA
			VAL
		ALA(15)	
	34	6-16	ART THR PRO SER ASP LYS PRO VAL ALA LYS
			VAL
		LYS(15)	
	35	6-16	ARG THR PRO SER ASP LYS PRO VAL ALA ASP
			VAL
		ASP(15)	
	36	6-16	ARG THR PRO SER ASP LYS PRO VAL ALA D-HIS
			VAL
		D-HIS(15)	
	275	111-120	ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU

302	43-48	LEU ARG ASP ASN GLN LEU VAL VAL PRO SER SLU GLY LEU TYR LEU ILE
303	94-109	LEU SER ALA ILE LYS SER PRO LYS GLN ARG GLU THR PRO GLU GLY ALA
304	63-83	LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU LEU THR HIS THR ILE SER ARG ILE
305	132-150	LEU SER ALA GLU ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL
306	13-26	VAL ALA HIS VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU
307	22-40	ALA GLU GLY GLN LEU GLN TRP LEU ASN ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY
308	54-68	GLY LEU TYR LEU ILE TYR SER SLN VAL LEU PHE LYS GLY GLN GLY
309	73-94	HIS VAL LEU LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS VAL ASN LEU LEU
323	79-89	THR ILE SER ARG ILE ALA VAL SER TYR GLN THR
347	132-157	LEU SER ALA GLU ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL TYR PHE GLY ILE ILE ALA LEU

Endothelial Cell Clotting Assays

Endothelial cell procoagulant activity (PCA) induction by TNFα was determined using bovine aortic endothelial cells (BAE) according to the procedure of Bevilacqua et al., 1986 PNAS 83, 4522 with the following modifications: BAE cells were propagated in McCoys 5A medium supplemented with 10% FCS, penicillin, streptomycin and L-gutamine in standard tissue culture flasks and 24-well dishes. TNFα treatment of culture (3μg/ml) was for 4 hours at 37^{o}C in the presence of growth medium after which the cells were washed and scrape-harvested before being frozen, thawed and sonicated. Total cellular PCA was determined in a standard one-stage clotting assay

using normal donor platelet poor plasma to which 100µl of $CaCl_2$ and 100µl of cell lystate was added. Statistical significance was determined by unpaired t-test.

Neutrophil Activation Studies

In these experiments, neutrophils were prepared from blood of healthy volunteers by the rapid single step method (Kowanko and Ferrante 1987 Immunol 62, 149). To 100µl of 5 x 10^6 neutrophils/ml was added 100µl of either 0, 10, 100µg of peptide/ml and 800µl of lucigenin (100µg). The tubes were immediately placed into a light proof chamber (with a 37°C water jacket incubator) of a luminometer (model 1250; LKB Instruments, Wallac, Turku, Finaldn). The resultant light output (in millivolts was recorded). The results are recorded as the maximal rate of chemiluminescence production.

Effects of peptides on neutrophil chemiluminescence induced by either TNF or LPS: Neutrophils of 96-99% purity and)99% viability were prepared from blood of normal healthy volunteers by centrifugation (400g for 30 min) through Hypaque-Ficoll medium of density 1.114. Following centrifugation the neutrophils formed a single band above the erythrocytes and 1 cm below the mononuclear leukocyte band. These were carefully recovered and washed in medium 199. To assess the lucigenin-dependent chemiluminescence response 100ul of 5 x 10^6 neutrophils/ml was added 100ul of either 0,1,10,100ug of peptide/ml and TNF or LPS and 800ul of lucigenin (100ug). The tubes were immediately placed into a light proof chamber with a 37°C water jacket incubator of a luminometer. The resultant light output (in millivolts) was recorded. The results are recorded as the maximal of chemiluminescence production. In experiments which examined the ability of the peptides to prime for the response to fMLP, 100ul of 5 x 10^5 neutrophils /ml preincubated in peptide and LPS or TNF for 20 mins was

added to 100ul of diluent or fMLP (5×10^{-6}M) before the addition of 700ul of lucigenin (100ug). The chemiluminescence was measured as above. Neutrophils from at least three individuals were used in triplicate determinations of anti-TNF or LPS activity. Results were deemed positive if at least 50% inhibition of chemiluminescence was obtained in at least two thirds of cases.

WEH1-164 Cytoxicity

Bioassay of recombinant TNF activity was performed according to the method described by Espevik and Nissen-Meyer. (Espevik and Nissen-Meyer 1986 J. Immunol. Methods 95 99-105)

Tumour Regression Experiments

Subcutaneous tumours were induced by the injection of approximately 5×10^5 WEH1-164 cells. This produced tumours of diameters of 10 to 15mm approximately 14 days later. Mice were injected i.p. with recombinant human TNF (10μg and 20μg) and peptide (1mg) for four consecutive days. Control groups received injections of PBS. Tumour size was measured daily throughout the course of the experiment. Statistical significance of the results was determined by unpaired Student T-test.

Radioreceptor assays

WEH1-164 cells grown to confluency were scrape harvested and washed once with 1% bovine serum albumin in Hanks balanced salt solution (HBSS, Gibco) and used at 2×10^6 cells pre assay sample. For the radioreceptor assay, the cells were incubated with varying amounts of either unlabelled TNFα($1\text{-}10^4$ ng per assay sample) or peptide ($0\text{-}10^5$ ng per assay sample) and ^{125}I-TNF (50,000cpm) for 3 hours at 37°C in a shaking water bath. At the completion of the incubation 1ml of HBSS/BSA was added to the WEH1-164 cells, the cells spun and the bound ^{125}I in the cell pellet counted. Specific binding

was calculated from total binding minus non-specific binding of triplicate assay tubes. 100% specific binding corresponded to 1500 cpm.

In Vivo Studies of TNF Toxicity

Mice were administered with either TNF (200μg), Peptide 1 (10mg) and TNF (200μg)+Peptide 1 (10mg) via intravenous injection. Blood glucose levels and appearance of the animals was evaluated at 15, 30, 60, 120, 180 minutes after injection. Appearance parameters which were evaluated included ruffling of fur, touch sensitivity, presence of eye exudate, light sensitivity and diarrhoea.

Infection of mice with malaria parasites and treatment with TNF+ Peptide 1

All the mice used were male, CBA/CaH stain and 6-8 weeks old. P. vinkei vinkei (Strain V52, from F.E.G. Cox, London) has undergone several serial passages in CBA mice, after storage in liquid nitrogen, before use in these experiments. Infections were initiated by intraperitoneal injection of 10^6 parasitized erythrocytes. Mice were treated with TNF(7μg) ± peptide (8.3 mg) administered iv.

Assays for blood glucose

Nonfasting blood glucose levels were determined on a Beckman Glucose Analyzer 2 (Beckman Instruments) or on a Exectech blood glucose sensor (Clifford Hallam Pty. Ltd).

Reactive Nitrogen Intermediates (RNI)

RNI levels in blood were determined by the method of Rockett et al (1991) in-vivo induction of TNF, LT and IL-1 implies a role for nitric oxide in cytokine-induced malarial cell-mediated immunity and pathology. J. Immunol. in press.

TNF and LPS Lethality Experiments: balb/C or balbC x swiss F1 mice carrying Meth A ascites tumours elicited by prior I.P. inoculation of 0.5μl pristane 7 days before I.P. injection of tumour cells. Nine to ten days after

inoculation with the tumour cells 25 ug of human recombinant TNF was subcutaneously administered and a short time later 1mg of either test peptide, bovine serum albumen, phosphate buffered saline or neutralizing anti-TNF MAb 47 was administered at a separate subcutaneous site. The number of surviving animals was then observed at 18 hours and 24 hours post TNF treatment. In experiments which assessed the effects of 1-related peptides on on LPS lethality the mice were administered 500ug E.coli LPS and peptide or other treatment in a similar manner. In LPS experiments polymyxin B, an LPS inhibitor, replaced MAb 47 as a positive control. The number of animals surviving was assessed at intervals up to 64 hours after LPS challenge.

Experiments in D-galactosamine sensitized mice: Female Bablb/C mice were co-injected intraperitoneally with 16 mg D-galactosamine and 2ug human recombinant TNF. The mice were then injected subcutaneously with either test peptide, phosphate buffered saline or neutralizing anti-TNF monoclonal antibody 47. The number of surviving animals was assessed at intervals up to 48 hours after TNF challenge.

RESULTS

The results obtained with each of the peptides are summarised in Table 4. A single * indicates heightened activity in that test whilst a double ** indicates activity at low concentrations of peptide but not high concentrations.

TABLE 4

	IN VIVO			IN VITRO NEUTROPHIL			
	TNF TOXICITY		LPS TOXICITY		TNF		LPS
PEPTIDE	METH A	D-GAL	METH A	DIRECT	PRIMING	DIRECT	PRIMING
1	+	+	+	+	+	+	+
2	+*	+	+	+*			
8	-		-	+			
9	-		-	+**			
10	+*	-	-	+			
11	-			-			
12	+			-			
16	-			-			
17	-		+	-			
13	-		-	+			
14	-		+	+			
15	-		-	-			
18	-	-					
19	+		+	+	+	+	+
20	-		-	-			
21	+*		+	+	+	+	+
22		+	+	+	+		
23	+	+	+	+			
24	-		-	-			
25	+/-		-	+			
26	-		-	+			
4	-			+			
5	-		-	+			
6	-			-			
3	-						
28	+	-	+				
29	-	-	+				
30	+*	+	+				
31	+	+	-				
32	-		-				
33	-		+*				
34	-		+*				
36	-		-				
35	+		+				
27	-		-				
7	-		+	+*			

TNF administered at a dose of 200μg was found to be toxic in mice according to the parameters studied. In particular, blood glucose levels had fallen by 120 minutes (Fig 7) Peptide 1 alone in 2 of the 3 mice studied did not reduce blood glucose levels. Mouse 1 in this group recovered normal blood glucose levels within by 180 minutes. Mice in the group treated with a combination of TNF and Peptide 1 showed no reduction in blood glucose levels at 120 min and a small decrease at 180 min.

