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<p>(21) International Application Number: PCT/DK98/00157          (22) International Filing Date: 15 April 1998 (15.04.98)          (30) Priority Data:              0418/97                   15 April 1997 (15.04.97)           DK              60/044.187               24 April 1997 (24.04.97)           US          (71) Applicant (for all designated States except US): FARMA-          CEUTISK LABORATORIUM FERRING A/S [DK/DK];          Indertofte 10, DK-2720 Vanløse (DK).          (72) Inventors; and          (75) Inventors/Applicants (for US only): JENSEN, Martin, Roland          [DK/DK]; Sjølskrænten 6, DK-2840 Solte (DK), MOURIT-          SEN, Søren [DK/DK]; Lindevangsvej 24, DK-3460 Birk-          erød (DK), ELSNER, Henrik [DK/DK]; Svend Gøngevej          36, DK-2700 Brønshøj (DK), DALUM, Iben [DK/DK]; Ol-          gasvej 13, DK-2970 Hørsholm (DK).          (74) Agent: HOFMAN-BANG &amp; ROUTHARD, LEHMANN &amp;          A/S; Hans Bekkevolds Allé 7, DK-2900 Hellerup (DK).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR,          BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE,          GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ,          LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW,          MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,          TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO          patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian          patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European          patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR,          IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF,          CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b>  <i>With international search report.</i></p>	
<p>(54) Title: MODIFIED TNF<math>\alpha</math> MOLECULES, DNA ENCODING SUCH MODIFIED TNF<math>\alpha</math> MOLECULES AND VACCINES COM-          PRISING SUCH MODIFIED TNF<math>\alpha</math> MOLECULES AND DNA</p>		
<p>(57) Abstract</p> <p>A modified human TNF<math>\alpha</math> molecule capable of raising neutralizing antibodies towards wild-type human TNF<math>\alpha</math> following administration of said modified TNF<math>\alpha</math> molecule to a human host, wherein at least one peptide fragment of the human TNF<math>\alpha</math> molecule has been substituted by at least one peptide known to contain an immunodominant T cell epitope or a truncated form of said molecule containing an immunodominant epitope at one or both flanking regions of the human TNF<math>\alpha</math> molecule comprising at least one TNF<math>\alpha</math> B cell epitope, wherein the substitution introduces a substantial change in the amino acid sequence of the front <math>\beta</math>-sheet, in any one of the connecting loops and/or in any one of the B', I or D strands of the back <math>\beta</math>-sheet. The modified human TNF<math>\alpha</math> molecules or DNA encoding them may be formulated as vaccines against TNF<math>\alpha</math> optionally with pharmaceutically acceptable adjuvants, for the prevention or treatment of chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel diseases, cancer, disseminated sclerosis, diabetes, psoriasis, osteoporosis or asthma. Human body fluids may be tested for the presence of TNF<math>\alpha</math> by contact with a composition containing the modified TNF<math>\alpha</math>.</p>		

Modified TNF $\alpha$  molecules, DNA encoding such modified TNF $\alpha$  molecules and vaccines comprising such modified TNF $\alpha$  molecules and DNA

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### Field of the Invention

The present invention relates to human cytokine Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ) molecules which has been modified so that they are capable of raising neutralizing antibodies towards wild-type human TNF $\alpha$  following administration of the modified TNF $\alpha$  molecule to the human host. The invention also relates to human TNF $\alpha$  vaccines based on said modified TNF $\alpha$  molecules. Further aspects of the invention will appear from the discussion below.

### 10 Background of the invention

Physiologically, the vertebrate immune system serves as a defense mechanism against invasion of the body by infectious objects such as microorganisms. Foreign proteins are effectively removed via the reticuloendothelial system by highly specific circulating antibodies, and viruses and bacteria are attacked by a complex battery of cellular and humoral mechanisms including antibodies, cytotoxic T lymphocytes (CTL), natural killer cells (NK), complement, etc. The leader of this battle is the T helper (T<sub>H</sub>) lymphocyte which, in collaboration with the Antigen Presenting Cells (APC), regulate the immune defense via a complex network of cytokines.

T<sub>H</sub> lymphocytes recognize protein antigens presented on the surface of the APC. They do not recognize, however, native antigen per se. Instead, they appear to recognize a complex ligand consisting of two components, a "processed" (fragmented) protein antigen (the so-called T cell epitope) and a Major Histocompatibility Complex class II molecule (O. Werdelin et al., Imm. Rev. 106, 181 (1988)). This recognition eventually enables the T<sub>H</sub> lymphocyte specifically to help B lymphocytes to produce specific antibodies towards the intact protein antigen (Werdelin et al., supra). A given T cell only recognizes

a certain antigen-MHC combination and will not recognize the same or another antigen presented by a gene product of another MHC allele. This phenomenon is called MHC restriction.

5 Fragments of self-proteins are also presented by the APC, but normally such fragments are ignored or not recognized by the T helper lymphocytes. This is the main reason why individuals generally do not harbour autoantibodies in their serum eventually leading to an attack on the individual's own proteins (the so-called self- or autoproteins).  
10 However, in rare cases the process goes wrong, and the immune system turns towards the individual's own components, which may lead to an autoimmune disease.

The presence of some self-proteins is inexpedient in situations where they, in elevated levels, induce disease symptoms. Thus, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) is known to be able to cause cachexia in cancer patients and patients suffering from other chronic diseases (H.N. Langstein et al. Cancer Res. 51, 2302-2306, 1991). TNF $\alpha$  also  
20 plays important roles in the inflammatory process (W.P. Arend et al. Arthritis Rheum. 33, 305-315, 1990) and neutralization of TNF $\alpha$  by the use of monoclonal antibodies has thus been demonstrated to be beneficial in patients with chronic inflammatory diseases, such as rheumatoid arthritis,  
25 Elliott et al., Lancet 1994, 344:1105-10 and Crohn's disease, van Dulleman et al., Gastroenterology 109(1):129-135(1995). There is therefore a need for a method for the induction of neutralizing antibodies against such TNF $\alpha$  proteins, and the present invention  
30 comprises a vaccine against TNF $\alpha$  which provide this property.

## TUMOUR NECROSIS FACTOR

### 1. General Background

Tumour necrosis factor (TNF) is a member of the cytokine family of regulatory proteins (see Walsh, G. and Headon, D.E., Protein Biotechnology, 1994, John Wiley & Sons Ltd. England, p. 257-267) which also include the interferons and the interleukins. Two forms of TNF are now recognized, TNF $\alpha$  and TNF $\beta$ , respectively. Although both proteins bind the same receptors and elicit broadly similar biological responses, they are distinct molecules and share less than 30% homology. The original protein termed tumour necrosis factor, referred to as TNF, is more properly termed TNF $\alpha$ ; it is also known as cachectin. TNF $\beta$  is also referred to as lymphotoxin.

TNF $\alpha$  is produced by a wide variety of cell types, most notably activated macrophages, monocytes, certain T lymphocytes and NK cells, in addition to brain and liver cells. The most potent known inducer of TNF $\alpha$  synthesis, is a complex biomolecule termed lipopolysaccharide (LPS). It contains both lipid and polysaccharide components, and is also referred to as endotoxin. Lipopolysaccharide itself is devoid of any anti-tumour activity. The serum of animals injected with lipopolysaccharide was found to contain a factor toxic to cancerous cells, and this factor, produced by specific cells in response to lipopolysaccharide, was termed tumour necrosis factor. Various other agents such as some viruses, fungi and parasites also stimulate the synthesis and release of this cytokine. Furthermore, TNF $\alpha$  may act in an autocrine manner, stimulating its own production.

Native human TNF $\alpha$  is a homotrimer, consisting of three identical polypeptide subunits tightly associated around a threefold axis of symmetry as will be further explained

below. This arrangement resembles the assembly of protein subunits in many viral capsid proteins. The individual polypeptide subunits of human TNF $\alpha$  are non-glycosylated and consist of 157 amino acids. The molecule has a molecular weight of 17300 Da and contains a single intrachain disulphide linkage. Human TNF $\alpha$  is synthesized initially as a 233 amino acid precursor molecule. Proteolytic cleavage of the -76 to -1 presequence including a signal sequence releases native TNF $\alpha$ . TNF $\alpha$  may also exist in a 26000 Da membrane-bound form. Three TNF $\alpha$  monomeric subunits associate noncovalently to form a trimer as further explained below.

TNF $\alpha$  induces its biological effects by binding specific receptors present on the surface of susceptible cells. Two distinct TNF $\alpha$  receptors have been identified. One receptor (TNF-R55) has a molecular weight of 55000 Da, whereas the second receptor (TNF-R75) has a molecular weight of about 75000 Da. These two distinct receptor types show no more than 25% sequence homology. TNF-R55 is present on a wide range of cells, whereas the distribution of the TNF-R75 receptor is more limited. Both are transmembrane glycoproteins with an extracellular binding domain, a hydrophobic transmembrane domain and an intracellular effector domain.

The exact molecular mechanisms by which TNF $\alpha$  induces its biological effects remain to be determined. Binding of TNF $\alpha$  to its receptor seems to trigger a variety of events mediated by G-proteins in addition to the activation of adenylate cyclase, phospholipase A and protein kinases. The exact biological actions induced by TNF $\alpha$  may vary from cell type to cell type. Other factors, such as the presence of additional cytokines, further modulate the observed molecular effects attributed to TNF $\alpha$  action on sensitive cells.

The TNF $\alpha$  gene has been cloned and inserted in a variety of recombinant expression systems, both bacterial and eukaryotic. The resultant availability of large quantities of purified, biologically active TNF $\alpha$  has facilitated clinical evaluation a number of diseases, most notably cancer. Many such trials, using TNF $\alpha$  either alone or in combination with interferons, yielded, however, very disappointing results. Large quantities of TNF $\alpha$  can not be administered to patients owing to its toxic - if not lethal - side-effects.

As mentioned above prolonged production of inappropriately elevated levels of TNF $\alpha$  has also been implicated in the development of cachexia, the wasting syndrome often associated with chronic parasitic or other infections, and with cancer. TNF $\alpha$  is also involved in the metastasis and growth of certain tumours as well as in induction of anaemia. Furthermore, TNF $\alpha$  is also directly involved in the development of certain chronic inflammatory disorders in humans, including rheumatoid arthritis and Crohn's disease where administration of monoclonal anti-TNF $\alpha$  antibodies has been shown to be beneficial. TNF $\alpha$  is also involved in osteoporosis and Psoriasis. In addition, it has been shown in animal models that administration of anti-TNF $\alpha$  antibodies may decrease or prevent rejection of grafted or transplanted tissues.

## 2. Structure of TNF $\alpha$

### I. Introduction

The three-dimensional structure of human tumour necrosis factor (TNF $\alpha$ ) has been solved (see "Tumor Necrosis Factors, Structure, Function and Mechanism of Action" edited by Bharat B. Aggarwal and Jan Vilcek, 1992 Marcel Dekker, Ind., New York, Chapter 5 "Crystal structure of TNF $\alpha$ ", by Jones, E.Y. Stuart, D.I. and Walker N.P.C.). The bic-

logical action of TNF $\alpha$  is dependent on its interaction with its receptors. These interactions are governed by the precise arrangement of the correctly folded tertiary structure. Thus, to understand how the TNF $\alpha$  molecule performs its biological function at the level of amino acid interactions, one must not only know the amino acid sequence, but also the three-dimensional structure.

## II. Three-dimensional structure

Biologically active TNF $\alpha$  has been shown by analytical ultracentrifugation, small angle x-ray scattering, and gel electrophoresis to be in a trimer conformation in solution, and cross-linking studies have indicated that this is the active form of the protein (Smith and Baglioni, 1987, J. Biol. Chemistry 252, 6951-6954). Binding assays showed that the trimer was at least 8-fold more active than the monomer. The experimental evidence indicated that both natural and recombinant TNF $\alpha$  exist predominantly as a trimer under physiological conditions. Analysis of circular dichroism spectra placed TNF $\alpha$  in the all-sheet class of proteins (Davis et al., Biochemistry 1987, 26, 1322-1326 who reported results of structure analyses of purified recombinant TNF $\alpha$  by sulfhydryl titration, gelfiltration and circular dichroism.). Several different crystal forms have been reported for human recombinant TNF $\alpha$ . All the reported crystal forms exhibit crystallographic and/or non-crystallographic threefold symmetry indicative of the presence of TNF $\alpha$  as a trimer within the crystal. The TNF $\alpha$  trimers lie in loosely packed arrays perforated by 100 Å diameter solvent channels. Only a small proportion of the molecular surface is involved in crystal packing contacts. Such contacts could slightly perturb a few side chains and perhaps even short portions of inherently flexible main chain from their preferred solution conformations.

### A. Main-Chain Fold of the TNF $\alpha$ Monomer

The overall shape of a single 157-amino-acid subunit of the TNF $\alpha$  trimer is wedgelike with a height of approximately 55Å and a maximum breadth of 35Å near the base.

5 The main-chain topology is illustrated in Fig. 1a-c; it is essentially a  $\beta$ -sandwich structure formed by two antiparallel  $\beta$ -pleated sheets. The main-chainfold conforms to that of the classic jellyroll motif (Fig. 1c) (common in viral capsid protein). The nomenclature adopted in Fig. 1

10 for the labels of the secondary structural units follows the established convention for viral structures. The standard eight  $\beta$ -strands (B to I) are all present but with an insertion between B and C that adds a short strand onto the edge of both  $\beta$ -sheets and truncates the

15 N-terminal half of C, so that each  $\beta$ -pleated sheet contains five antiparallel  $\beta$ -strands, the back  $\beta$ -sheet comprising  $\beta$ -strands B', B, I, D and G and the front sheet comprising  $\beta$ -strands C', C, H, E and F.