As shown in Fig. 6, 10μg of Peptide 2 given to mice treated with 200μg of recombinant human TNF abrogated TNF toxicity as indicated by the inhibition of blood glucose changes evident in mice treated with TNF alone.

When general appearance of treated mice was considered it was noted that all 3 TNF only treated mice had ruffled fur, touch sensitivity and light sensitivity. One mouse in this group also had diarrhoea. Mice treated with Peptide 1 alone showed only slight touch sensitivity with one mouse showing slight ruffling of the fur at 180 mins. Mice treated with a combination of TNF and Peptide 1 showed ruffling of the fur and slight touch sensitivity at 180 mins but failed to show either light sensitivity or onset of diarrhoea. In addition, Peptide 1 and related peptides prevented death in acute models of TNF tethality (Figs. 12 & 13).

Peptide 1 failed to either activate the respiratory burst of human neutrophils (Table 5) or to induce procoagulant activity on bovine aortic endothelial cells, and hence is free of these negative aspects of TNF activity in acute or chronic inflammation. However, Peptide 1 and related peptides inhibited both the TNF and LPS-induced respiratory burst of human neutrophils (Figs. 15, 19, 18, 21). Further, several peptides inhibited priming of the neutrophil response to a bacterially-derived peptide EMLP (Figs. 16, 17, 20, 22).

TABLE 5

Peptide	Concentration ug/10^6 cells)				
	0	1	10	100	500
275	1.02	0.99	0.69	0.43	0.80
1	0.34	0.93	0.74	0.55	1.10
302	0.37	0.15	0.18	0.29	
303	0.37	0.22	0.17	0.22	
304	0.37	0.18	0.43	2.56	2.76
305	0.37	0.27	0.36	0.24	
306	0.37	0.27	0.35	0.23	
307	0.37	0.35	0.37	0.42	
323	0.37	0.23	0.17	0.47	
308	0.37	0.91	1.80	49.52	
309	0.37	0.38	0.98	13.44	

Results are expressed as mV of lucigenin dependent chemiluminescence and represent peak of response i.e. the maximal cell activity attained.

The results shown in Fig. 3 clearly show one of the desirable effects of TNFα, i.e. tumour regression, is unaffected by Peptide 1. Further, Peptide 1 does not inhibit binding of TNF to tumour cell receptors (Fig 4). Table 6 indicates that Peptide 1 is devoid of intrinsic anti-tumour activity. The ability of Peptide 1 to prevent high plasma RNI levels in TNFα treated malaria primed mice is also strongly indicative of the therapeutic usefulness of this peptide (Fig 5). Peptide 1 also inhibits the TNF-induced decrease in blood glucose levels evident in mice treated with TNF alone (Fig 2). Further in the experiments involving mice infected with malaria parasites; of the three mice treated with TNFα alone one died and the other two were moribund. In contrast in the

group of three mice treated with TNFα and Peptide 1 all survived and none were moribund. This very marked result also strongly indicates the potential usefulness of this peptide as a therapeutic.

Peptide 1 inhibits not only the TNF-induced hypoglycaemia in sensitized mice but also in ascites tumour-bearing mice (Fig 8). Further, tumour-bearing mice treated with TNF + Peptide 1 fail to develop the cachexia or weight loss associated with TNF treatment (Fig 9).

As will be seen from the above information the peptide of the present invention are capable of abrogating TNF and/or LPS toxicity in vivo and neutrophil activation by LPS or TNF in vitro. This peptide has utility in the treatment of numerous disease states which are due to the deleterious effects of TNF and/or LPS.

TABLE 6

In vitro cytotoxicity of TNF and synthetic TNF peptides on WEHI 164 fibrosarcoma cells

TNF/PEPTIDE	% VIABLE CELLS*
TNF#	26.6
275+	100
1	100
302	48.7
304	100
305	72.7
306	100
307	100
308	42.2
309	92.8

* %Viability was determined by comparison with untreated control cells. Results shown are the means of quadruplicate determinations.

\# TNF was at 50 units per culture which is equivalent to 3ug (12ug/ml)

\+ Each peptide was tested at 50ug/culture (200ug/ml)

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:-

1. A linear or cyclic peptide of the general formula:-

X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9

in which

X_1 is null, Cys or R_1

X_2 is null, Cys, R_1 or A_1-A_2-A_3-A_4-A_5

in which A_1 is Val or Ile or Leu or Met or His

A_2 is Arg or Cys or His

A_3 is Ser or Thr or Ala

A_4 is Ser or Thr or Ala

A_5 is Ser or Thr or Ala

X_3 is Cys, R_1 or A_6-A_7

in which A_6 is Arg or Cys or His or Absent

A_7 is Thr or Ser or Ala

X_4 is Cys, R_1 or A_8-A_9

in which A_8 is Pro or an Nα-alkylamino acid

A_9 is Ser or Thr or Ala

X_5 is Cys, R_1 or A_{10}

in which A_{10} is Asp or Ala or Cys or Glu or Gly or Arg or His

X_6 is Cys, R_2 or A_{11}-A_{12}-A_{13}

in which A_{11} is absent or Cys or Arg or His or Asp or Glu

A_{12} is Pro or an Nα-alkylamino acid

A_{13} is Val or Ile or Phe or Tyr or Trp or His or Leu or His or Met

X_7 is null, Cys, R_2 or A_{14}-A_{15}

in which A_{14} is Ala or Val or Gly or Ile or Phe or Trp or Tyr or Leu or His or Met

A_{15} is absent or His or Arg or Glu or Asn or Ala or Lys or Asp or Phe or Tyr or Tap or Glu or Gln or Ser or Thr or Gly

X_8 is null, Cys, R_2, A_{16}, A_{16}-A_{17}, A_{16}-A_{17}-A_{18} or A_{16}-A_{17}-A_{18}-A_{19}-A_{20}-A_{21}-A_{22}-A_{23}-A_{24}-A_{25}-A_{26}

in which A_{16} is Val or Ile or Leu or Met or His
A_{17} is Val or Ile or Leu or Met or His
A_{18} is Ala or Gly
A_{19} is Asp or Glu
A_{20} is Pro or an Nα-alkylamino acid
A_{21} is Gln or Asn
A_{22} is Ala or Gly
A_{23} is Glu or Asp
A_{24} is Gly or Aln
A_{25} is Gln or Asn
A_{26} is Leu or Ile or Val or Met or His

X_9 is null, Cys or R_2

R_1 is R-CO, where R is H, straight, branched or cyclic alkyl up to C20, optionally containing double bonds and/or substituted with halogen, nitro, amino, hydroxy, sulfo, phospho or carboxyl groups (which may be substituted themselves), or aralkyl or aryl optionally substituted as listed for the alkyl and further including alkyl, or R_1 is glycosyl, nucleosyl, lipoyl or R_1 is an L- or D-α amino acid or oligomers thereof consisting of up to 5 residues R_1 is absent when the amino acid adjacent is an unsubstituted desamino-derivative.

R_2 is
$-NR_{12}R_{13}$, wherein R_{12} and R_{13} are independently H, straight, branched or cyclic alkyl, aralkyl or aryl optionally substituted as defined for R_1 or N-glycosyl or N-lipoyl
$-OR_{14}$, where R_{14} is H, straight, branched or cyclic alkyl, aralkyl or aryl, optionally substituted as defined for R_1
-O-glycosyl, -O-lipoyl or
- an L- or D-α-amino acid or a oligamu thereof consisting of up to 5 residues
or R_2 is absent, when the adjacent amino acid is a decarboxy derivative of cysteine or a homologue thereof or the peptide in a N-C cyclic form.

with the proviso that:

when X_6 is Cys or R_2 then X_5 is A_{10}, X_4 is A_8-A_9, X_3 is A_6-A_7 and X_2 is A_1-A_2-A_3-A_4-A_5

when X_5 is Cys or R_1 then X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15}, X_8 is A_{16}-A_{17}-A_{18} and A_{11} is absent

when X_4 is Cys or R_1 then X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15} and X_8 is A_{16}-A_{17}-A_{18}

when X_2 is A_1-A_2-A_3-A_4-A_5 then X_8 is not A_{16}

when X_1 is null, X_2 is Cys or R_1, X_3 is A_6-A_7, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15} and X_8 is A_{16} then A_{16} is not D-His.

X_1 is always and only null when X_2 is R_1, Lys or Null

X_2 is always and only null when X_3 is Cys or R_1

X_3 is always and only null when X_6 is Cys or R_2

X_7 is always and only null when X_7 is Cys, R_2 or Null

X_8 is always and only null when X_8 is Cys, R_2 or Null

X_9 is always and only null when X_8 is Cys, R_2 or Null

when X_1 and R_2 are null, X_3 is R_1, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15}, X_8 is R_2 and A_{14} is Ala and A_{15} is absent then R_1 is acetyl and R_2 is NH_2.

2. A linear or cyclic peptide as claimed in claim 1 in which:-

X_1 is H, X_2 is A_1-A_2-A_3-A_4-A_5, X_3 is A_6-A_7, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15}, X_8 is A_{16}-A_{17}-A_{18} and X_9 is OH.

3. A linear or cyclic peptide as claimed in claim 1 in which:-

X_1 is null, X_2 is H or Ac, X_3 is A_6-A_7, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is A_{11}-A_{12}-A_{13}, X_7 is A_{14}-A_{15}, X_8 is A_{16}-A_{17}-A_{18} and X_9 is OH or NH_2.

4. A linear or cyclic peptide as claimed in claim 1 in which:-

X_1 is H, X_2 is A_1-A_2-A_3-A_4-A_5, X_3 is A_6-A_7, X_4 is A_8-A_9, X_5 is A_{10}, X_6 is OH and X_6, X_7 and X_8 are null.

5. A linear or cyclic peptide as claimed in claim 1 in which the peptide is selected from the group consisting of:-

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Ala-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Lys-Asp-Pro-Val-Ala-His-Val-Val-Ala;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Arg-Val-Val-Ala;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Gln-Val-Val-Ala;

Ac-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-NH2;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Ala-Val;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Lys-Val;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val;

Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val;

Pro-Ser-Asp-Lys-Pro-Val-Ala-His;

Pro-Ser-Asp-Lys-Pro-Val;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Val-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-Asn-Pro-Gln-Ala-Glu-Gly-Gln-Leu;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp;

Ac-Pro-Ser-Asp-Lys-Pro-Val-Ala-NH2;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Asp-Val;

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-Asn-Pro-Gln-Ala-Glu-Gly-Gln-Leu;

Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;

Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val;

Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;
Pro-Sir-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;
Pro-Val-Ala-His-Val-Val-Ala; and
Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Val-His-Val.

6. A peptide as claimed in claim 5 in which the peptide is

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;
Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala;
Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp;
Arg-Thr-Pro-Ser-Ala-Lys-Pro-Val-Ala-His-Fal-Val-Ala;
Arg-Thr-Pro-Ser-Lys-Asp-Pro-Val-Ala-His-Val-Val-Ala;
Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Arg-Val-Val-Ala;
Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Gln-Val-Val-Ala; or
Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Asp-Val.

7. A pharmaceutical composition for use in treating subjects suffering from acute or chronic inflammation, the composition comprising a therapeutically effective amount of a peptide as claimed in any one of claims 1 to 6 and a pharmaceutically acceptable sterile carrier.

8. A composition as claimed in claim 7 in which the composition is for administration topically, as a nasal spray, ocularly, intraveneously, intraperitoneally, intramuscularly, subcutaneously or for oral delivery.

9. A composition as claimed in claims 7 or 8 in which the composition provides slow release of the active peptide.

10. A method of treating a subject suffering from acute or chronic inflammation, the method comprising administering to the subject the composition as claimed in any one of claims 7 to 9.

11. A method as claimed in claim 10 in which the subject is suffering from toxic shock, adult respiratory distress

syndrome, hypersensitivity pneumonitis, systemic lupus erythromatosis, cystic fibrosis, asthma, bronchitis, drug withdrawal, schistosomiasis, sepsis, rheumatoid arthritis, acquired immuno-deficiency syndrome, multiple sclerosis, leperosy, malaria, systemic vasculitis, bacterial meningitis, cachexia, dermatitis, psoriasis, diabetes, neuropathy associated with infection or autoimmune disease, ischemia/reperfusion injury, encephalitis, Guillame Barre Syndrome, atherosclerosis, chronic fatigue syndrome, TB, other viral and parasitic diseases and OKT3 therapy.

12. A method of ameliorating or reducing the adverse side effects in a subject receiving cytotoxic drugs, cytokines, immunopotentiating agents, radiation therapy and/or chemotherapy comprising administering to the subject the composition as claimed in any one of claims 7 to 9.

13. An anti-idiotypic antibody to the peptide as claimed in any one of claims 1 to 6, the anti-idiotypic antibody being characterised in that it is capable of abrogating TNF and/or LPS toxicity.

14. A compound the three dimensional structure of which is similar as a pharmacophore to the three dimensional structure of the peptide as claimed in any one of claims 1 to 6, the compound being characterised in that it binds to one or more antibodies raised against the peptides as claimed in any one of claims 1 to 6 and that the compound is capable of abrogating TNF and/or LPS toxicity.