The N terminus is highly flexible. This region, as far as

20 residue 10 (see fig. 1b), is rather independent of the rest of the molecule, with the first few residues free to sample a variety of conformations in the solvent. In contrast, the C terminus is embedded in the base of the back  $\beta$ -sheet and forms an integral part of this relatively

25 flat secondary structural unit. The gradation in  $\beta$ -strand lengths and the insertion between  $\beta$ -strands B and C conspire to produce a front surface formed almost entirely of loops, and it is this "masked" side of the  $\beta$ -sandwich, which in the trimer is presented to the solvent. The

30 crystallographic data yield a measure of the relative flexibility of the various parts of the structure. The  $\beta$ -strands form a fairly inflexible scaffold; in particular, the back  $\beta$ -sheet is situated at the core of the trimer and consequently is particularly rigid. As would be ex-



pected, it is the loops that adorn the outer solvent-accessible surface of the molecule, which exhibit high levels of flexibility/mobility. Overall, there is a general decrease in rigidity as the core becomes more  
5 loosely packed in the upper half of the molecule.

#### B. General Topology of the TNF $\alpha$ Trimer

Three TNF $\alpha$  monomeric subunits associate noncovalently to form a compact, conical trimer having a length of about 55 Å and a maximum breadth of 50 Å. The  $\beta$ -strands of the  
10 three individual  $\beta$ -sandwiches lie approximately parallel (the tilt is about 30°) to the threefold axis of the trimer. The interaction between subunits related by the three-fold axis is through a simple edge-to-face packing of the  $\beta$ -sandwich; the edge of the  $\beta$ -sandwich, consisting  
15 of strands F and G from one subunit, lies across the back  $\beta$ -sheet [GDIBB'] of a threefold related subunit (see Fig. 2). The carboxy termini lie close to the threefold axis. The edge-to-face mode of packing produces an extremely tight association between the subunits. Thus the core of  
20 the trimer is completely inaccessible to solvent.

#### C. Amino Acid-Type Distribution

The overall distribution of residue types in the three-dimensional structure of TNF $\alpha$  echoes the general rule for proteins: namely, that hydrophobic residues cluster in  
25 the core of the molecule while charged residues decorate the surface. Thus the core of the TNF $\alpha$  sandwich has the expected filling of tightly intercalating large apolar residues.

The energetics of the system do not favour the existence  
30 of TNF $\alpha$  in a monomeric state. For a large interface area composed of complementary residues (e.g., polar residues matched against polar residues) the loss of solvent-

accessible surface area confers a considerable energetic advantage to formation of the oligomer (i.e., the trimer). The exposure to solvent of the large patch of strongly hydrophobic residues normally buried in the lower portion of the trimeric interface would also act to destabilize the TNF $\alpha$  monomer.

### 3. Probes of structure-function

#### A. TNF $\alpha$ /Antibody Interactions

It has been observed that antibodies raised against TNF $\alpha$  from one species (e.g., human) do not cross-react with TNF $\alpha$  from another species (e.g., mouse) despite a sequence identity in excess of 80% and the ability of TNF $\alpha$  to bind to the TNF $\alpha$  receptors of other species. If the degree of sequence variation is mapped onto the three-dimensional structure, it is immediately apparent that the most sequence-variable regions of the molecule correspond to the antibody-accessible surface loops. The regions of highly conserved residues within the sandwich or at the trimeric interface are effectively invisible to antibodies. Thus the epitope for an antibody against TNF $\alpha$  will always contain some residues that will vary between species, thus abolishing antibody binding. This implies that the characteristics of the interaction between TNF $\alpha$  and its receptor must somehow differ from those required for binding of an antibody to TNF $\alpha$ .

#### B. Site-Directed Mutagenesis

The role of various specific residues and regions of the TNF $\alpha$  molecule with regard to its biological (cytotoxic) activity and receptor binding has been probed by replacement of those residues by different amino acids or deletion of part of the sequence using the techniques of

site-directed mutagenesis (Jones et al, op.cit. p. 113-119).

The deletion of up to eight residues from the N-terminus without any deleterious effect on biological activity serves to emphasize the nonessential nature of this region for overall molecular stability. N-terminal residues appear to exert an indirect, second-order effect on the biological efficacy of the TNF $\alpha$  trimer.

Non-conservative substitutions of the normally highly conserved residues which form the tightly packed core of the  $\beta$ -sandwich distort the structure and hence abrogate the biological activity of TNF $\alpha$  (Yamagishi et al., Protein Engineering, Vol. 3, No. 8, p. 713-719 (1989)). Many such mutated proteins (muteins) fail to form a stable, correctly folded molecule. Some conservative substitutions are permitted within the hydrophobic patch at the bottom of the threefold axis; however, there appears to be much greater leeway in the more loosely packed region near the top of the trimer. In particular, Cys 69 and Cys 101, which form the disulphide bridge between two connecting loops at the loosely packed top of the molecule, are relatively insensitive to changes (see Fig. 1a). Generally, however, in order to retain some biological activity of TNF $\alpha$  the mutations near the central axis of TNF $\alpha$  must be highly conservative, preserving the overall shape of TNF $\alpha$ .

The residues on the surface of the molecule have a considerably greater freedom to mutate without incurring disastrous structural penalties as witnessed by the proliferation of variations of residues in this category between species. Thus drastic reductions in biological activity of TNF $\alpha$  due to substitutions in this area points to the direct involvement of such residues in the func-

tional interaction of the TNF $\alpha$  trimer with its receptor. Residues comprising Arg 31, Arg 32 and Ala 33 situated in the connecting loop between the B and B' strand of the back  $\beta$  sheet, Ser 86 and Tyr 87 situated in the connect-  
5 ing loop between the E and F strands of the front  $\beta$ -sheet, and Glu 146 situated in the connecting loop between the H strand of the front  $\beta$  sheet and the I-strand of the back  $\beta$ -sheet appear to be such amino acid residues (see Fig.3). They appear to fall into two distinct  
10 "hotspot" regions on the front and the back sides of the TNF $\alpha$  monomer. The distribution of all deleterious mutations regardless of structural category further reinforces this picture. The existence of these "hot spots" for sensitivity of biological function to mutation has  
15 been reviewed by Yamagishi et al., op.cit., and Goh et al. Protein Engineering, Vol. 4, No. 4, p. 385-389 (1991)

Studies on the mutation of human TNF $\alpha$  polypeptides have been reported in a number of patent applications.

20 Thus, Yamagishi et al. (EP 251 037) discloses numerous site-specific mutated TNF $\alpha$  molecules which are soluble and have the cytotoxic and antitumour activity characteristic for human TNF $\alpha$  in vitro and/or in vivo.

Other muteins with TNF $\alpha$  activity are described in WO  
25 90/07579. Muteins with higher binding affinity for the human p75-TNF receptor than for human p55-TNF receptor are described in EP-A-619 372.

None of these citations, which are incorporated herein by  
reference discloses a modification of the TNF $\alpha$ -molecule  
30 in order to abrogate the biological activity of TNF $\alpha$  and provide the ability to induce antibodies against wild-type human TNF $\alpha$ .

However, they provide useful background information with regard to TNF $\alpha$  and its biological effect. Also production and expression of TNF $\alpha$ -analogs and their formulation are disclosed as are receptor-binding assays.

5 4. Summary

A rich variety of data may now be brought to bear on the specific relationship of structure to function for TNF $\alpha$ . All available evidence points to the importance of the trimer as the stable natural unit. It is apparent that  
10 the two hotspot regions situated on separate sides of the TNF $\alpha$  monomer are brought close to each other in terms of neighbouring subunits in the trimer. Thus a region of functional importance consisting of residues 31 to 35, 84 to 87, and 143 to 148 appears to be located at the inter-  
15 face between two subunits on the lower half of the trimer. Yamagishi et al. op.cit report loss of receptor binding ability as well as cytotoxicity for the mutation of Asp 143 to Tyr, and Tsujimoto et al., *J. Biochem.* 101, p. 919-925 (1987) report a similar effect for Arg 31 and  
20 Arg 32 to Asn and Thr. Thus the site may be associated directly with receptor binding as well as cytotoxicity. It is interesting that the receptor binding region of TNF $\alpha$  appears to lie at the interface between two subunits.

25 In summary, the detailed three-dimensional structure for TNF $\alpha$  serves to explain a wide range of observations on antibody binding, oligomerization, and site-directed mutagenesis. When the structure is considered in combination with recent, extensive site-directed mutants, a re-  
30 gion of biological importance with regard to receptor binding is apparently at the subunits on the lower half of the trimer.

Description of the prior art

In WO 95/05849, some of the present inventors disclose a method for the modification of self-proteins so as to induce antibody response against the unmodified self-protein, wherein a self-protein analog is provided by molecular biological means. More specifically one or more peptide fragments of the self-protein are substituted by one or more peptide fragments containing immunodominant, foreign T-cell epitopes.

Before the invention which lead to WO 95/05849, it was known to conjugate peptides or proteins, including self-proteins, with carrier proteins comprising a T-cell epitope. WO 92/19746 discloses a recombinant polypeptide comprising the LHRH sequence, one or more T-cell epitopes and a purification site, which is useful in animal immunocastration vaccines.

It is stated as preferable in WO 95/05849 that the immunodominant T-cell epitope is inserted so that flanking regions from the original protein comprising at least 4 amino acids are preserved on either side of the inserted epitope. In other words the epitope should not be conjugated with the self-protein as a fusion protein. Preferably, the substitution should be made so as to essentially preserve the tertiary structure of the original protein. Apart from that no specific guidance is provided as to the optimal intramolecular position of the inserted epitope in order to create the most powerful antibody response against the unmodified self-protein. Presumably, this will vary from self-protein to self-protein but based on the general guidance in the specification the most appropriate position(s) can be determined without undue experimentation by selecting peptides comprising appropriate immunodominant epitopes, exchanging peptide

sequences of essentially the same length in various parts of the self-protein molecule and determining the raised antibody response by suitable assay techniques.

It is probably very difficult by using current modelling  
5 tools, to predict whether substitution of one or more  
peptide fragments in a protein results in a change in the  
tertiary structure of the original protein. Therefore, in  
a drug development program's search for lead compounds,  
it must be considered as a standard screening procedure  
10 early in the development phase to evaluate which modified  
self-proteins have preserved the tertiary structure of  
the original protein. This can be done using several ex-  
perimental techniques as have been described in numerous  
text books on protein characterization, e.g. fluorescence  
15 spectroscopy, near-UV circular dichroism, Fourier trans-  
formed infrared spectroscopy and multidimensional NMR  
techniques ("Physical Methods to Characterize Pharmaceu-  
tical Proteins", Pharmaceutical Biotechnology Vol. 7.  
Eds. J.H. Herron, W. Jiskoot & D.J.A. Crommelin, Plenum  
20 Press, New York (1995)). Ideally, in a screening process  
for lead compounds, two or more of the experimental tech-  
niques mentioned above should be combined in order to  
evaluate whether a change in the tertiary structure has  
occurred or not.

Brief description of the drawings

- Figure 1(a) illustrates the crystal structure of native TNF $\alpha$  monomer. The figure is a diagrammatic sketch of the subunit fold,  $\beta$  strands are shown as thick arrows in the amino-to-carboxy direction and connecting loops are depicted as thin lines. The disulfide bridge is denoted by a lightning flash and a region of high flexibility is crosshatched. The trimer threefold axis would be vertical for this orientation.
- 5
- 10 Figure 1(b) is a C $\alpha$  chain trace of the TNF $\alpha$  monomer crystal structure. This detailed representation should be used in conjunction with Figure 1(a) to give the precise alignment of the amino acid sequence with the clearer but stylized representation of the subunit fold.
- 15 Figure 1(c) shows the TNF $\alpha$  structure as a jelly roll motif. The insertion between  $\beta$  strands B and C is shown in dashed lines; the connection between B and C would run straight across at the top of the molecule.
- Figure 2 shows the edge-to-face packing of  $\beta$  sandwiches in the TNF trimer. The view, down the threefold axis, shows a narrow slab of the trimer with  $\beta$  strands represented by ribbons running into and out of the page.
- 20
- Figure 3(a) illustrates the DNA sequence encoding tumor necrosis factor (TNF $\alpha$ ) having the amino acid sequence shown in Fig. 3(b)
- 25
- The DNA sequence is available from Gen Bank under accession no. M10988, SEQ ID NO:339737.
- The sequence has been described by Wang et al., Science 228, 149-154 (1985). The sequence includes codons encoding the -76--1 presequence of human TNF $\alpha$ .
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The complete gene sequence including introns has been described by Nedwin et al., *Nucleic Acids, Res.* 13(17) 6361-6373 (1985), Shirai et al., *Nature* 313(6005), 803-806 (1985) and Dennica et al., *Nature* 312 (5996), 724-729  
5 (1984).

Figure 3(b) shows the amino acid sequence of human TNF $\alpha$  including the -76--1 presequence.

Figure 4(a) schematically illustrates the substitutions of immunodominant epitopes P2 and P30 in a wild type TNF $\alpha$  (WT) to form the TNF $\alpha$  analogs TNF2-1 to 2-7 and TNF30-1  
10 to 30-5.

Figure 4(b) shows the exact locations of the substitutions in the WT sequence for the individual TNF $\alpha$  analogs.

Figures 5(a) and 5(b) show the structure of the analogs based on Fig. 1(a), where the individual substitutions by P2 and P30 in the strands of the  $\beta$ -sheets and the connection loops, respectively, are marked in black.  
15

Figure 6 shows the biological activity of the TNF $\alpha$  analogs in the L929 assay (Meager, A., Leung, H., & Woolley, J. *Assays for Tumour Necrosis Factor and related cytokines.*, *J. Immunol. Meth.* 116, 1-17 (1989)) compared with recombinant TNF $\alpha$ .  
20

Figure 7 shows the anti-human TNF $\alpha$  antibody response in rabbits to vaccination with the modified TNF $\alpha$  molecules in rabbits.  
25

Figure 8 shows the ability of P2/P30 modified human TNF $\alpha$  molecules to induce neutralizing antibodies as measured  
30 in the L929 cell assay.

Figure 9 shows the ability - when administered to rabbits - of P2/P30 modified human TNF $\alpha$  molecules to induce neutralizing antibodies as measured in receptor assay.

Figure 10 shows the Peripheral Blood Mononuclear Cell (PBMC) response in three donors towards TT and the P2 and P30 peptides.