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FIG. 1

VRSSSRTPSD10**KPVAHVVA**NP20QAEGQLQWLN30RRA

NALLANG40VELRDNQLVV50PSEGLYLIYS60QVLFKGQGCP70STHVLL

THTI80SRIAVSYQTK90VNLLSAIKSP100CQRETREGAE110AKPWYEPI

YL120GGVFQLEKGD130RLSAEINRPD140YLDFAESGQV150YFGIIAL157

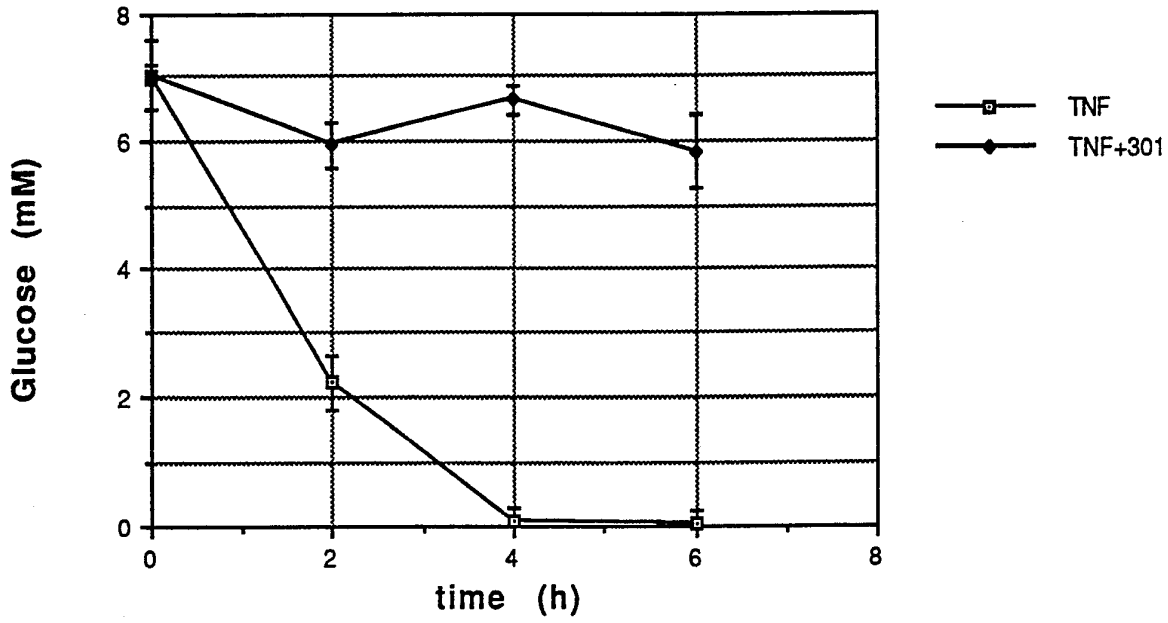


Fig 2

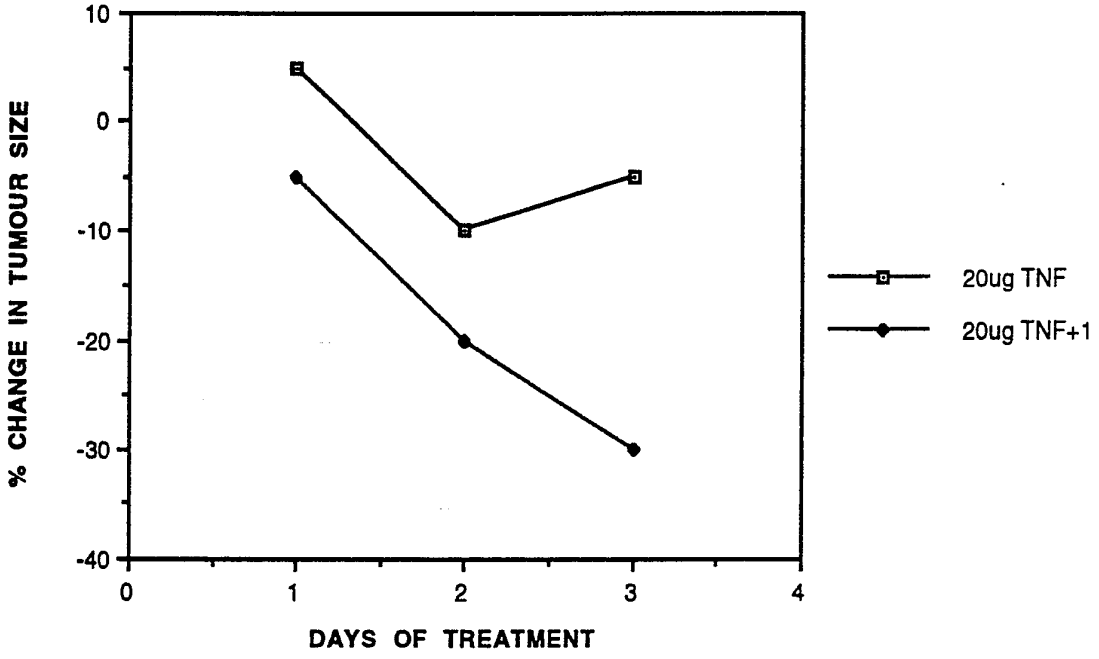
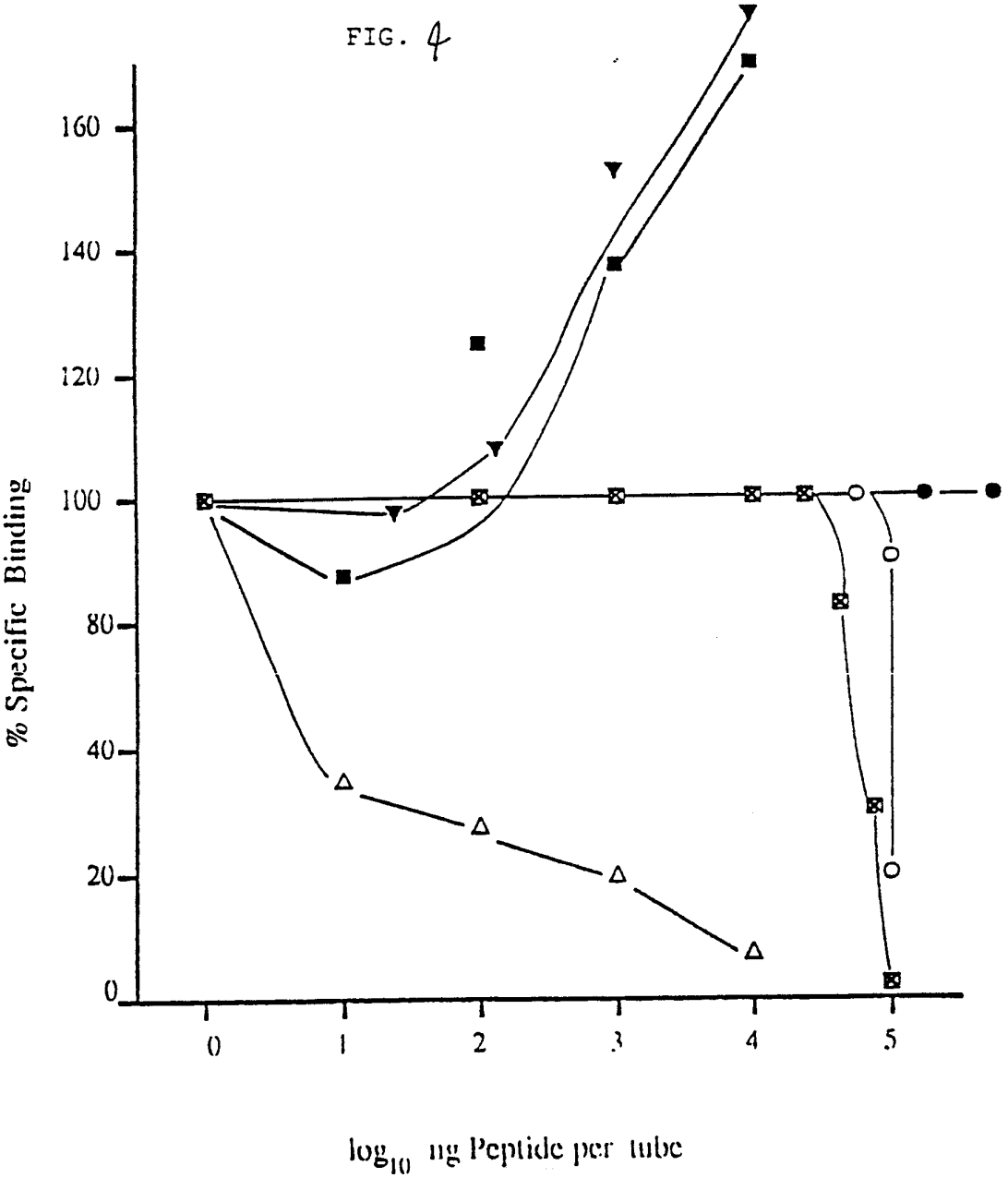

10
0
-10
-20
-30
-40
% CHANGE IN TUMOUR SIZE
0
1
2
3
4
DAYS OF TREATMENT
20ug TNF
20ug TNF+1

Fig 3

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FIG. 4


% Specific Binding
160
140
120
100
80
40
20
0
0
1
2
3
4
5
log10 ng Peptide per tube

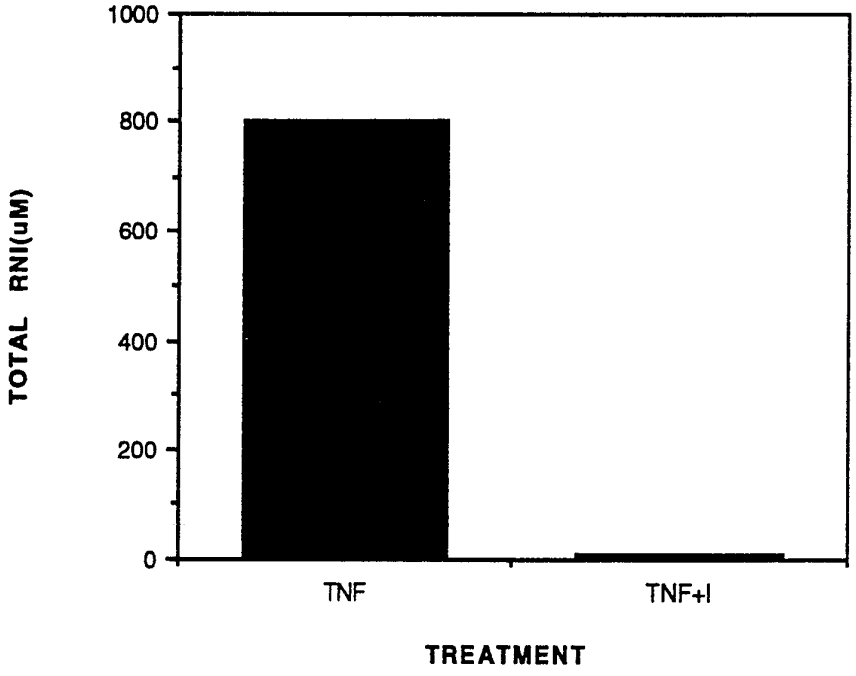


Fig 5

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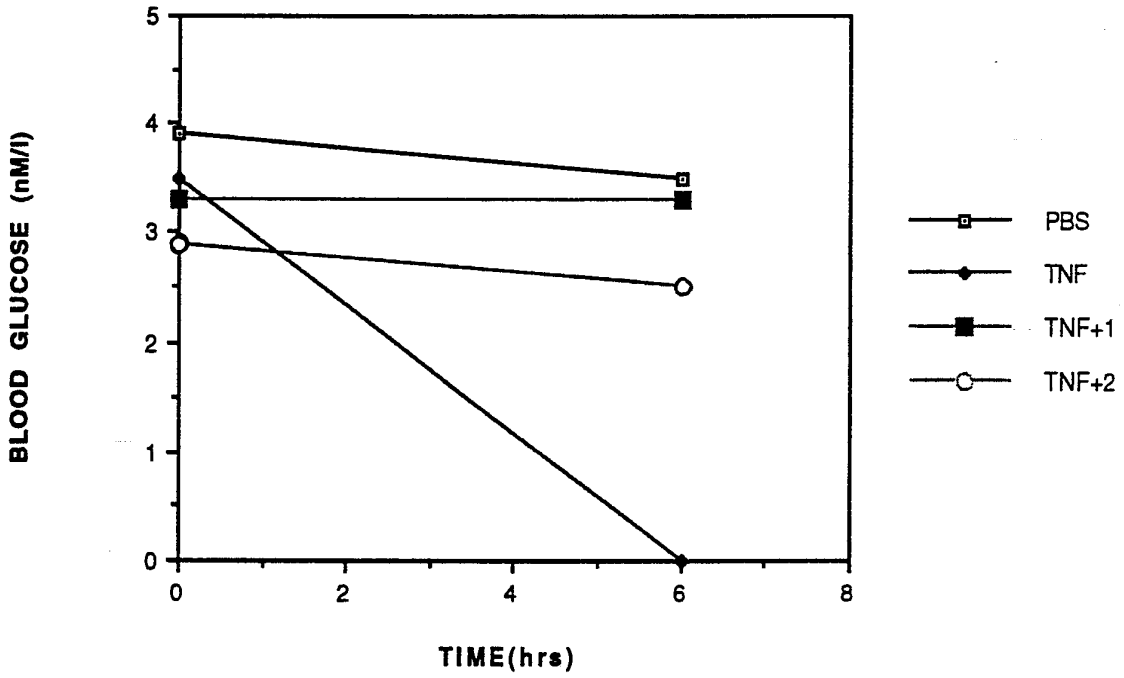


Fig 6

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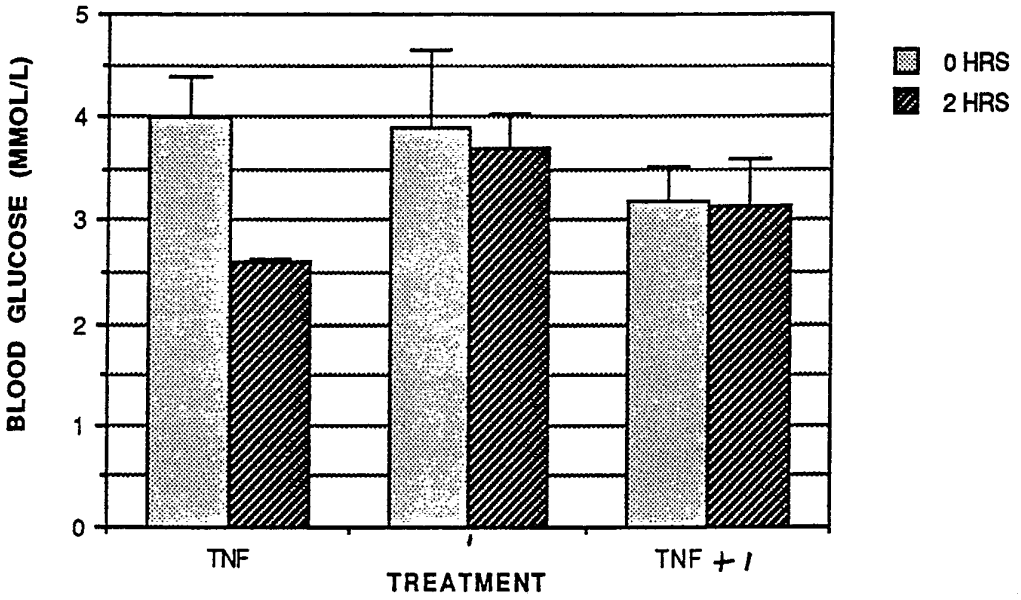


Fig 7

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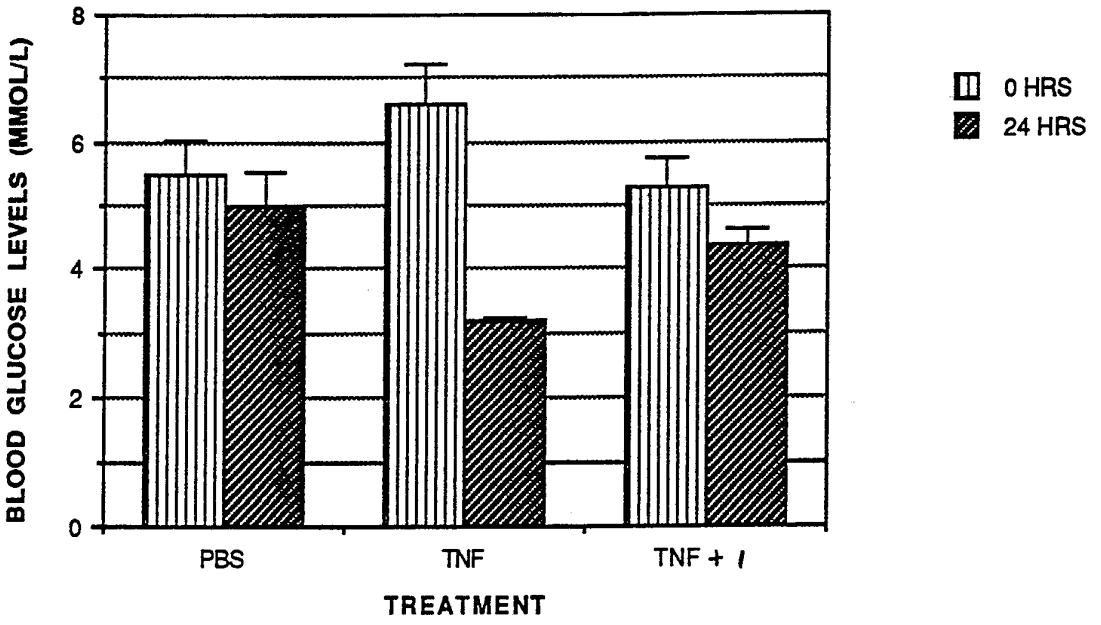

BLOOD GLUCOSE LEVELS (MMOL/L)
8
6
4
2
0
PBS
TNF
TNF + I
TREATMENT
0 HRS
24 HRS

Fig 8

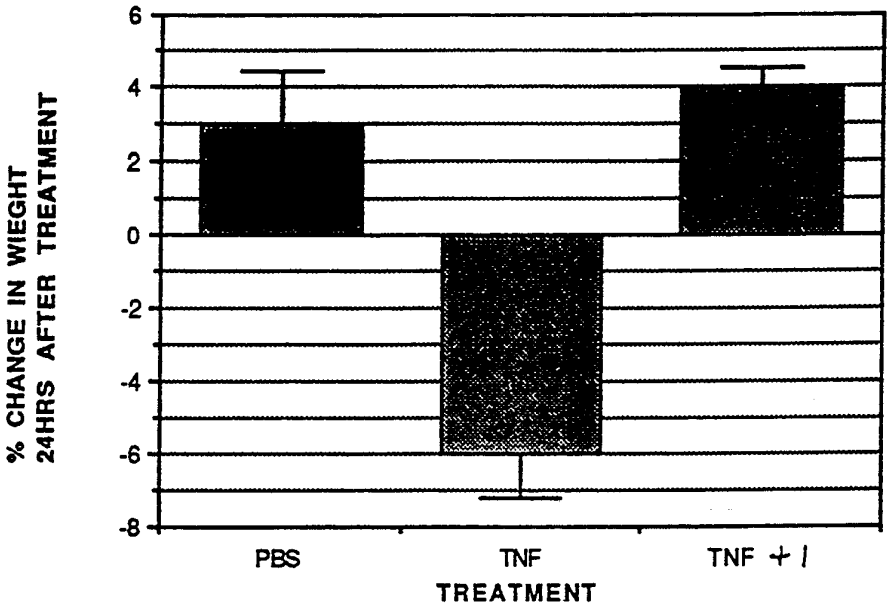

% CHANGE IN WIEGHT
24HRS AFTER TREATMENT
6
4
2
0
-2
-4
-6
-8
PBS
TNF
TNF + I
TREATMENT

Fig 9

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Fig 10

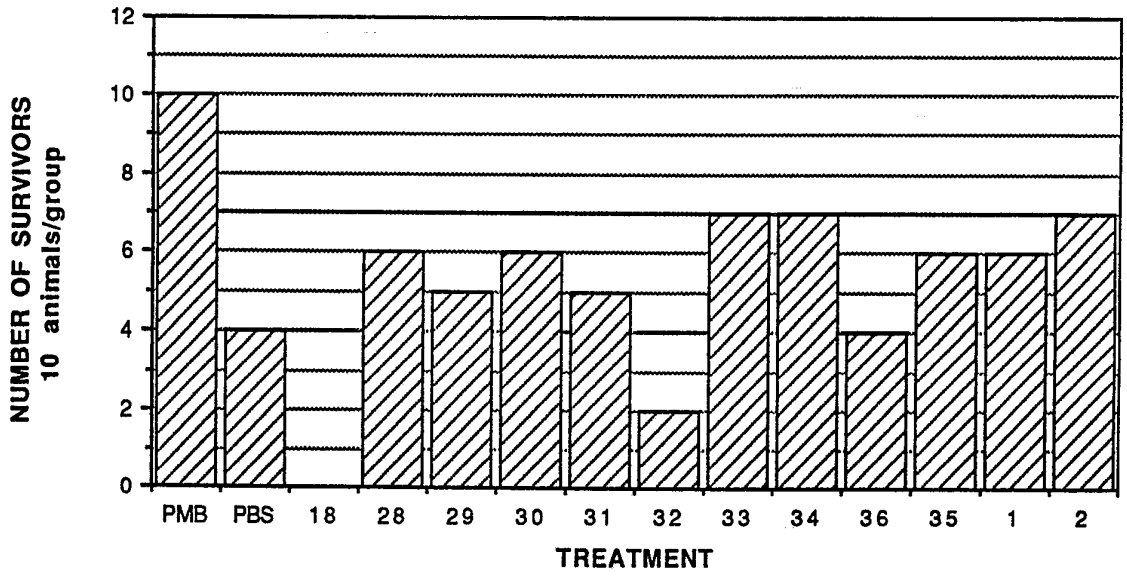

NUMBER OF SURVIVORS
10 animals/group
12
10
8
6
4
2
0
PMB
PBS
18
28
29
30
31
32
33
34
36
35
1
2
TREATMENT