Figure 11 shows the polyclonal proliferation response in two donors using the different P2 and P30 modified TNF $\alpha$  molecules.

Figure 12 shows the Proliferation Indexes (PIs) calculated from 34 experiments for the P2 and P30 modified TNF $\alpha$  molecules.

Figure 13 shows the PBMC response against P2 and P30 modified TNF $\alpha$  proteins in P2 and P30 specific responders, respectively.

Figure 14 shows a similar PBMC response in two other donors.

Figure 15 shows the influence of flanking amino acids on the T cell recognition of P2 and P30.

Figure 16 shows the mutation strategy used for the preparation of the modified TNF $\alpha$  molecules.

#### Summary of the invention

The purpose of the present invention is to provide guidelines as to how a particular self-protein within the general scope of the above-mentioned WO 95/05849, viz. human TNF $\alpha$  should be modified in order to be biologically inactive as well as be able to induce a strong neutralizing antibody response towards wild-type, biologically active TNF $\alpha$ . In the present context "biologically inactive" re-

fers to the activities of the wild-type TNF $\alpha$ , mainly its cytotoxic activity.

From the discussion of the tertiary structure of TNF $\alpha$  given above, it is recalled that the biologically active TNF $\alpha$  is a trimer of three subunits. Due to the "edge-to-face" packing the "back  $\beta$ -sheet" represents the "hidden" area of contact between the subunits which is completely inaccessible to solvent. Significant substitutions in this area will almost inevitably deprive the TNF $\alpha$  molecule of all biological activity. On the contrary the "front  $\beta$ -sheet" and the connecting regions provide the accessible surface area which includes the areas interacting with the TNF $\alpha$  receptors. Antibodies towards these areas would therefore probably be able to interfere with receptor binding and would hence possess TNF $\alpha$  neutralizing properties.

A person skilled in the art who wanted to construct a detoxified and yet immunogenic TNF $\alpha$  molecule according to WO 95/05849 would therefore as the first choice insert the immunodominant T cell epitope in the back  $\beta$ -sheet of the TNF $\alpha$  monomer. Modifications of this area would thus most probably interrupt the biological activity of TNF $\alpha$  and leave the receptor-accessible front  $\beta$ -sheet free for interaction with antibodies. This is also consistent with the discussion of the site-directed mutagenesis in the tightly packed core of the  $\beta$ -sandwich discussed above.

However, surprisingly it is not so. As it will appear from the test results below, the result was quite the contrary, since substitutions involving the B and G strands of the back  $\beta$ -sheet surprisingly provided TNF $\alpha$  analogs which were unable to induce neutralizing antibodies against TNF $\alpha$ .

Thus, the present invention seeks to provide a modified human TNF $\alpha$  molecule capable of raising neutralizing antibodies towards wild-type human TNF $\alpha$  following administration of said modified TNF $\alpha$  molecule to a human host, wherein at least one peptide fragment of the human TNF $\alpha$  molecule has been substituted by at least one peptide known to contain an immunodominant T cell epitope or a truncated form of said molecule containing an immunodominant epitope and one or both flanking regions of the human TNF $\alpha$ -molecule comprising at least one TNF $\alpha$  B cell epitope, wherein the substitution introduces a substantial change in the amino acid sequence of the front  $\beta$ -sheet, in any one of the connecting loops and/or in any one of the B', I or D strands of the back  $\beta$ -sheet. By inference, substitution in the B and G strands of the back  $\beta$ -sheet should be avoided.

Within the present context, "substantial change" is intended to mean a change which goes beyond a mere conservative substitution of the individual amino acids, and beyond the point mutations which do not alter the secondary and/or tertiary structure of the wild-type TNF $\alpha$ . In other words, the T cell epitope introduced in the TNF $\alpha$  sequence should preferably introduce a sequence which is of very low homology with the wild-type TNF $\alpha$  sequence.

In accordance with the present invention is also provided, a modified human TNF $\alpha$  molecule capable of raising neutralizing antibodies towards wild-type human TNF $\alpha$  following administration of said modified TNF $\alpha$  molecule to a human host, wherein at least one peptide fragment of the human TNF $\alpha$  molecule has been substituted by at least one peptide known to contain an immunodominant T cell epitope or a truncated form of said molecule containing an immunodominant epitope and one or both flanking regions of the human TNF $\alpha$ -molecule comprising at least one TNF $\alpha$  B

cell epitope, wherein said modified TNF $\alpha$  molecule is substantially free from TNF $\alpha$  activity.

The term "substantially free from TNF $\alpha$  activity" is in the present context intended to mean that the administration to a human being of such a modified TNF $\alpha$  molecule will not result in significant adverse effects due to the known cytokine effects exerted by native TNF $\alpha$ . In other words, the modified TNF $\alpha$  molecules of the invention are pharmaceutically acceptable substances.

10 In order to test whether the modified human TNF $\alpha$  molecule according to the invention is substantially free from TNF $\alpha$  activity, the L929 bioassay as defined herein can be applied. Furthermore, in order to confirm the immunogenic character of the modified TNF $\alpha$  molecules, antibodies  
15 raised against the modified TNF $\alpha$  molecule in a suitable host will significantly inhibit the activity of wild-type TNF $\alpha$  in the L929 bioassay as defined herein, and/or said antibodies will significantly inhibit the binding of wild-type TNF $\alpha$  to the 55 kD TNF $\alpha$  receptor 1 (TNF $\alpha$ -R55) or  
20 the to the 75 kD TNF $\alpha$  receptor (TNF $\alpha$ -R75).

These suitable hosts can for instance be a primate such as a Rhesus or Cynomuleus monkey, a rodent such as a rat or mouse or guinea pig, or a lagomorph such as a rabbit.

The assays suitable for evaluating the potential of the modified molecules according to the invention are carried  
25 out using antibody or antiserum in a concentration relative to the assay setup and ought to reflect physiological conditions with respect to the involved reactants, or it ought to be possible to extrapolate to physiological  
30 conditions from the results obtained from the assay. In other words, the results from the assay must indicate that a physiological concentration of antibodies against TNF $\alpha$  in vivo is able to reduce the TNF $\alpha$  activity to an

extent of at least 10%, 15%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100%. The person skilled in the art will readily know how to determine such conditions.

5 It is to be understood that a "significant inhibition of the wild-type TNF $\alpha$ " may suitably be such which results in reduction or elimination of the adverse effects of wild-type TNF $\alpha$ . Such inhibition may suitably be at least 10%, at least 15%, at least 25%, at least 30%, at least 35%,  
10 at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or even 100%.

On this background the modified human TNF $\alpha$  molecules according to the present invention are characterized in  
15 that the substitution has been made in regions of the TNF $\alpha$  molecule, which involves the strands of the front  $\beta$ -sheets and/or the connecting loops so as essentially preserve the  $\beta$ -sheet structure of any of the strands of the  
20 back  $\beta$ -sheet.

Although it has not yet been fully verified experimentally, it must be assumed that this property is common to the strands forming the back  $\beta$ -sheet, so that preferably the substitutions should be made in regions of the TNF $\alpha$   
25 molecule which do not comprise any complete strand of the back  $\beta$ -sheet. In view of the discussion above of the functional importance of the residues 31-35 it can be assumed that the connecting loops between the individual strands of the back  $\beta$ -sheet should preferably also be  
30 avoided.

However, it is permissible that the substitution is made in regions of the TNF $\alpha$  molecule which only involve a segment of the D strand of the back  $\beta$ -sheet.

According to a preferred embodiment of the invention the substitution comprises at least a segment of the H strand of the front  $\beta$ -sheet and of the connecting loop to the I strand of the back  $\beta$ -sheet, preferably amino acids 132 to 146. According to another embodiment of the invention the substitution comprises segments of the H and I strands and the entire connecting loop, preferably amino acids 132 to 152. According to yet another presently preferred embodiment of the invention the substitution comprises a segment of the D strand of the back  $\beta$ -sheet, at least a segment of the E strand of the front  $\beta$ -sheet and the entire connecting loop, preferably amino acids 65 to 79 or 64 to 84.

According to a further embodiment of the invention the substitution comprises the entire C' and C strands of the front  $\beta$ -sheet and a segment of the D strand of the back  $\beta$ -sheet, preferably amino acids 40 to 60.

According to a still further embodiment of the invention the substitution comprises at least a segment of the E strand of the front  $\beta$ -sheet and of one or both of the connecting loops, preferably amino acids 76 to 90.

The inserted T cell epitope should preferably be promiscuous and known to be immunogenic in a majority of human HLA class II types. Applicable epitopes can be derived e.g. from Tetanus toxoid, preferably epitope P2 and/or P30, Panina-Bordignon et al., Eur. J. Immunol. 19:2237-42, 1989. Also epitopes derived from diphtheria toxoid may be used.

The preferred modified human TNF $\alpha$  molecules (TNF $\alpha$  analogs) as referred to above with reference to the location of the substitution are shown in the enclosed sequence listing as SEQ ID NO:8 and SEQ ID NO:16.

Other applicable TNF $\alpha$  analogs are listed as SEQ ID NO:4, 10, 14 and 16.

The invention also relates to truncated analogs of the above-mentioned modified TNF $\alpha$  analogs according to the present invention. Thus, truncated analogs of TNF $\alpha$  molecules containing a promiscuous and immunodominant T cell epitope and one or both flanking regions comprising at least one TNF $\alpha$  B cell epitope, preferably comprising at least five amino acids, would also constitute a possible active ingredient in a TNF $\alpha$  vaccine according to the invention. The T cell epitope would induce the proliferation of T cells when presented to MHC class II molecules by the APC, while the B cell epitope would potentially be recognized by the immunoglobulin receptors on B cells, and would subsequently be presented by MHC class I molecules on these cells. This constitutes the basis for raising an immune response towards wild-type TNF $\alpha$ , harbouring the B cell epitope, according to WO 95/05849 and the present invention.

B cell epitopes could be identified in the TNF $\alpha$  both theoretically and experimentally. Algorithms for identification of potential linear B cell epitopes have been published, and this would form the basis of an experimentally based investigation of the nature of these potential epitopes. Antibodies raised in a heterologous system (e.g. rabbits) in response to injections of such truncated TNF $\alpha$  molecules comprising T cell epitopes could be analyzed for *in vitro* capability to bind native human TNF $\alpha$ , preferentially in neutralizing manner. Panels of monoclonal antibodies known to be neutralizing could be screened *in vitro* for capability to bind the potential B cell epitopes of TNF $\alpha$ . Both of these are strategies for identifying the possible and best B cell epitopes.



The above-mentioned TNF $\alpha$ -analogs are believed to remain in monomer form, since the modification probably destroys the inherent trimerization tendency of the wild-type TNF $\alpha$ .

- 5 However, the invention further relates to dimers, oligomers, especially trimers or multimers of the claimed modified TNF $\alpha$  molecules provided they are devoid of TNF $\alpha$ -activity and also isolated DNA molecules that code for the claimed modified TNF $\alpha$  molecules.
- 10 The isolated DNA molecules encoding the preferred TNF $\alpha$  analogs have the sequences listed as SEQ ID NO:7 and 15, and the DNA molecules encoding the other applicable analogs are listed as SEQ ID NO:3, 9, 13 and 15.

15 The invention further comprises vectors comprising the isolated DNA molecules encoding the analogs and expression vectors comprising said DNA molecules operatively linked to a suitable expression control sequence.

Another aspect of the invention is a host transformed with an expression vector for said analog.

- 20 Said host may be any of the hosts commonly used for expression, e.g. a strain of bacteria, yeast or fungi or insect, mammalian or avian cell lines.

25 The invention further relates to a method of producing the claimed TNF $\alpha$  analogs, whereby host cells transformed with an expression vector for the analog is grown under suitable conditions permitting production of the analog, and the produced analog is recovered.

30 If desired, the modified TNF $\alpha$  molecules according to the invention may be expressed as or form part of a fusion protein with a suitable adjuvant molecule, preferably an

immunologically active adjuvant, such as GM-CSF, HSP70 or an interleukin, e.g. interleukin 2.

More specifically, the modified TNF $\alpha$  molecules are produced by substituting the appropriate gene segments encoding peptide containing or constituting immunodominant T cell epitopes into the gene encoding the native human TNF $\alpha$  molecule. Subsequently the modified TNF $\alpha$  gene is expressed in an appropriate eukaryotic or prokaryotic expression vector. The expressed modified TNF $\alpha$  molecules are purified and refolded as described below.

Although it may be preferred to insert the whole T cell epitope, it may in some cases be advantageous to insert a peptide sequence comprising both the epitope as well as flanking regions from the protein from which the epitope is derived.

According to the invention the modified human TNF $\alpha$  molecules may be used in vaccines against TNF $\alpha$ . Strategies in formulation development of vaccines based on purified proteins, such as modulated self-proteins, generally correspond to formulation strategies for any other protein-based drug product. Potential problems and the guidance required to overcome these problems - as for instance preservation of tertiary structure - are dealt with in several textbooks, e.g. "Therapeutic Peptides and Protein Formulation. Processing and Delivery Systems" Ed. A.K. Banga; Technomic Publishing AG, Basel 1995. The use of an adjuvant, e.g., aluminium hydroxide, aluminium phosphate (Adju-Phos), calcium phosphate, muramyl dipeptide analog, or some of the more recent developments in vaccine adjuvants such as biodegradable microparticles and Iscom's is a formulation challenge familiar to a pharmaceutical scientist working in this area.

Preparation of the vaccines according to the invention which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 5 4,596,792; and 4,578,770. all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The 10 preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, 15 and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

20

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for 25 example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5 to 10, preferably 30 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, 35 tablets, pills, capsules, sustained release formu-

lations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired which in turn depends on the level of TNF $\alpha$  in the patient. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1  $\mu$ g to 1000  $\mu$ g, such as in the range from about 1  $\mu$ g to 300  $\mu$ g, and especially in the range from about 10  $\mu$ g to 50  $\mu$ g. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral appli-

cation on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and, to a lesser degree, the size of the person to be vaccinated.

Some of the modified TNF $\alpha$  molecules according to the present invention are sufficiently immunogenic in a vaccine, but for some of the others the immune response will be enhanced if the vaccine further comprises an adjuvant substance.

Various methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70 $^{\circ}$ C to 101 $^{\circ}$ C for 30 second to 2 minute periods respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. According to the invention DDA (dimethyldioctadecylammonium bromide) is an interesting candidate for an adjuvant, but also QuilA and RIBI are interesting possibilities. Further possibilities are monophosphoryl lipid A (MPL), and muramyl dipeptide (MDP). Other suitable adjuvants are aluminium hydroxide, aluminium phosphate (Adju-Phos), calcium phosphate, muramyl dipeptide analog. Some of the more recent devel-

opments in vaccine adjuvants such as biodegradable microparticles and Iscom's may also suitably be used.

Another highly interesting (and thus, preferred) possibility of achieving adjuvant effect is to employ the technique described in Gosselin *et al.*, 1992 (which is hereby incorporated by reference herein). In brief, the presentation of a relevant antigen such as a modified TNF $\alpha$  molecule of the present invention can be enhanced by conjugating such antigen to antibodies (or antigen binding antibody fragments) against the Fc $\gamma$  receptors on monocytes/macrophages. Especially conjugates between antigen and anti-Fc $\gamma$ RI have been demonstrated to enhance immunogenicity for the purposes of vaccination.

Other possibilities involve the use of immune modulating substances such as lymphokines (e.g. IFN- $\gamma$ , IL-2 and IL-12) or synthetic IFN- $\gamma$  inducers such as poly I:C in combination with the above-mentioned adjuvants.

In many instances, it will be necessary to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain the desired levels of protective immunity.

One reason for admixing the polypeptides of the invention with an adjuvant is to effectively activate a cellular immune response. However, this effect can also be achieved in other ways, for instance by expressing the effective antigen in a vaccine in a non-pathogenic microorganism. A well-known example of such a microorganism is

*Mycobacterium bovis* BCG.

It is preferred that the non-pathogenic microorganism is a bacterium, e.g. selected from the group consisting of  
5 the genera *Mycobacterium*, *Salmonella*, *Pseudomonas* and *Escherichia*. It is especially preferred that the non-pathogenic microorganism is *Mycobacterium bovis*.

The incorporation of one or more copies of a nucleotide  
10 sequence encoding the molecule according to the invention in a mycobacterium from a *M. bovis* BCG strain will enhance the immunogenic effect of the BCG strain. The incorporation of more than one copy of a nucleotide sequence of the invention is contemplated to enhance the  
15 immune response even more, and consequently an aspect of the invention is a vaccine wherein at least 2 copies of a DNA sequence encoding a molecule is incorporated in the genome of the microorganism, such as at least 5 copies. The copies of DNA sequences may either be identical en-  
20 coding identical molecule or be variants of the same DNA sequence encoding identical or homologues of a polypeptide, or in another embodiment be different DNA sequences encoding different polypeptides where at least one of the polypeptides is according to the present invention.

25 The living vaccine of the invention can be prepared by cultivating a transformed non-pathogenic cell according to the invention, and transferring these cells to a medium for a vaccine, and optionally adding a carrier, vehicle and/or adjuvant substance.  
30

Apart from their use as starting points for the synthesis of molecule of the invention and for hybridization probes (useful for direct hybridization assays or as primers in  
35 e.g. PCR or other molecular amplification methods) the nucleic acid fragments of the invention may be used for

effecting *in vivo* expression of antigens, i.e. the nucleic acid fragments may be used in so-called DNA vaccines. Recent research have revealed that a DNA fragment cloned in a vector which is non-replicative in eukaryotic cells may be introduced into an animal (including a human being) by e.g. intramuscular injection or percutaneous administration (the so-called "gene gun" approach). The DNA is taken up by e.g. muscle cells and the gene of interest is expressed by a promoter which is functioning in eukaryotes, e.g. a viral promoter, and the gene product thereafter stimulates the immune system. These newly discovered methods are reviewed in Ulmer et al., 1993, which hereby is included by reference.

The efficacy of such a "DNA vaccine" can possibly be enhanced by administering the gene encoding the expression product together with a DNA fragment encoding a polypeptide which has the capability of modulating an immune response. For instance, a gene encoding lymphokine precursors or lymphokines (e.g. IFN- $\gamma$ , IL-2, or IL-12) could be administered together with the gene encoding the immunogenic protein, either by administering two separate DNA fragments or by administering both DNA fragments included in the same vector.

The vaccines may be used for preventing/treating any of the diseases described above, the pathophysiology of which is characterised by TNF $\alpha$  release, in particular chronic inflammatory diseases. As examples can be mentioned rheumatoid arthritis and inflammatory bowel diseases (IBD). The latter includes ulcerative colitis and Crohn's disease, in particular Crohn's colitis. Other examples are cancer, cachexia, often related to cancer, disseminated sclerosis, diabetes, psoriasis, osteoporosis and asthma. Cancers, which can preferably be treated or prevented according to the invention can be histogeneti-



cally classified as malignant epithelial tumours, including carcinomas and adenocarcinomas, and as malignant non-epithelial tumours, including liposarcomas, fibrosarcomas, chondrosarcomas, osteosarcomas, leiomyosarcomas, rhabdomyosarcomas, gliomas, neuroblastomas, medulloblastomas, malignant melanoma, malignant meningioma, various leukemias, various myeloproliferative disorders, various lymphomas (Hodgkin's lymphoma and non-Hodgkin lymphoma), haemangiosarcoma, Kaposi's sarcoma, lymphangiosarcoma, malignant teratoma, dysgerminoma, seminoma, and chorionic carcinoma.

Carcinomas and adenocarcinomas are the most abundant (accounting for approximately 90% of deaths from cancer) and are therefore interesting target diseases to treat/prevent according to the invention. The most important carcinomas and adenocarcinomas are those of the airways (especially of bronchial origin), of the breast, of the colorectum and of the stomach. However, also carcinomas and adenocarcinomas of the prostate, the ovary, of the lymphoid tissue and bone marrow, of the uterus, of the pancreas, of the esophagus, the urinary bladder, and the kidney cause a significant number of deaths and are therefore of interest.

Preferably the vaccines will be given as a preventive treatment, but in view of the chronic nature of these diseases and their tendency to remission and recurvency, they may also be administered to patients, where one or more of the above-mentioned diseases has been diagnosed, and may serve to maintain the patient in a state of remission.

Based on earlier studies in mice, it is believed that the modified TNF $\alpha$  analogs according to the invention can also be administered as part of a curative treatment of the

above-mentioned diseases in an acute state or at least with a view to bringing the patient in remission and maintain a steady state condition.

At present no specific effective dose range can be stated, since the vaccines have not yet been tested in human beings susceptible to any of the diseases.

At any rate the administered dose will be prescribed by the responsible doctor.

According to one embodiment of the invention the vaccine comprises a mixture of two differently modified TNF $\alpha$  molecules containing two different T cell epitopes e.g. P2 and P30 which are derived from tetanus toxoid. This mixture optionally contains appropriate amounts of a pharmaceutically acceptable adjuvant

According to yet another aspect of the invention, the vaccines do not comprise the modified human TNF $\alpha$  molecules as such, but rather a construct comprising non-infectious non-integrating DNA sequence encoding said molecules operatively linked to a promoter sequence which can control the expression of said DNA sequence in humans, in an amount sufficient that uptake of said construct occurs, and sufficient expression occurs to induce a neutralizing antibody response against TNF $\alpha$ .

The utility of this type of vaccines, the so-called DNA vaccines, is illustrated e.g. in US patents nos. 5.589.466 and 5.580.859, both of which are incorporated herein by reference, in particular in relation to the methods of administration.

The DNA vaccines may comprise a viral expression vector, such as a retroviral expression vector.

Generally, the vaccines according to the invention may be adapted for oral or parenteral, in particular subcutaneous, intramuscular or intradermal administration.

The invention further comprises the use of antibodies raised by administering a vaccine according to the invention, preferably monoclonal antibodies, particularly a diagnostic use.

The invention further relates to a method of testing human body fluids for the presence of TNF $\alpha$  which comprises contacting a composition containing modified TNF $\alpha$  according to the invention with a sample of human body fluid and determine, whether said antibodies bind to TNF $\alpha$  in said sample.

The invention also relates to a diagnostic method for TNF $\alpha$ -related diseases employing an in vitro immunoassay to detect TNF $\alpha$  in human body fluids.

Said methods may involve the use of a sandwich assay, ELISA assay or equivalent assay, which can be unamplified or amplified, e.g. using avidin/biotin technology.

The vaccines/recombinant proteins can be characterized using the following assays familiar to a person skilled in the art:

SDS-Page gels stained with coomassie or silver (information on size and purity),

IEF (Isoelectric focusing) (information on isoelectric point),

Endotoxin LAL assay (information on purity),

Host cell protein (information on purity),

- Mass spectroscopy (information on molecular mass),
- SE-HPLC with UV detection profile (information on molecule weight distribution),
- N-terminal sequence (information on identity),
- 5 Circular Dichroism (information on tertiary structure),
- SE-HPLC with mallas detection (information on tertiary structure by light scattering),
- Amino acid composition (information on identity),
- Immunogenecity in heterologous species (mouse, rat or  
10 rabbit),
- Reactivity to antibodies in Western Blots,
- NMR spectroscopy,
- Crystal structure,
- Complete amino acid sequence determination.

15 EXPERIMENTAL PART WITH DESCRIPTION OF PREFERRED EMBODIMENTS

1. Introduction

In the previous application WO 95/05849 it was shown that  
T cell epitope modified murine TNF $\alpha$  molecules could induce  
20 high titers of antibodies cross-reactive with native  
(wild-type) murine TNF $\alpha$ . These antibodies were able to  
interfere with TNF $\alpha$  and its receptor *in vitro* as well as  
*in vivo*. Beneficial effects of immunization against TNF $\alpha$   
were demonstrated in several animal models of TNF $\alpha$ -  
25 induced disease such as experimental cachexia, collagen  
arthritis and experimental allergic encephalomyelitis

(EAE). These animal experimental results were obtained despite the fact that the two modified murine TNF $\alpha$  molecules used (denominated MR 103 and MR 106) were not optimized to be immunogenic in the MHC class II haplotypes of DBA/1 and SJL mice. These mouse strains were used for the collagen arthritis and the EAE experiments, respectively. In another experiment a differently modified murine TNF $\alpha$  molecule (MR 105) was shown to provide a stronger immune response than the recombinant murine TNF $\alpha$  conjugated to E.coli proteins using formaldehyde.

MR 103, MR 105 and MR 106 were mouse molecules and based on the prior application WO 95/05849 no specific conclusions could be drawn with regard to the immunogenicity of appropriately T cell substituted human modified TNF $\alpha$  molecules nor about the potential ability of such molecules to induce TNF $\alpha$  neutralizing autoantibodies, since they were all active.

## 2. Development of human TNF $\alpha$ constructs

In general terms in a TNF $\alpha$  vaccine for human use the modified human TNF $\alpha$  molecules should fulfill the following requirements:

- a. They should be immunogenic in a large proportion of the population
- b. They should be optimally able to induce TNF $\alpha$  neutralizing antibodies
- c. They should not possess any remaining biological TNF $\alpha$  activity

Furthermore, in a selection process other practical parameters such as levels of recombinant expression, ease of purification, solubility etc. could also be considered.

### 2.1. Immunological promiscuity of the modified TNF $\alpha$ molecules

During the development of the human TNF $\alpha$  vaccine the aim was to produce modified human TNF $\alpha$  molecules which eventually will be immunogenic in the largest possible part of the human population which of course represents a large number of different HLA class II types. Therefore, instead of the MHC specific epitopes used in the previous animal experiments, promiscuous T cell epitopes were used. It was not known from the previous application WO 95/05849 how such epitopes could influence the capability of such molecules to induce neutralizing antibodies.

The two tetanus toxoid (TT) derived T cell epitopes, P2 and P30, which have been well characterized in the scientific literature were chosen. These epitopes are known to be immunodominant in TT and to be able to bind to at least 80 % of the HLA class II molecules in the human population.

Furthermore, by using these TT epitopes it was expected to be possible to test the immunogenicity of the TNF $\alpha$  constructs *in vitro* on peripheral blood mononuclear cells (PBMC) and T cell lines generated from TT immune blood donors.

The amino acid sequence of the P2 epitope is QYIKANSKFIGITEL and corresponds to TT amino acids 830-844, and the sequence of the P30 epitope is FNNFTVSFWLRVPKVSASHLE and corresponds to TT amino acids 947-967. Substituting P2 and P30 into two different human TNF $\alpha$  molecules would exchange approximately 10 % and 15 %, respectively, of the native TNF $\alpha$  sequence. In case both epitopes were inserted into a single TNF $\alpha$  molecule, about 25 % of the molecule would be exchanged, and one could fear that this would interfere too much with the

remaining native parts of the TNF $\alpha$  molecule. It was therefore decided to develop two TNF $\alpha$  molecules, each containing either P2 or P30. Together, such two molecules would be expected to be immunogenic in at least 80 % of the human population. In addition, it is very likely that truncated molecules composed partly of the P2 or P30 epitope and partly of TNF $\alpha$  flanking regions also will contribute to the immunogenicity resulting in the constructs being immunogenic in almost 100 % of the population.

10 Although it was possible to induce antibodies with all murine TNF $\alpha$  constructs in all mouse strains tested so far, cf. discussion of MR 103, 105 and 106 above, one would a *priori* expect, that insertion of the foreign T cell epitope at certain positions in TNF $\alpha$  would be more  
15 beneficial than other positions with regard to the presentation of the epitope to T cells by MHC class II molecules. It was therefore decided to produce an array of differently modified human TNF $\alpha$  molecules with the P2 and P30 epitope inserted at different positions in the molecule, see fig 4.. Subsequently, all molecules were tested  
20 *in vitro* in T cell assays based on peripheral blood mononuclear cells (PBMC) or P2/P30 specific T cell lines isolated from a number of healthy TT immune blood donors.