Fig 11

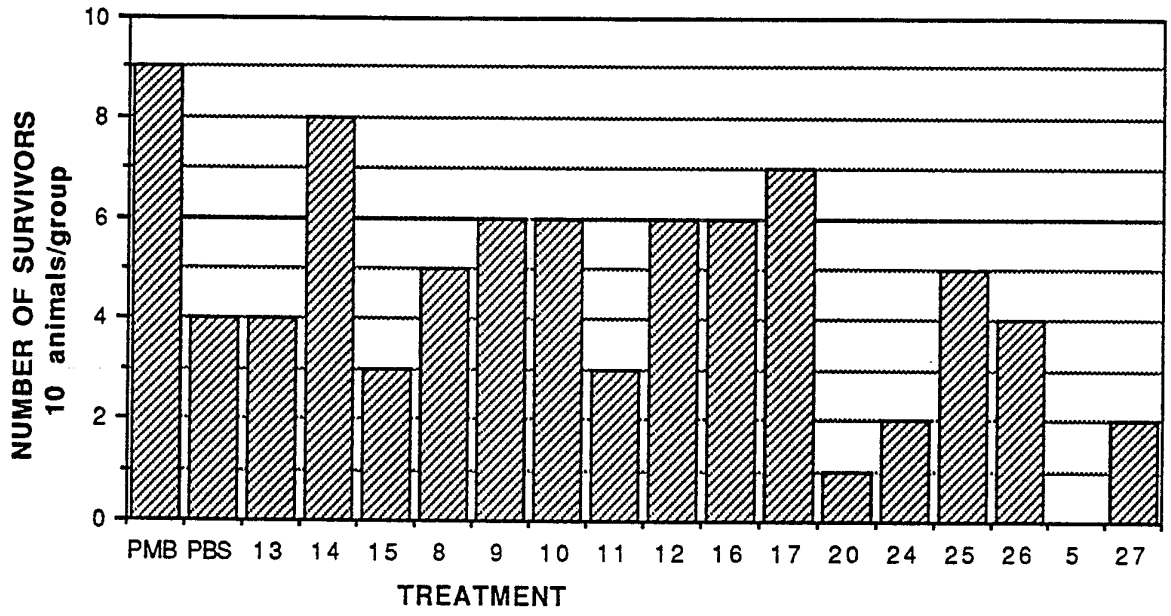
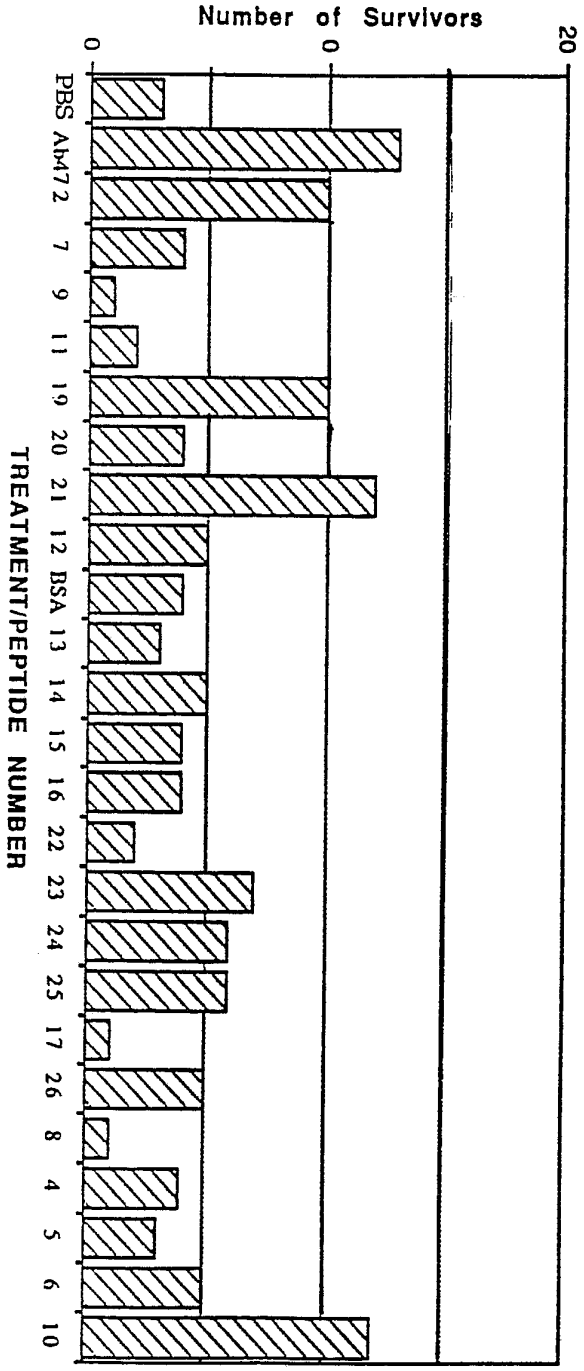

NUMBER OF SURVIVORS
10 animals/group
0
2
4
6
8
10
PMB PBS 13 14 15 8 9 10 11 12 16 17 20 24 25 26 5 27
TREATMENT

FIG 12



SUBSTITUTE SHEET

Fig 13

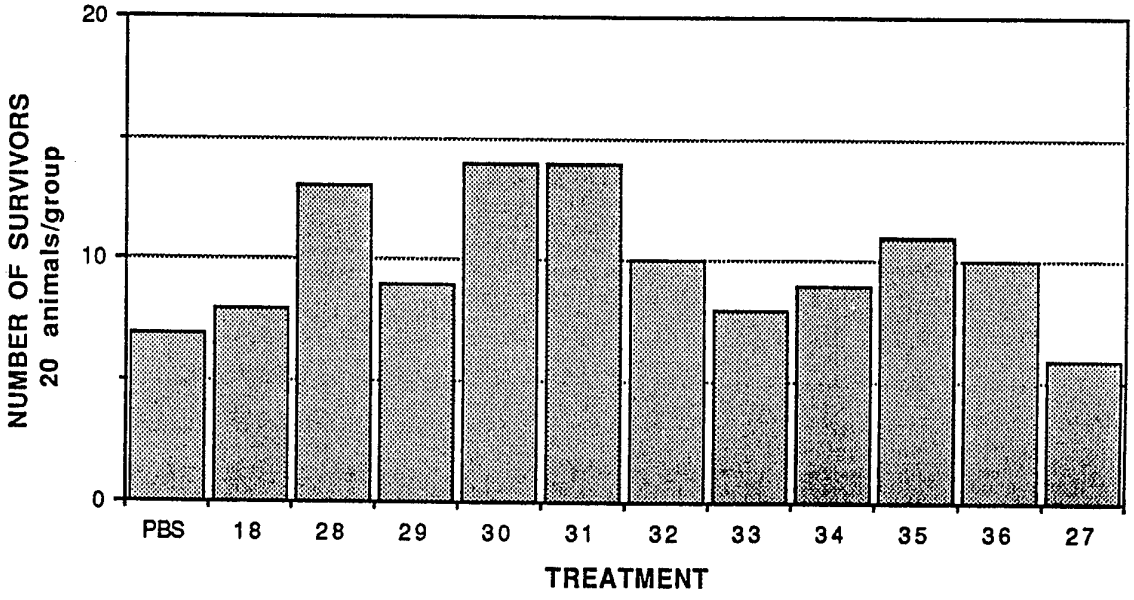
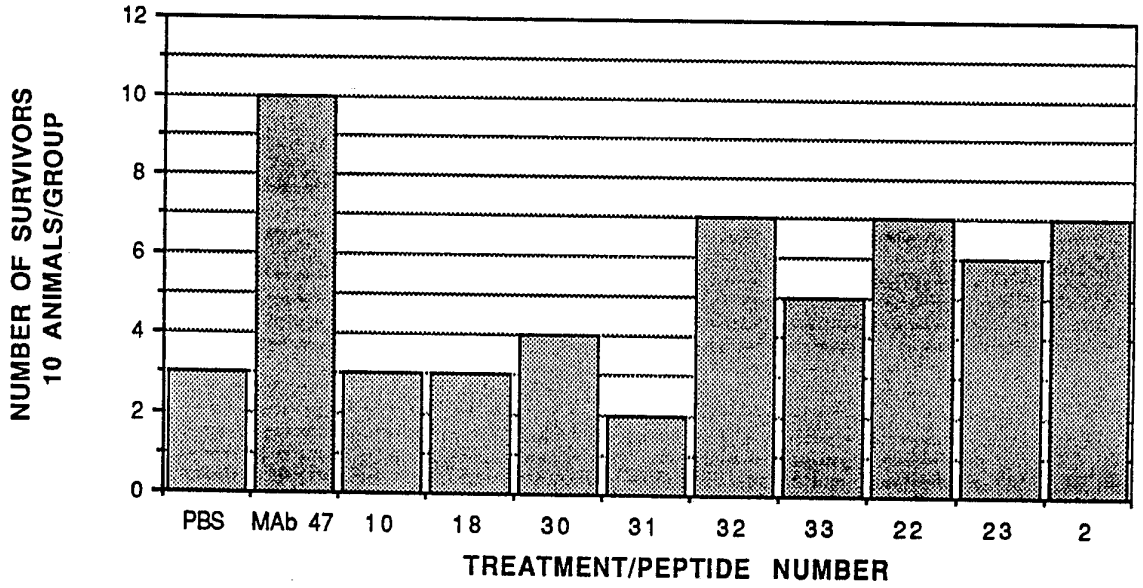

20
10
0
NUMBER OF SURVIVORS
20 animals/group
PBS
18
28
29
30
31
32
33
34
35
36
27
TREATMENT