Contrary to what was expected, however, it was shown that  
25 although minor quantitative differences were seen, the intramolecular position of the P2 and P30 epitopes was not essential for the ability to be processed by antigen presenting cells and subsequently presented to TT specific T cells. Thus, P2 inserted in positions 132 to 146,  
30 65 to 79 and 76 to 90 and P30 inserted in positions 40 to 60 and 132 to 152 (TNF2-5, 2-3, 2-7, 30-2, 30-5) were all processed and presented to T cells. So from the discussion above it is very likely, that these molecules even-

tually will be universally immunogenic in the human population.

2.2. The ability of the modified human TNF $\alpha$  molecules to induce neutralizing antibodies

5 As mentioned above, it was not possible from the previous mouse studies described in WO 95/05849 to predict which position would be most appropriate in order to be able to induce neutralizing TNF $\alpha$  antibodies since the three analogues MR 103, MR 105 and MR 106 were all able to induce  
10 antibodies despite their different areas of substitution. An array of different human TNF $\alpha$  molecules with P2 or P30 inserted at different positions was therefore produced. The substitutions were randomly distributed over the entire molecule. The antibodies induced in rabbits upon injection of these molecules were subsequently tested in  
15 biochemical as well as biological *in vitro* assays for their ability to interfere with TNF $\alpha$  biological activity.

It has been shown in non-published observations by the present inventors that depending on the intramolecular  
20 position of the inserted epitope a different overall specificity of the induced autoantibodies was observed. Quite contrary to what would be expected based on the structural data of the TNF $\alpha$  molecule it was observed, that substitutions made in the front  $\beta$ -sheet with either  
25 P2 or P30 totally deprived the molecules of biological TNF $\alpha$  activity, but at the same time preserved the ability of the modified molecules to induce TNF $\alpha$  neutralizing antibodies.

The molecules containing P2 or P30, in the positions mentioned above, were shown to be particularly effective at  
30 inducing neutralizing antibodies. Any of these molecules are therefore potential candidates for use in human TNF $\alpha$  vaccines.



2.3 The biological activity of the different TNF $\alpha$  constructs

It would obviously not be feasible to use a molecule which is as toxic as TNF $\alpha$  in a vaccine. The modified TNF $\alpha$  molecules would therefore have to be non-toxic i.e. devoid of any residual TNF $\alpha$  activity.

All mutant TNF $\alpha$  proteins were therefore tested *in vitro* in TNF $\alpha$  dependent bioassays as well as receptor binding assays in order to examine whether they are non-toxic. It was shown clearly that the modified human TNF $\alpha$  molecules (TNF2-5, 2-3, 2-7, 30-2, and 30-5,) all were deprived of TNF $\alpha$  biological activity. So all the necessary requirements of these molecules to be part of a universally, non-toxic vaccine capable of inducing anti-human TNF $\alpha$  neutralizing antibodies were fulfilled

EXAMPLE 1Genetic construction work

It was decided to produce 10 different modified human TNF $\alpha$  molecules - five containing the P2 and five containing the P30 epitope. The epitopes were distributed at different positions within the molecule. The genetic constructions were made by using various standard restriction enzyme and PCR based mutagenesis techniques. The genetic constructs are shown schematically in Fig. 4a and 4b. DNA sequences encoding the modified TNF $\alpha$  molecules and the corresponding amino acid sequences are incorporated as SEQ ID NO:1 - SEQ ID NO:20. The constructions of the mutant human TNF2-5 gene, the cloning and mutation strategy, and the subsequent expression, isolation and purification of the TNF2-5 analog is explained below by way of example.

## Construction and production of TNF2-5:

Genetic construction of the mutant human TNF2-5 gene, cloning and mutation strategy.

The genetic construction of the gene encoding the mutant human TNF2-5 analog was based upon traditional PCR based mutagenesis techniques, as were all the other genetic constructions.

The native DNA sequence of human TNF $\alpha$  encoding the soluble part of this molecule was obtained by traditional PCR cloning using synthetically synthesized primers I and II (table 1 and SEQ ID NO:21 and 22) from a human commercially available cDNA library, CLONTECH Laboratories, Palo Alto, CA, USA (Fig. 16,1). The native gene was inserted into a commercial *E.coli* expression vector pET28c available from Novagen, Madison, WI 53711, USA, in such a

way that the gene could be transcribed in frame from an IPTG inducible promoter.

The genetic construction of the TNF $\alpha$  mutant analog TNF2-5 was performed by a PCR mutagenesis technique applied to the native DNA sequence. The nucleic acid sequence encoding the T cell epitope was incorporated into a synthetically synthesized 75-mer oligonucleotide (Primer "mut2-5", table 1 and SEQ ID NO:27) between the two 3'- and 5'-annealing stretches of TNF homologous DNA, the "mut" primer is thus capable of annealing to the native human TNF $\alpha$  gene sequence at the defined site selected for TNF2-5, (see Fig 16,2a). In the "mut" oligonucleotide the number of codons encoding the T cell epitope exactly matched the number of TNF-codons omitted between the two 3'- and 5'- annealing stretches of TNF homologous DNA. The mutagenesis primer was used to produce a PCR product containing the DNA encoding the T cell epitope and the TNF $\alpha$  sequence below (or 3') to the inserted epitope, (Fig. 16, 2a). The stretch of TNF $\alpha$  DNA above (or 5') to the point of insertion of the epitope was provided by a second PCR product using primers I and III (Table 1, SEQ ID NO:23) (Fig. 16, 2b). The two PCR products are eventually joined together in a final PCR reaction, (Fig. 16, 3) using the two most distal primers, (I, II) from the two reactions. The complete mutant TNF2-5 DNA sequence is then introduced into a commercial *E.coli* expression vector in analogy to the expression cloning of the native gene in such a way that the gene could be transcribed from an IPTG inducible promoter from transformed cells.

The "mut" primers used for construction of the other analogs (TNF2-1, 2-3, 2-4, 2-7, 30-1, 30-2, 30-3, 30-4 and 30-5) are identified as SEQ ID NO:23-26 and 28-33, respectively.

Table 1

	Primer I	HumanTNF-alpha FW.	24'-mer.
		NcoI-site	
	5'-GAC AAG CCC ATG GTC AGA TCA TCT-3'		
5			
	Primer II	HumanTNF-alpha Rev	30'-mer.
		XbaI-site.	
	5'-TCT CTA GAG GGC AAT GAT CCC AAA GTA GAC-3'		
10	Primer "mut2-5"	Mutant oligo" P2-5 (tt830-44),	75'-mer.
	5'-G AAG GGT GAC CGA CAG TAC ATT AAG GCC AAT TCG AAG TTC ATT GGC ATC ACT GAG CTG TCT GGG CAG GTC TAC TT-3'		
	Primer III	HumanTNF-alpha Rev 2'nd.	21'-mer.
15	5'-CCC AAA GTA GAC CTG CCC AGA-3'		

Cultivation of Recombinant Bacteria, harvest and dissolve inclusion bodies.Protein purification of the TNF2-5 analog

20 The production of the TNF2-5 protein was analogous to the production of the other recombinant TNF molecules.

1. Inoculate 20 ml TB medium containing 50 µg/ml Carbenicillin with the transformed *E.coli* strain carrying the IPTG inducible plasmid vector harbouring the TNF gene encoding the recombinant protein, grow the *E.coli* over  
25 night at 37°C with shaking.

2. Dilute the over night culture 1:25 in 250 ml TB medium with 50 µg/ml Carbenicillin, and grow the culture until OD<sub>450</sub> is 1. Induce the expression of the recombi-  
30 nant protein by adding IPTG to a final concentration of 1 mM. Grow over night with vigorous shaking at 37 °C.

3. Harvest the recombinant cells from the medium by centrifugation at 3500 x g. Wash the pellet once in BSB buffer. Use 150 ml BSB per 50 g wet weight bacteria.
4. Sonicate 4 times 30 seconds at maximal amplitude until the bacterial suspension is completely homogeneous. The sonication is performed using a MSE Soniprep 150 sonicator mounted with a 9.5 mm *standard probe* (Soniprep 05 38 121-1154)
5. Add 8  $\mu$ l PMSF (50 mM) and 80  $\mu$ l lysosyme solution (10 mg/ml) per gram of cell pellet. Incubate 30 min at RT
6. Add 4 mg deoxycholic acid per gram pellet, mix and store at 37 °C
7. When the solution has become viscous add 20  $\mu$ l DNase (1 mg/ml) per gram pellet and MgCl<sub>2</sub> to a final conc. of 5 mM, mix and store at room temperature for 30 min.
8. Sonicate in ice 5 times 30 seconds with 30 seconds intervals at maximum amplitude, until the solution has become fluid and non-viscous.
9. Centrifuge at 20.000 x g for one hour, preserve the supernatant for later checking of the washing procedure to check if all the inclusion bodies have been precipitated.
10. Resuspend the pellet in MilliQ water (1 ml H<sub>2</sub>O per gram of E. coli), shake 1 hour.
11. Centrifuge at 20.000 x g for one hour, preserve the supernatant to check if all the inclusion bodies have been precipitated.

12. Resuspend the pellet in 1 M urea dissolve in 1 ml per gram of E. coli, shake 1 hour.
- 13 Centrifuge at 20.000 x g for one hour, preserve the supernatant to check if all the inclusion bodies have  
5 been precipitated.
- 14 Resuspend the pellet in 1 M guanidine dissolved in 1 ml per gram of E. coli, shake 1 hour.
- 15 Centrifuge at 20.000 x g for one hour, preserve the supernatant to check if all the inclusion bodies have  
10 been precipitated.
- 16 Resuspend the pellet in 25 ml 6 M guanidine + 20 mM Tris pH 8.0, agitate over night.
- 17 Centrifuge at 20.000 x g for one hour, preserve the supernatant containing the recombinant protein inclu-  
15 sion bodies, preserve the pellet to check if all the inclusion bodies have been dissolved
- 18 The protein solution is extensively dialyzed against MilliQ water, and subsequently the solution is freeze dried.
- 20 19 The freeze dried material is solubilized in 20 mM Tris, 6 M guanidine, 30 % 2-propanol (pH 8.0) at a concentration of 20 mg/ml. It is allowed to solubilize overnight under gentle agitation. The presence of mono-  
25 mers is examined on a superdex 200 column (XK 16, Pharmacia, diameter: 1.6 cm, height: 750 cm.). Run in running buffer at 1 ml/min. Compared to standards in the same buffer.
- 20 Protein purification is performed by gel filtra-  
tion on a superdex 200 column (XK26, Pharmacia; height:  
30 100 cm, diameter: 2.6 cm) which is equilibrated with

equilibration buffer. Run in the equilibration buffer. A sample volume of about 1% of total column volume is applied.

21 Refolding of the recombinant protein is performed by dialysis. The protein is diluted to 0.1 mg/ml in equilibration buffer, and this solution is placed in a boiled dialysis bag and dialyzed against: 20 mM Tris, 4 M urea (pH 8.5) with three changes, one night over at room temperature. The dialysis bag is transferred to a Tris buffer (20 mM Tris, 150 mM NaCl (pH 8.0)). Change three times of which the first one takes place at room temperature. Overnight in the cold room.

Refolding is evaluated on a Superose 12 column equilibrated in Tris buffer (20 mM Tris, 150 mM NaCl (pH 8.0)). Compare to standards.

Storage. The recombinant proteins are stored freeze dried.

Large scale production of the modified TNF $\alpha$  molecules may be carried out as described below:

20

Starting Material:

500 Liter fermented *E.coli* culture, diafiltrated with water to approx. 50 Liter

25

1) Washing of the cells

A) Thaw the frozen cell slurry

B) Centrifuge the cells for 10 minutes at 4000 x g

30 C) Re-suspend the pellet in 50 mM Tris, 150 mM NaCl, pH 8.0

Repeat step B) and C) three times

## 2) Homogenizing the cells

- 5 A) Homogenize the cells by a Rannie homogenizer, 5 cyclic at 700 bar.
- B) Centrifuge the inclusion bodies for 30 minutes at 16.500\*g
- 10 C) Wash the inclusion bodies three times with 3M guanidine, 1M NaCl, 5 mM EDTA, 20% Sucrose, 50 mM Tris, pH 8. Centrifuge the inclusion bodies for 5 minutes at 16.500\*g

## 3) Solubilizing of inclusion bodies

- 15 Re-suspend the pellet in 6 M guanidine, 10 mM DTT, 150 mM NaCl, 5% Ethanol, 50 mM Tris, pH 8. Use approx. 1 ml buffer/100 mg pellet

## 4) Ultrafiltration of the inclusion bodies

- 20 A) Remove rough impurities by a 0.45 $\mu$  filter
- B) Remove high molecular components by a 30 KD filter
- C) Concentrate the inclusion bodies by a 5 KD filter.

## 25 5) Buffer change

Prepare the sample for ion-exchanger chromatography. Change the buffer to 6 M Urea, 1 mM DTT in 50 mM Tris, pH 8 by diafiltration

30

## 6) SP-Sepharose purification

Load the protein on a SP-Sepharose column. Wash the column with four volumes of A-buffer and Elute the protein with 100% B-buffer and Pool all the fractions with TNF $\alpha$

35



A-buffer: 6 M Urea, 1 mM DTT, 50 mM Tris/Cl, pH 8.

B-buffer: 6 M Urea, 1 M NaCl, 1 mM DTT, 50 mM Tris/Cl, pH 8.

5 7) Refolding of human TNF $\alpha$

Dilute the protein pool to approx. 0.1 mg TNF $\alpha$ /ml in 6 M Urea, 1 mM DTT, 50 mM NaCl, 5% EtOH in 20 mM Tris/Cl pH 8.8 and remove the Urea stepwise by going to following buffer composition - by diafiltration.

10

A) 2 M Urea, 1 mM DTT, 150 mM NaCl in 20 mM Tris/Cl, pH 8.8 - overnight at 5°C

B) 1M Urea, 1 mM DTT, 150 mM NaCl in 20 mM Tris/Cl pH 8.8 - eight hours at 5°C

15 C) 1 mM DTT, 150 mM NaCl in 20 mM Tris/Cl pH 8.8 - overnight at 5°C

D) 150 mM NaCl in 20 mM Tris/Cl pH 8.8 - overnight at 5°C

8) Storage of the human TNF $\alpha$  analogs

20

Concentrate the protein to 1.0 mg/ml and store the sample at -20°C.