FIG 14


NUMBER OF SURVIVORS
10 ANIMALS/GROUP
12
10
8
6
4
2
0
PBS
MAb 47
10
18
30
31
32
33
22
23
2
TREATMENT/PEPTIDE NUMBER

15/22

FIG 15

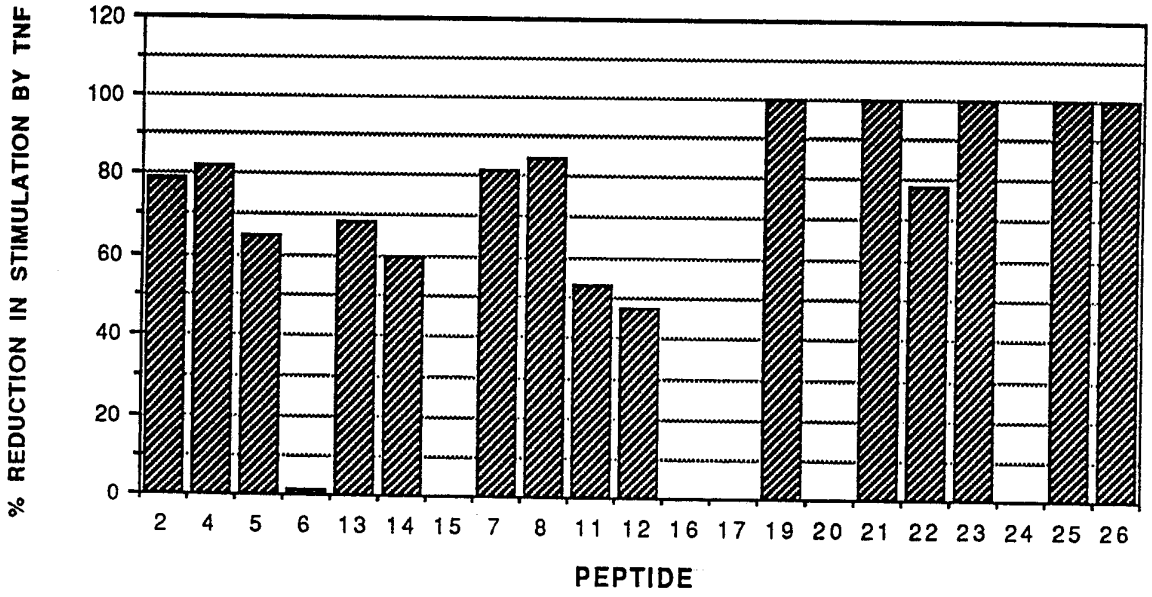
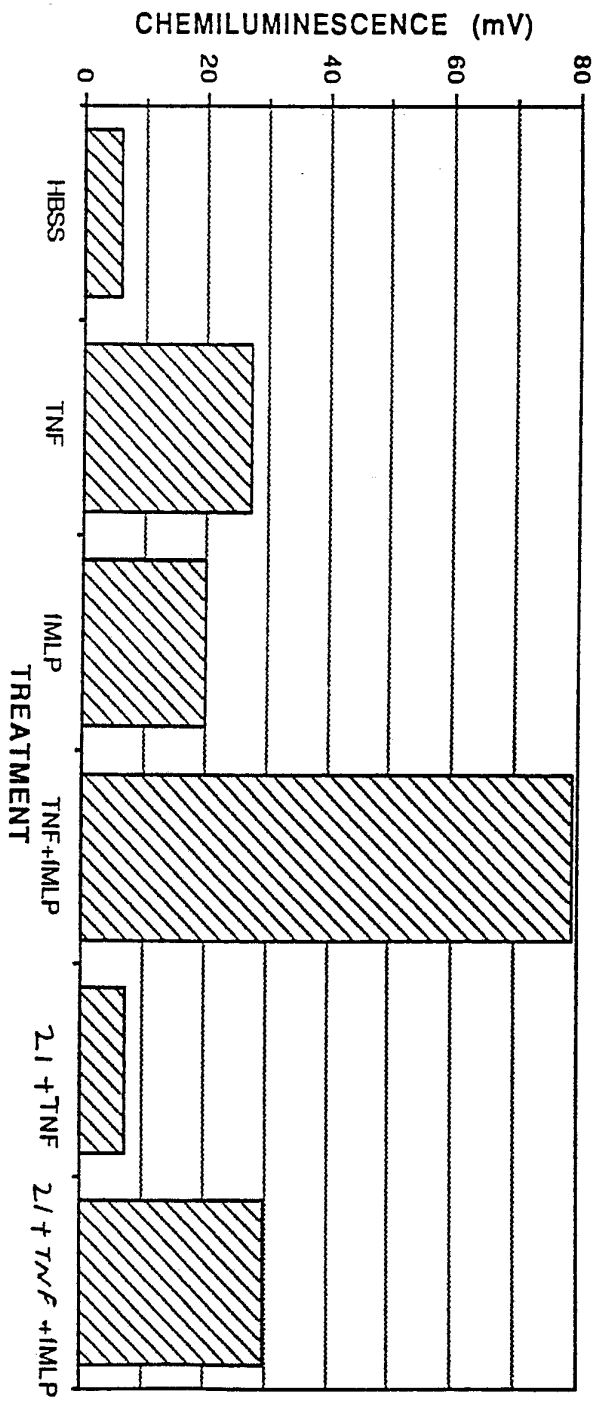

% REDUCTION IN STIMULATION BY TNF
120
100
80
60
40
20
0
2 4 5 6 13 14 15 7 8 11 12 16 17 19 20 21 22 23 24 25 26
PEPTIDE

FIG 16 16/22



SUBSTITUTE SHEET

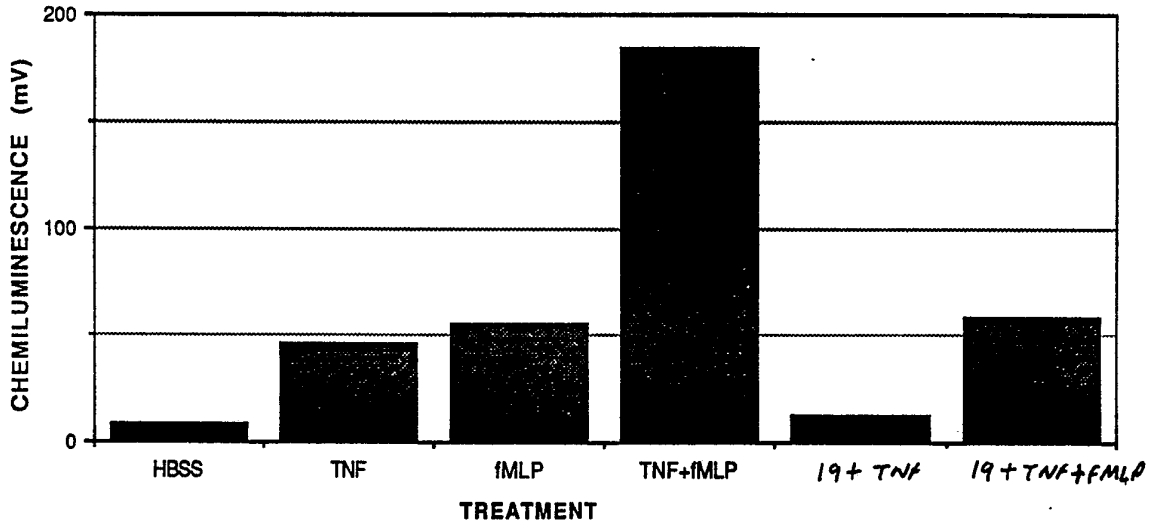

200
100
0
CHEMILUMINESCENCE (mV)
HBSS
TNF
fMLP
TNF+fMLP
19+ TNF
19+TNF+FMLP
TREATMENT

FIG 17

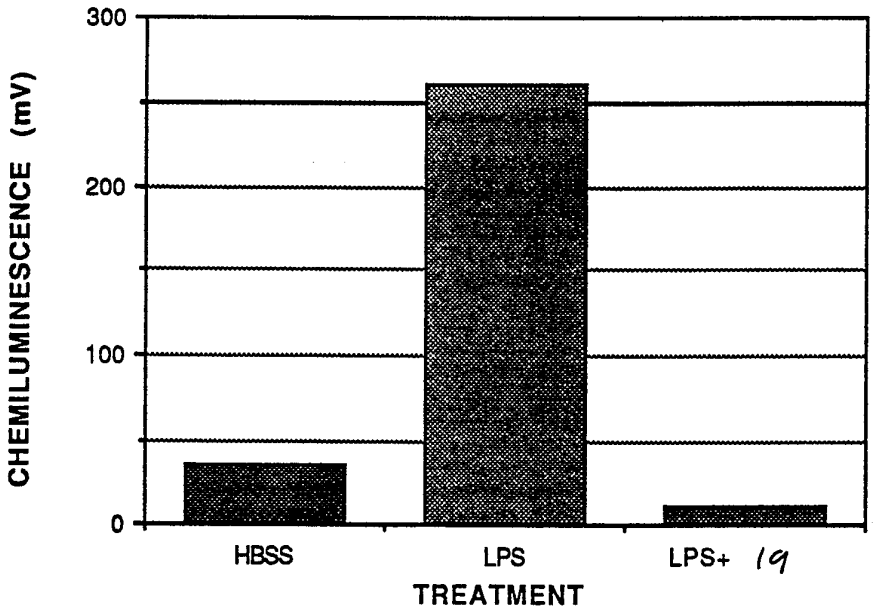
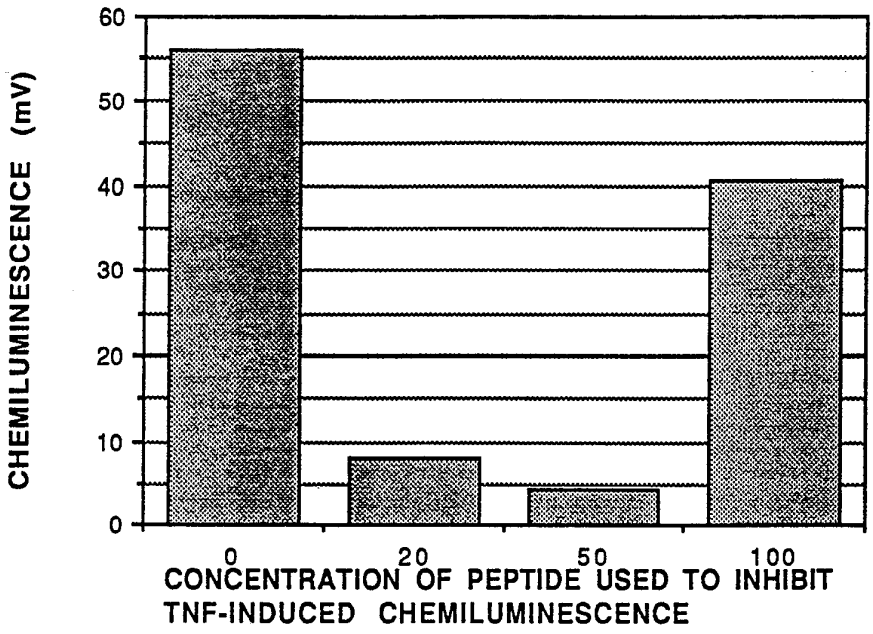

300
200
100
0
CHEMILUMINESCENCE (mV)
HBSS
LPS
LPS+ 19
TREATMENT

FIG 18

FIG. 19


CHEMILUMINESCENCE (mV)
60
50
40
30
20
10
0
0
20
50
100
CONCENTRATION OF PEPTIDE USED TO INHIBIT
TNF-INDUCED CHEMILUMINESCENCE

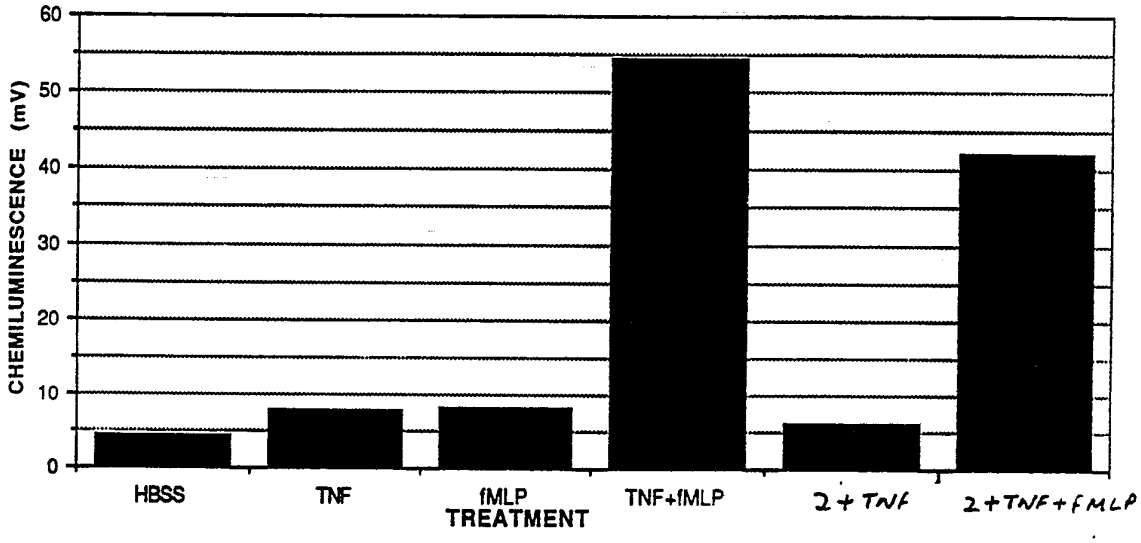
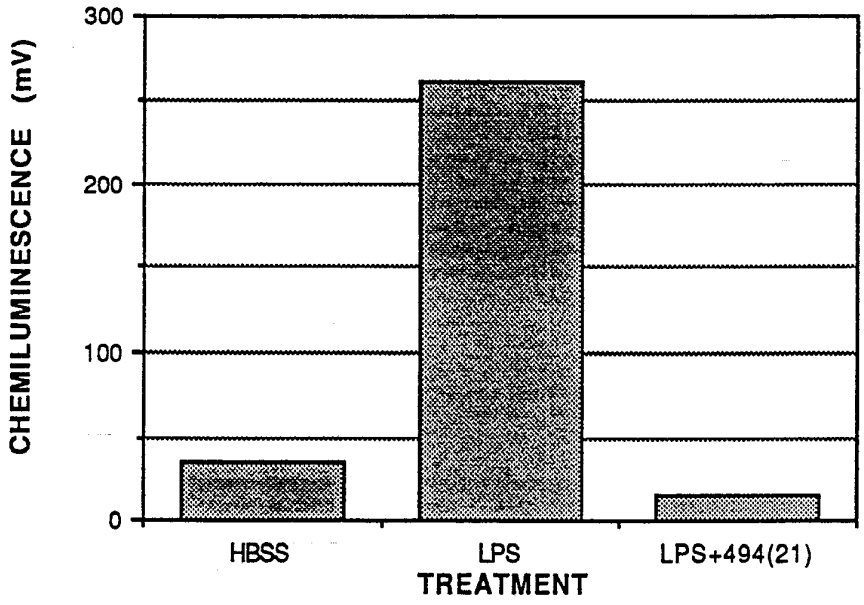

CHEMILUMINESCENCE (mV)
60
50
40
30
20
10
0
HBSS
TNF
fMLP
TNF+fMLP
2+TNF
2+TNF+fMLP
TREATMENT

FIG 20

FIG 21


CHEMILUMINESCENCE (mV)
300
200
100
0
HBSS
LPS
LPS+494(21)
TREATMENT

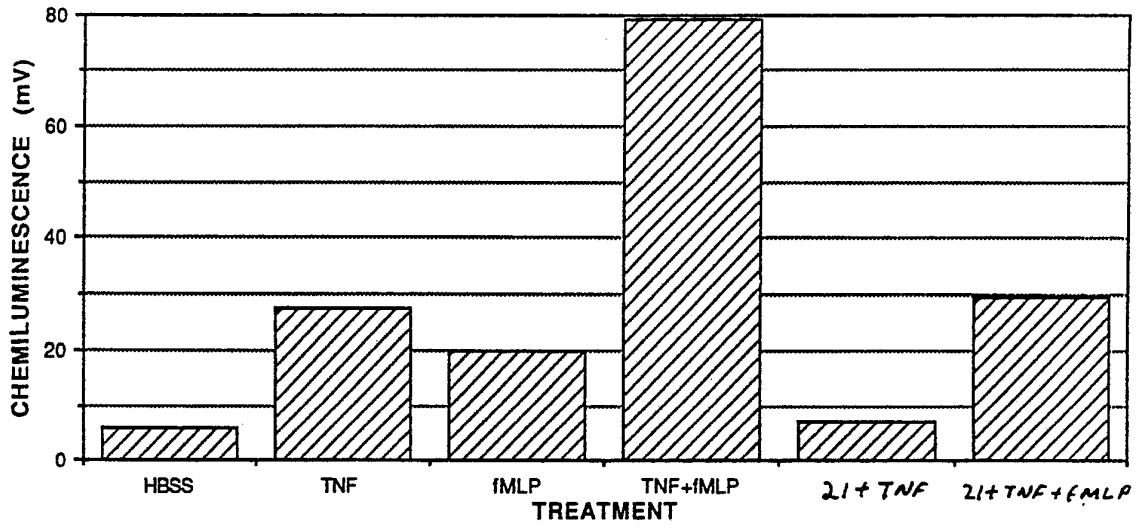

80
60
40
20
0
CHEMILUMINESCENCE (mV)
HBSS
TNF
fMLP
TNF+fMLP
2I + TNF
2I+TNF+fMLP
TREATMENT

FIG. 22

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] C07K 7/06, 7/08, 7/10, A61K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07K 7/06, 7/08, 7/10, C07C 103/52.
Chem. Abs. online	Keywords: Tumo(u)r Necrosis Factor OR TNF
CAS online registry	PROTEIN SEQUENCE SEARCH

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

AU: IPC As Above

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]
A	Derwent Abstract Accession no. 90-143138/19, Classes B04 and D16, JP,A, 02-088598 (SOMA G) 28 March 1990 (28.03.90)	1-14
A	Derwent Abstract Accession no. 91-152432/21, Classes B04 and D16, JP,A, 03-087196 (TEIJIN K K) 11 April 1991 (11.04.91)	1-14
A	Derwent Abstract Accession no. 91-145993/20, Classes B04 and D16, JP,A, 03-083587 (TEIJIN K K) 9 April 1991 (09.04.91)	1-14
A	Derwent Abstract Accession no. 91-145992/20, Classes B04 and D16, JP,A, 03-083586 (TEIJIN K K) 9 April 1991 (09.04.91)	1-14

* 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
28 August 1992 (28.08.92)	- 7 SEP 1992 (07.09.92)
International Searching Authority	Signature of Authorized Officer
AUSTRALIAN PATENT OFFICE	A BESTOW

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.