TB medium

25

Dissolve Terrific Broth (GIBCO BRL 22711-22) in MiliQ water according to the manufacturers instruction. Autoclave at 121 °C for 20 min.

30 Carbenicillin x 100 stock solution (50mg/ml)

Carbenicillin disodium salt (Sigma C1389) is dissolved in MiliQ water at a concentration of 50 mg/ml. The solution is filtersterilized through a 0.2  $\mu$ m filter (Sterifix

35 0409 9206)

IPTG x 100 stock solution 100mM

isopropyl-beta-D-thiogalactopyranoside (IPTG, USB 17884)  
1.19 g IPTG is dissolved in MilliQ water ad 50 ml. The so-  
5 lution is filtersterilized through a 0.2 µm filter  
(Sterifix 0409 9206)

BSB buffer

10 Bacterial Suspension Buffer  
50 mM TRIS (Trisma base, Sigma T1503)  
0.5 M NaCl (Ridel-de.Haën 31434)  
5 mM DTT (DL-dithiothretiol, Sigma D-0632)  
pH 8.0

15

PMSF

50 mM, phenylmethylsulfonylfluride, SIGMA # P-7626,  
dissolved in 2-propanol

20

Lysosyme solution

10 mg/ml Grade III lysozyme from chicken egg white, (EC  
3.2.1.17) SIGMA # L-7001

25

Deoxycholic acid

(7-deoxycholic acid) Sigma # D-6750

30 DNase

1 mg/ml Dnase I, Deoxyribonuclease I, (EC.3.1.21.1) Boe-  
hringer Cat # 1284932

## UREA

Urea (GibcoBRL 15716-020)

## 5 Guanidine

Guanidine hydrochloride (Sigma G4505)

## 2-Propanol

10

## Running buffer

20 mM TRIS, (Trisma base, SigmaT1503)

8 M urea, (GibcoBRL 15716-020)

15 0.1%  $\beta$ -mercaptoethanol

pH 8.0

## Equilibration buffer

20 20 mM TRIS, (Trisma base, SigmaT1503)

8m urea, (GibcoBRL 15716-020)

0.1%  $\beta$ -mercaptoethanol

EXAMPLE 2Expression, purification and refolding of P2 and P30 modified TNF $\alpha$  molecules

It is well established that recombinant proteins behave  
5 differently during expression, purification and refolding. All proteins were expressed in *E. coli* and expression levels ranging from 2-20 % were obtained. All the proteins were recognized in Western blotting experiments using a commercially available polyclonal rabbit-anti human TNF $\alpha$  antibody.  
10

The TNF $\alpha$  constructs were subsequently expressed one by one in 250 ml cultures in batch sizes of 3-4 l. All modified TNF $\alpha$  proteins were expressed as inclusion bodies and more than 85 % pure and refolded protein preparations  
15 were produced as described above.

The protein content was determined with standard BCA analysis. All protein purifications were performed in at least three separate runs for each molecule, and in all cases the separate protein batches were tested and found  
20 similar. The proteins were stored in concentrations from 0.2 - 1.0 mg/ml in PBS at -20 °C until use.

Standard quality control analyses included SDS gel electrophoresis with both Coomassie blue staining and silver staining of the individual protein preparations. In all  
25 cases the proteins were found to be of the expected size and to be more than 85 % pure.

Molecules with epitopes inserted at position 4 (TNF2-4 and TNF30-4) were only expressed at relatively low levels (app. 2 %). These molecules were furthermore very difficult to purify and especially their refolding was troublesome. Both molecules, but in particular TNF30-4, were  
30

not very soluble in PBS and tended to precipitate during storage.

EXAMPLE 3.

Biological activity of the different TNF $\alpha$  constructs

5 The direct biological activity of the purified TNF $\alpha$  molecules was tested in the L929 bioassay which is a standard *in vitro* assay for determination of biological TNF $\alpha$  activity. As seen in Fig. 6 none of the P2 or P30 modified TNF $\alpha$  molecules were able to kill L929 cells in the concentration range tested (up to 60 mg/ml). A commercially available recombinant TNF $\alpha$  molecule were fully active at  
10 about 0.6 ng/ml. The wild type TNF $\alpha$  preparation was also fully active in the entire concentration range tested.

EXAMPLE 4.

15 The ability of the modified TNF $\alpha$  molecules to induce neutralizing antibodies

Rabbits were immunized with each of the ten constructs to see whether these were able to induce antibodies capable of neutralizing biologically active human TNF $\alpha$  *in vitro*.  
20 Groups of three rabbits were used and each rabbit received 100 mg TNF $\alpha$  s.c. in Freund's Complete Adjuvant and subsequently 100 mg approximately every third week in Freund's Incomplete Adjuvant.

During the entire immunization period of 18 weeks blood  
25 samples were collected at regular intervals and the sera were tested for anti-TNF $\alpha$  activity in a conventional ELISA assay where commercially available, pure and non-modified human TNF $\alpha$  was used as the antigen.

All rabbits developed anti-TNF $\alpha$  antibodies during the immunization period and the maximum level of antibody  
30

titers varied within a decade within each group. The average antibody titers are shown in Fig. 7. TNF2-5, TNF2-7, TNF2-3, TNF2-1 and WT-TNF $\alpha$  induced a titer of one hundred within 2-4 weeks whereas TNF2-4 gave a remarkably slower response. Similar kinetics were observed for the TNF30 proteins where a slower response also was obtained with TNF30-4.

The ability of these sera to interfere with human TNF $\alpha$  and its receptor was tested in the L929 assay as well as in a solid phase receptor binding assay.

In the L929 assay dilutions of immune sera as well as a non-immune control serum were added to commercially available human TNF $\alpha$  prior to the addition of the mixture to L929 cells. Sera from all three rabbits in each group were tested in duplicates and the average values were calculated. Since normal serum tended to increase the background values of the color detecting system these were subtracted from all values and the relative inhibition in percent was calculated. The results for the inhibitory capacity at week 14 in the immunization schedule of all rabbits are shown in Fig. 8.

TNF2-5 which does not comprise substitutions in any segment of the back  $\beta$ -sheet strands is clearly superior compared to the other constructs and this molecule was comparable to the fully toxic WT-TNF $\alpha$  molecule with regard to the ability to induce neutralizing antibodies (data not shown). TNF2-3 which comprises a small substituted segment in the D  $\beta$ -strand and TNF2-7 which does not comprise any substituted segment of the back  $\beta$ -sheet strands, were also able to induce inhibitory antibodies, and the neutralizing antibody titer towards TNF2-7 increased significantly during the following three weeks (data not shown). TNF2-4 and TNF2-1, which comprise the G

and B  $\beta$ -strands, respectively, of the back  $\beta$ -sheet, were unable to induce neutralizing antibodies in the L 929 assay.

5 The results with regard to the TNF30 proteins were more heterogeneous although TNF30-3 seemed to be best at week 14. TNF30-1 and TNF30-4, which comprise substitutions in the G and B  $\beta$ -strands of the back  $\beta$ -sheet, respectively, did not induce neutralizing antibodies in the L929 assay.

10 In the solid phase receptor binding assay recombinant human 55 kD TNF $\alpha$  receptor 1 (TNF-R55) was immobilized on microtiter plates and commercially available biotinylated human TNF $\alpha$  was added in an appropriate dilution. Specific binding to the receptor was subsequently detected with horse radish peroxidase labeled streptavidin and a chromogenic substrate. When testing sera from immunized rabbits these were added to the biotinylated human TNF $\alpha$  solution prior to the addition of the mixture to the TNF-R55 coated microtiter plate. Sera from all three rabbits in each group were tested and the average values  
15 were calculated. Serum from a non-immunized rabbit was used as negative control. The background values were very low and the assay is highly sensitive. The results are shown in Fig. 9.  
20

25 It can be seen that the results obtained from the L929 assay and the solid phase receptor binding assay, respectively, are almost identical with regard to the TNF $\alpha$  2 constructs. In the solid phase assay the difference between TNF30-2, 30-3 and 30-5 was not as pronounced as observed in the L929 assay. The solid phase assay is, however, more reproducible due to its biochemical rather than cellular character, and normal serum values were not  
30 subtracted in this assay.

The relative amounts of serum (in percent) by which half maximum inhibition of TNF $\alpha$  binding was achieved (IC<sub>50</sub> values) were calculated for TNF2-3, TNF2-5, TNF2-7, TNF30-2, TNF30-3, TNF30-5 and WT-TNF $\alpha$  for each of the corresponding antisera. Assuming that a similar curve shape would appear for antisera raised against TNF2-1, TNF2-1, TNF30-1 and TNF30-4 extrapolations were performed, and IC<sub>50</sub> values were also calculated for these sera. The results are shown in Table II.



Table II.

IC50 values of rabbit-anti human TNF $\alpha$ sera in the solid phase assay										
2-1	2-3	2-4	2-5	2-7	WT	30-1	30-2	30-3	30-4	30-5
>100%	2.02%	>100%	0.53%	1.38%	0.43%	>100%	0.82%	0,88%	>74%	1.69%

It can be seen that TNF2-5 is equivalent to wild type (WT) mouse TNF $\alpha$  with regard to the ability to induce neutralizing anti-mouse TNF $\alpha$  antibodies in rabbits. TNF2-1, TNF2-4, TNF30-1 and TNF30-4 which all comprise substitutions in the B or G strand of the back  $\beta$ -sheet are very-poor or even totally unable to induce such antibodies. This indicates surprisingly that although the strands B and G of the back  $\beta$ -sheet are not involved in receptor binding, the mutations represented in this area anyhow lead to disturbance of the regions of human TNF $\alpha$  involved in receptor binding. TNF30-2 and TNF30-3 seem to induce neutralizing antibodies equally well.

#### EXAMPLE 5.

The ability of the modified TNF $\alpha$  molecules to stimulate T cells from P2 and P30 immune healthy blood donors

Since it was expected that the localization of the P2 and P30 epitopes in the modified TNF $\alpha$  molecules also would affect the antigen processing and presentation of the inserted epitopes - and thus their immunogenicity - all 10 molecules were tested in different T cell assays. A polyclonal Peripheral Blood Molecular Cell (PBMC) assay was used, and a number of P2 and P30 specific T cell lines were established using conventional immunological methods to test TNF $\alpha$  peptides and recombinant proteins with inserted P2 and P30 epitopes.

Initially 28 healthy volunteers (donors) were tested in a conventional PBMC proliferation assay for their ability to respond to TT and the P2 and P30 peptides. On the basis of these results 15 individuals capable of responding significantly to TT, P2 as well as P30 peptides were selected for further experiments. Although the response levels varied much, this clearly confirmed that P2 and

P30 are promiscuous as well as immunodominant T cell epitopes.

In addition to the 28 donors, seven further donors were also selected. For reasons explained below some of these were selected for their ability to respond either to P30 or to P2. Several of these were furthermore vaccinated with TT in order to be able to give a strong T cell response. Some of the second group of donors were also used to raise P2 or P30 specific T cell lines in order to study the antigen presentation of P2 and P30 modified synthetic TNF $\alpha$  peptides as well as the modified molecules. In Fig. 10 representative examples of the polyclonal PBMC proliferative response in three donors towards TT as well as the P2 and P30 peptides are shown.

All 10 recombinant proteins were tested in triplicate in at least 6 different concentrations starting from about 100 mg/ml, and all PBMC experiments were repeated 2-3 times for each individual. Intracellular incorporation of <sup>3</sup>H-labelled thymidine was used to assess T cell proliferation, and the experiments were harvested and counted in a 96-well format. The maximum proliferation indexes (PI) from each titration curve was calculated as the relation between the average number of CPMs in the experimental wells divided by the average number of CPMs obtained in the antigen free wells (PBS only). The T cell proliferation data were made in triplicate and Con A as well as the P2 and P30 were used as negative and positive controls, respectively. In Fig. 11 three examples are shown from experiments with two different blood donors using the different P2 and P30 modified TNF $\alpha$  molecules.

JH only responds to P2, whereas SR responds to both P2 and P30. This is also reflected in the proliferative responses to the P2 and P30 modified TNF $\alpha$  proteins which to

a varying extent almost all are able to stimulate the PBMCs.

In Fig. 12 the Proliferation Indexes (PIs) calculated from 34 experiments are shown. Although it is very difficult to quantitatively compare PIs between different experiments due to varying PBS backgrounds it seems clear that all constructs more or less are capable of eliciting a proliferative response.

Among the second group of donors some individuals were vaccinated against TT 1-2 months before the experiments. The PIs obtained from these persons (HB, MR, KG, and MW) were all among the highest PIs observed for all the modified TNF $\alpha$  constructs and the data were easy to evaluate. Three other individuals (DL, ID, and LS) were vaccinated more than 5 years back, and they all showed PI values below average. This supports that the observed PIs were antigen specific. No data regarding the last TT vaccination dates were available for the first group of donors, but their immunization status was clearly very variable.

It is not possible to select a preferred TNF $\alpha$  2 or TNF $\alpha$  30 protein merely based on the average size of the PI values. This may be due to the heterogeneous vaccination status of the individual used and the variable nature of PBMC assays obtained from individuals with variable responder status. It was anyway surprising, that P2 and P30 at all the inserted positions in TNF $\alpha$  seemed to be able to be processed and presented to T cells. In order to exclude the possibility that despite repeated affinity chromatography, gel filtration and dialysis, the antigen preparations could still contain significant concentrations of non-specific mitogens, some further experiments were performed.

Donors from the second group known to be non-responders to P2 and responders to P30 as well as donors with the opposite response pattern were tested in PBMC assays. In Fig. 13 the response to P2, P30 as well as the TNF $\alpha$  2 and TNF $\alpha$  30 proteins are shown for DL and ID.

It can be seen that specific responses are obtained to the respective T cell epitopes and the respectively modified TNF $\alpha$  molecules. However, no significant proliferative responses of DL against TNF $\alpha$  30 proteins were observed (upper panel) and no significant proliferative responses of ID to TNF $\alpha$  2 proteins were observed (lower panel) supporting that non-specific mitogens were absent in the purified TNF $\alpha$  preparations.

This possibility was even further examined by the use of P2 and P30 specific T cell lines isolated from the second group of donors, which had been cultured for at least six weeks in at least three rounds of stimulation with the respective synthetic P2 and P30 peptides. In Fig. 14 the results of two such experiments are shown.

It can be seen that the P2 and P30 specific T cell lines were only stimulated by their corresponding P2 and P30 proteins. Furthermore, it can be seen again that all TNF $\alpha$  constructs are able to induce T cell proliferation emphasizing that although antigen processing may be quantitatively important for presentation of P2 and P30, it does not seem to be a significant qualitative limiting factor for antigen presentation.

It has been reported in the literature that flanking regions of T cell epitopes can influence the binding of antigenic peptides to MHC class II molecules. Since none of the positions in TNF $\alpha$ , which were chosen for insertion of P2 or P30, seemed to be prohibitive for antigen presentation, it was investigated whether the different flanking

TNF $\alpha$  sequences of the inserted epitopes could influence the T cell response to P2 and P30 epitopes as a result of differential binding to the human HLA class II molecules. The peptides shown in Table III, which represent the inserted epitopes as well as the flanking human TNF $\alpha$  amino-  
5 acids were therefore synthesized. These are designated PP2-5, PP30-3, etc. The amino acid sequences are shown in the sequence listing as SEQ ID NO:34 to SEQ ID NO:42 designated Pep2-1 to Pep30-5.

Table III.

Synthetic peptides representing P2 and P30 and their flanking TNF $\alpha$ regions			
2-1	SRTPSQYIKANSKFIGITELQLQWL	30-1	SRTPSFNNFTVSFWLRVPKVSASHLERPANA
2-3	SQVLFQYIKANSKFIGITELISRIA	30-2	ALLANFNNFTVSFWLRVPKVSASHLEQVLFK
2-4	AEAKPQYIKANSKFIGITELGDRLS	30-3	YSQVLFNNFTVSFWLRVPKVSASHLEVSYQT
2-5	EKGDRQYIKANSKFIGITELSGQVY	30-4	QPETPFNNFTVSFWLRVPKVSASHLEKGDRL
2-7	ND	30-5	EKGDPFNNFTVSFWLRVPKVSASHLEGIIAL

These peptides were used for stimulation of P2 and P30 specific T cell lines. The results from stimulation of the P30 lines are shown in Fig. 15. It can be seen that the P2 specific T cell lines from MR and KG shows parallel stimulation patterns when stimulated either with the peptide or with the corresponding TNF $\alpha$  2 protein. It is clear that TNF2-5 is a good potential antigen and these data further supports that the observed T cell proliferations are antigen specific. There was generally no qualitative differences in the stimulation pattern when the P30 specific T cell lines from MR and KG were stimulated either with peptides or proteins (data not shown). The P30 specific T cell line from HC preferably recognized with TNF30-3 and reacted to a minor extent with TNF30-2.

#### 15 CONCLUSION

Ten differently modified human TNF $\alpha$  proteins have been produced and characterized. They were constructed to contain two well known promiscuous T cell epitopes P2 and P30 in order to be potentially immunogenic in at least 85% of the populations.

All proteins could be expressed and purified although TNF2-4 and TNF30-4 at low levels. Mutations at this position in TNF $\alpha$  also seems to interfere with refolding which results in proteins with poor solubility. No biological TNF $\alpha$  activity could be detected in any of the modified TNF $\alpha$  molecules.

Rabbits were immunized with all ten proteins as well as with native TNF $\alpha$ . After 2-3 months of immunization it was possible to detect high titers of strongly cross-reactive antibodies towards human, non-modified TNF $\alpha$  in all sera.

The ability of these antibodies to interfere with the biological activity of native TNF $\alpha$  was tested in two dif-



ferent *in vitro* assays - the L929 bioassay and a solid phase receptor binding assay. Both assays showed essentially the same TNF2-1, TNF2-4, TNF30-1 and TNF30-4 was not able to induce significant neutralizing antibodies, whereas TNF2-5 was superior compared to the other constructs. TNF30-2 and TNF30-3 were equally efficient at inducing neutralizing antibodies and twice as good as TNF30-5 which was reasonably good.

Somewhat surprisingly it was not possible to see significant differences between the abilities of the molecules to stimulate PBMC to proliferate. It could be demonstrated that this was not due to the presence of mitogens in the antigen preparations and based on these data, it was concluded that the locations chosen for P2 and P30 all allowed presentation of the respective epitopes. The specificity of the responses was further documented by using testing the modified TNF $\alpha$  proteins using epitope specific T cell lines as well as synthetic peptides representing the inserted epitopes as well as the flanking TNF $\alpha$  sequences. From these experiments it was clearly demonstrated that TNF2-5, TNF30-2 and TNF30-3 (among other constructs) were the most powerful potential immunogens.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Farmaceutisk Laboratorium Ferring A/S
- (B) STREET: Indertoften 10
- (C) CITY: Vanloese
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2720

(ii) TITLE OF INVENTION: Modified human TNF-alpha molecules, DNA encoding them, and vaccines containing said modified TNF-alpha or DNA

(iii) NUMBER OF SEQUENCES: 42

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:1..477
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/codon\_start= 1
  - /function= "Antigen"
  - /product= "TNF-alpha analog"
  - /evidence= EXPERIMENTAL
  - /gene= "tnfp2-1"
  - /standard\_name= "TNF2-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG	GTC	AGA	TCA	TCT	TCT	CGA	ACC	CCG	AGT	CAG	TAC	ATT	AAA	GCC	AAT	48
Met	Val	Arg	Ser	Ser	Ser	Arg	Thr	Pro	Ser	Gln	Tyr	Ile	Lys	Ala	Asn	
1				5				10						15		
TCT	AAA	TTC	ATC	GGT	ATA	ACT	GAG	CTG	CAG	CTC	CAG	TGG	CTG	AAC	CGC	96
Ser	Lys	Phe	Ile	Gly	Ile	Thr	Glu	Leu	Gln	Leu	Gln	Tyr	Leu	Asn	Arg	
			20					25						30		

```

CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG      144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
      35                                40                                45

CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC      192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
      50                                55                                60

TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC      240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
      65                                70                                75                                80

ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT      288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
      85                                90                                95

GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC      336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
      100                               105                               110

AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG      384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
      115                               120                               125

AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC      432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
      130                               135                               140

TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG      477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
      145                               150                               155
    
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(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 159 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Gln Tyr Ile Lys Ala Asn
 1                                5                                10                                15

Ser Lys Phe Ile Gly Ile Thr Glu Leu Gln Leu Gln Trp Leu Asn Arg
      20                                25                                30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
      35                                40                                45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
      50                                55                                60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
      65                                70                                75                                80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
      85                                90                                95
    
```

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala  
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu  
 115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp  
 130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu \*  
 145 150 155

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:1..477
- (D) OTHER INFORMATION:/codon\_start= 1  
 /function= "Antigen"  
 /product= "TNF-alpha analog"  
 /gene= "tnfP2-3"  
 /standard\_name= "TNF2-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Ile Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC CAG TAC ATA AAG GCC AAC TCC AAG TTT ATC GGC ATC ACC GAG CTC	240
Phe Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu	
225 230 235	

ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC	432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC AAT GCC CTC TAG	477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 159 amino acids

(B) TYPE: amino acid

TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
1 5 10 15	
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
20 25 30	
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
35 40 45	
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
50 55 60	
Phe Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu	
65 70 75 80	
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
85 90 95	
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
100 105 110	
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
115 120 125	
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
130 135 140	
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
145 150 155	

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:1..477
- (D) OTHER INFORMATION:/codon\_start= 1  
/function= "Antigen"  
/product= "TNF-alpha analog"  
/gene= "tnfP2-4"  
/standard\_name= "TNF2-4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATG GTC AGA TCA TCT TCT CCA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235	
ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG CCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC CAG TAT ATC AAG GCC AAT TCG AAA TTC ATC GGC ATC ACG GAG	384
Lys Pro Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu	
275 280 285	
CTC GGT GAC CGA CTC AGC GCT GAG ATC AAT CCG CCC GAC TAT CTC GAC	432
Leu Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	

TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG 477  
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu \*  
 305 310 315

## (2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 159 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His  
 1 5 10 15  
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg  
 20 25 30  
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln  
 35 40 45  
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu  
 50 55 60  
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr  
 65 70 75 80  
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser  
 85 90 95  
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala  
 100 105 110  
 Lys Pro Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu  
 115 120 125  
 Leu Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp  
 130 135 140  
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu \*  
 145 150 155

## (2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 477 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..477

(D) OTHER INFORMATION:/function= "Antigen"

/product= "TNF-alpha analog"

/gene= "tnfp2-5"

/standard\_name= "TNF2-5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
CTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC ACC TCC ACC CAT GTG CTC CTC ACC CAC ACC	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235	
ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA CAG TAC ATT AAG GCC AAT TCG AAG TTC ATT GGC ATC	432
Lys Gly Asp Arg Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile	
290 295 300	
ACT GAG CTG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Thr Glu Leu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu +	
305 310 315	

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 159 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein



## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His  
 1 5 10 15  
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg  
 20 25 30  
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln  
 35 40 45  
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu  
 50 55 60  
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr  
 65 70 75 80  
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser  
 85 90 95  
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala  
 100 105 110  
 Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu  
 115 120 125  
 Lys Gly Asp Arg Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile  
 130 135 140  
 Thr Glu Leu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu \*  
 145 150 155

## (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 477 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Homo sapiens
- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION:1..477  
 (D) OTHER INFORMATION:/codon\_start= 1  
 /function= "Antigen"  
 /product= "INF-alpha analog"  
 /gene= "tnfp2-7"  
 /standard\_name= "TNF2-7"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CAG TAC ATC AAA	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Gln Tyr Ile Lys	
225 230 235	
GCT AAC TCC AAA TTC ATC GGC ATC ACC GAA CTG GTT AAC CTC CTC TCT	288
Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC	432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 159 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
1 5 10 15	
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
20 25 30	
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
35 40 45	

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu  
 50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Gln Tyr Ile Lys  
 65 70 75 80

Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Val Asn Leu Leu Ser  
 85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala  
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu  
 115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp  
 130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu \*  
 145 150 155

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 477 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION:1..477
  - (D) OTHER INFORMATION:/codon\_start= 1  
 /function= "Antigen"  
 /product= "TNF-alpha analog"  
 /gene= "tnfP30-1"  
 /standard\_name= "TNF30-1"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT TTC AAC AAT TTT ACC GTA 48  
 Met Val Arg Ser Ser Arg Thr Pro Ser Phe Asn Asn Phe Thr Val  
 160 165 170 175

AGC TTT TGG CTC CGT GTA CCT AAG GTG TCG GCC TCG CAC CTG GAG CGC 96  
 Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Arg  
 180 185 190

CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG 144  
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln  
 195 200 205



Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp  
 130 135 140  
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu \*  
 145 150 155

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 477 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 

- (A) ORGANISM: Homo sapiens

- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION:1..477
  - (D) OTHER INFORMATION:/codon\_start= 1  
 /function= "Antigen"  
 /product= "TNF-alpha analog"  
 /gene= "tnfP30-2"  
 /standard\_name= "TNF30-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT TTC AAC AAC TTC ACA GTT AGC TTC	144
Arg Ala Asn Ala Leu Leu Ala Asn Phe Asn Asn Phe Thr Val Ser Phe	
195 200 205	
TGG TTG AGG GTA CCA AAG GTC TCG GCC AGC CAC CTC GAG CAG GTC CTC	192
Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235	
ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	

```

AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG      384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
      275                                280                                285

AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC      432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
      290                                295                                300

TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG      477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
      305                                310                                315

```

## (2) INFORMATION FOR SEQ ID NO: 14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 159 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1                                5                                10                                15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
      20                                25                                30

Arg Ala Asn Ala Leu Leu Ala Asn Phe Asn Asn Phe Thr Val Ser Phe
      35                                40                                45

Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Gln Val Leu
      50                                55                                60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
      65                                70                                75                                80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
      85                                90                                95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
      100                                105                                110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
      115                                120                                125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
      130                                135                                140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
      145                                150                                155

```

## (2) INFORMATION FOR SEQ ID NO: 15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Homo sapiens
- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION:1..477  
 (D) OTHER INFORMATION:/codon\_start= 1  
 /function= "Antigen"  
 /product= "TNF-alpha analog"  
 /gene= "tnfP30-3"  
 /standard\_name= "TNF30-3"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAC AAC TTT ACC GTC TCC TTC TGG CTT CGG GTA CCC AAG GTC AGC	240
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser	
225 230 235	
GCT AGC CAC CTC GAG GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ala Ser His Leu Glu Val Ser Tyr Gln Thr Lys Val Asn Ile Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC	432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu +	
305 310 315	

## (2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 159 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```

Met Val Arg Ser Ser Ser Arg Thr Fro Ser Asp Lys Pro Val Ala His
 1                               10                               15
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20                               25                               30
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35                               40                               45
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50                               55                               60
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
 65                               70                               75                               80
Ala Ser His Leu Glu Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85                               90                               95
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100                              105                              110
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115                              120                              125
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130                              135                              140
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 145                              150                              155

```

## (2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 477 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:1..477



(D) OTHER INFORMATION:/function= "Antigen"  
 /product= "TNF-alpha analog"  
 /gene= "tnfp30-4"  
 /standard\_name= "TNF30-4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235	
ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA TTT AAT AAT TTC ACC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Phe Asn Asn Phe Thr	
260 265 270	
GTG TCC TTC TGG TTG CGC GTC CCT AAG GTA AGC GCT TCC CAC CTG GAG	384
Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu	
275 280 285	
AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC	432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 159 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg  
 20 25 30  
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln  
 35 40 45  
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu  
 50 55 60  
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr  
 65 70 75 80  
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Ile Ser  
 85 90 95  
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Phe Asn Asn Phe Thr  
 100 105 110  
 Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu  
 115 120 125  
 Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp  
 130 135 140  
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu  
 145 150 155

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 477 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (v) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 1..477
  - (D) OTHER INFORMATION: /codon\_start= 1  
 /function= "Antigen"  
 /product= "TNF-alpha analog"  
 /gene= "tnfp30-5"  
 /standard\_name= "TNF30-5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	

CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235	
ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA TTC AAC AAT TTC ACC GTA AGC TTC TGG CTT CGC GTC	432
Lys Gly Asp Arg Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val	
290 295 300	
CCT AAG GTG TCT GCG TCG CAC CTC GAA GGG ATC ATT GCC CTC TAG	477
Pro Lys Val Ser Ala Ser His Leu Glu Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 159 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
1 5 10 15	
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
20 25 30	
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
35 40 45	
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
50 55 60	
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
65 70 75 80	
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
85 90 95	

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala  
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu  
 115 120 125

Lys Gly Asp Arg Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val  
 130 135 140

Pro Lys Val Ser Ala Ser His Leu Glu Gly Ile Ile Ala Leu \*  
 145 150 155

## (2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI SENSE: NO
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (B) LOCATION:1..24
  - (C) IDENTIFICATION METHOD: experimental
  - (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha"  
 /product= "Primer binding to TNF-alpha gene"  
 /evidence= EXPERIMENTAL  
 /standard\_name= "TNF-alpha Primer I"  
 /label= Primer1
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GACAAGCCCA TGSTCAGATC ATCT

24

## (2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI SENSE: NO
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (B) LOCATION:1..30
  - (C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION:/function= "Primer for PCR cloning  
of DNA encoding TNF-alpha"  
/product= "Primer binding to TNF-alpha gene"  
/evidence= EXPERIMENTAL  
/standard\_name= "TNF-alpha Primer II"  
/label= Primer2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TCTCTAGAGG GCAATGATCC CAAAGTAGAC

30

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION:1..21
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/function= "Primer for PCR cloning  
of DNA encoding TNF-alpha"  
/product= "Primer binding to TNF-alpha gene"  
/evidence= EXPERIMENTAL  
/standard\_name= "TNF-alpha Primer III"  
/label= Primer3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CCCAAAGTAG ACCTGCCAG A

21

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: insertion\_seq
- (B) LOCATION:7..51
- (C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"  
 /evidence= EXPERIMENTAL  
 /organism= "Homo sapiens"  
 /standard\_name= "Primer "mut2-1""  
 /label= mut2-1  
 /note= "Primer "mut2-1" is a synthetically synthesised 69-mer oligonucleotide comprising DNA encoding the human T cell epitope P2 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

ACCCCGAGTC AGTACATTAA AGCCAATTCT AAATTCATCG GTAT/ACTGA GCTGCAGCTC 60  
 CAGTGGCTG 69

(2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 73 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Homo sapiens
- (ix) FEATURE:  
 (A) NAME/KEY: insertion\_seq  
 (B) LOCATION:1..59  
 (C) IDENTIFICATION METHOD: experimental  
 (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"  
 /evidence= EXPERIMENTAL  
 /organism= "Homo sapiens"  
 /standard\_name= "Primer "mut2-3""  
 /label= mut2-3  
 /note= "Primer "mut2-3" is a synthetically synthesised 73-mer oligonucleotide comprising DNA encoding the human T cell epitope P2 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCCAGTCCCT CTTCCAGTAC ATAAAGGCCA ACTCCAAGTT TATCGGCATC ACCGAGCTCA 60  
 TCAGCCGCAT CGC 73

(2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 75 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: insertion\_seq
  - (B) LOCATION:12..56
  - (C) IDENTIFICATION METHOD: experimental
  - (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"
    - /evidence= EXPERIMENTAL
    - /organism= "Homo sapiens"
    - /standard\_name= "Primer "mut2-4""
    - /label= mut2-4
    - /note= "Primer "mut2-4" is a synthetically synthesised 75-mer oligonucleotide comprising DNA encoding the human T cell epitope P2 between stretches of DNA homologous to stretches of the human TNF-alpha gene"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
AGTCGGTCAC CGAGCTCCGT GATGCCGATG AATTTCGAAT TGGCCTTGAT ATACTGGGGC      60
TTGGCCTCAG CCCCC                                           75
```

## (2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 75 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: insertion\_seq
  - (B) LOCATION:8..52
  - (C) IDENTIFICATION METHOD: experimental
  - (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"
    - /evidence= EXPERIMENTAL
    - /organism= "Homo sapiens"
    - /standard\_name= "Primer "mut2-5""
    - /label= mut2-5
    - /note= "Primer "mut2-5" is a synthetically synthesised 75-mer oligonucleotide comprising DNA encoding the human T cell epitope P2 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GAAGGGTGAC CGACAGTACA TTAAGGCCAA TTCGAAGTTC ATTGGCATCA CTGAGCTGTC 60  
 TGGGCAGGTC TACTT 75

## (2) INFORMATION FOR SEQ ID NO: 28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 80 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: insertion\_seq
  - (B) LOCATION: 14..58
  - (C) IDENTIFICATION METHOD: experimental
  - (D) OTHER INFORMATION: /function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"  
 /evidence= EXPERIMENTAL  
 /organism= "Homo sapiens"  
 /standard\_name= "Primer "mut2-7"  
 /label= mut2-7  
 /note= "Primer "mut2-7" is a synthetically synthesised 80-mer oligonucleotide comprising DNA encoding the human T cell epitope P2 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CACCCATGTG CTCCAGTACA TCAAAGCTAA CTCCAAATTC ATCGGCATCA CCGAACTGGT 60  
 TAACCTCCTC TCTGCCATCA 80

## (2) INFORMATION FOR SEQ ID NO: 29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 96 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens



## (ix) FEATURE:

- (A) NAME/KEY: insertion\_seq
- (B) LOCATION:10..72
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"  
/evidence= EXPERIMENTAL  
/organism= "Homo sapiens"  
/standard\_name= "Primer "mut30-1"  
/label= mut30-1  
/note= "Primer "mut30-1" is a synthetically synthesised 96-mer oligonucleotide comprising DNA encoding the human T cell epitope P30 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```

ACCCCGAGTT TCAACAATTT TACCGTAAGC TTTTGGCTCC GTGTACCTAA GGTGTCGGCC      60
TCGCACCTGG AGCGCCGGGC CAATGCCCTC CTGGCC                                96

```

## (2) INFORMATION FOR SEQ ID NO: 30:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

## (ix) FEATURE:

- (A) NAME/KEY: insertion\_seq
- (B) LOCATION:12..74
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"  
/evidence= EXPERIMENTAL  
/organism= "Homo sapiens"  
/standard\_name= "Primer "mut30-2"  
/label= mut30-2  
/note= "Primer "mut30-2" is a synthetically synthesised 100-mer oligonucleotide comprising DNA encoding human T cell epitope P30 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```

TCCTGGCCAA TTTCAACAAC TTCACAGTTA GCTTCTGGTT GAGGGTACCA AAGGTCTCGG      60
CCAGCCACCT CGAGCAGGTC CTCTTCAAGG GCCAAGGCTG                                100

```

## (2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 100 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: insertion\_seq
  - (B) LOCATION:12..74
  - (C) IDENTIFICATION METHOD: experimental
  - (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"  
 /evidence= EXPERIMENTAL  
 /organism= "Homo sapiens"  
 /standard\_name= "Primer "mut30-3"  
 /label= mut30-3  
 /note= "Primer "mut30-3" is a synthetically synthesised 100-mer oligonucleotide comprising DNA encoding human T cell epitope P30 between stretches of DNA homologous to stretches of the human TNF-alpha gene"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

```

CCCAGGTCCT CTTCAACAAC TTTACCGTCT CCTTCTGGCT TCGGGTACCC AAGGTCAGCG      60
CTAGCCACCT CGAGGTCTCC TACCAGACCA AGGTCAACCT                               100
  
```

## (2) INFORMATION FOR SEQ ID NO: 32:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 100 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: insertion\_seq
  - (B) LOCATION:15..77
  - (C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"  
 /evidence= EXPERIMENTAL  
 /organism= "Homo sapiens"  
 /standard\_name= "Primer "mut30-4"  
 /label= mut30-4  
 /note= "Primer "mut30-4" is a synthetically synthesised 100-mer oligonucleotide comprising DNA encoding human T cell epitope P30 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```
AGTCGGTCAC CCTTCTCCAG GTGGGAAGCG CTTACCTTAG GGACGCGCAA CCAGAAGGAC      60
ACGGTGAAT TATTAAATGG GGTCTCCCTC TGGCAGGGGG                                100
```

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 100 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: insertion\_seq  
 (B) LOCATION:14..76  
 (C) IDENTIFICATION METHOD: experimental  
 (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"  
 /evidence= EXPERIMENTAL  
 /organism= "Homo sapiens"  
 /standard\_name= "Primer "mut30-5"  
 /label= mut30-5  
 /note= "Primer "mut30-5" is a synthetically synthesised 100-mer oligonucleotide comprising DNA encoding human T cell epitope P30 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

```
GAAGGGTGAC CGATCAACA ATTACCGT AAGCTTCTGG CTTGCGTCC CTAAGGTGTC      60
TGGGTCGCAC CTCGAAGGGA TCATTGCCCT CTAGAGTCGA                                100
```

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 25 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION:1..25

(D) OTHER INFORMATION:/label= Pep2-1

/note= "Pep2-1 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P2 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Ser Arg Thr Pro Ser Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly  
1                   5                   10                   15  
  
Ile Thr Glu Leu Gln Leu Gln Trp Leu  
          20                   25

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION:1..25

(D) OTHER INFORMATION:/label= Pep2-3

/note= "Pep2-3 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P2 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Ser Gln Val Leu Phe Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly  
1                   5                   10                   15  
  
Ile Thr Glu Leu Ile Ser Arg Ile Ala  
          20                   25

## (2) INFORMATION FOR SEQ ID NO: 36:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: Peptide
  - (B) LOCATION:1..25
  - (D) OTHER INFORMATION:/label= Pep2-4  
 /note= "Pep2-4 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P2 and flanking portions of human TNF-alpha"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Ala	Glu	Ala	Lys	Pro	Gln	Tyr	Ile	Lys	Ala	Asn	Ser	Lys	Phe	Ile	Gly
1				5					10					15	
Ile	Thr	Glu	Leu	Gly	Asp	Arg	Leu	Ser							
			20					25							

## (2) INFORMATION FOR SEQ ID NO: 37:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: Peptide
  - (B) LOCATION:1..25
  - (D) OTHER INFORMATION:/label= Pep2-5  
 /note= "Pep2-5 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P2 and flanking portions of human TNF-alpha"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Glu	Lys	Gly	Asp	Arg	Gln	Tyr	Ile	Lys	Ala	Asn	Ser	Lys	Phe	Ile	Gly
1					5				10					15	

Ile Thr Glu Leu Ser Gly Gln Val Tyr  
 20 25

(2) INFORMATION FOR SEQ ID NO: 38:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 31 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: Peptide
  - (B) LOCATION:1..31
  - (D) OTHER INFORMATION:/label= Pep30-1  
 /note= "Pep30-1 is a synthetically prepared truncated form of a TNF-alpha analog comprising human T cell epitope P30 and flanking portions of human TNF-alpha"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:
 

Ser Arg Thr Pro Ser Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg  
 1 5 10 15

Val Pro Lys Val Ser Ala Ser His Leu Glu Arg Arg Ala Asn Ala  
 20 25 30

(2) INFORMATION FOR SEQ ID NO: 39:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 31 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
  - (A) NAME/KEY: Peptide
  - (B) LOCATION:1..31
  - (D) OTHER INFORMATION:/label= Pep30-2  
 /note= "Pep30-2 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P30 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

```

Ala Leu Leu Ala Asn Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
 1              5              10              15
Val Pro Lys Val Ser Ala Ser His Leu Glu Gln Val Leu Phe Lys
          20              25              30

```

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION:1..31
- (D) OTHER INFORMATION:/label= Pep30-3  
/note= "Pep30-3 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P30 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

```

Tyr Ser Gln Val Leu Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
 1              5              10              15
Val Pro Lys Val Ser Ala Ser His Leu Glu Val Ser Tyr Gln Thr
          20              25              30

```

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION:1..31

(D) OTHER INFORMATION:/label= Pep30-4  
 /note= "Pep30-4 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P30 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

```

Gln Arg Glu Thr Pro Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
1             5             10             15

Val Pro Lys Val Ser Ala Ser His Leu Glu Lys Gly Asp Arg Leu
20             25             30
  
```

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 31 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Homo sapiens

(ix) FEATURE:  
 (A) NAME/KEY: Peptide  
 (B) LOCATION:1..31  
 (D) OTHER INFORMATION:/label= Pep30-5  
 /note= "Pep30-5 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P30 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

```

Glu Lys Gly Asp Arg Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
1             5             10             15

Val Pro Lys Val Ser Ala Ser His Leu Glu Gly Ile Ile Ala Leu
20             25             30
  
```



P A T E N T   C L A I M S

1. A modified human TNF $\alpha$  molecule capable of raising neutralizing antibodies towards wild-type human TNF $\alpha$  following administration of said modified TNF $\alpha$  molecule to a human host, wherein at least one peptide fragment of the human TNF $\alpha$  molecule has been substituted by at least one peptide known to contain an immunodominant T cell epitope or a truncated form of said molecule containing an immunodominant epitope and one or both flanking regions of the human TNF $\alpha$  molecule comprising at least one TNF $\alpha$  B cell epitope, wherein the substitution introduces a substantial change in the amino acid sequence of any one of the strands of the front  $\beta$ -sheet, in any one of the connecting loops and/or in any one of the B', I or D strands of the back  $\beta$ -sheet.

2. A modified human TNF $\alpha$  molecule capable of raising neutralizing antibodies towards wild-type human TNF $\alpha$  following administration of said modified TNF $\alpha$  molecule to a human host, wherein at least one peptide fragment of the human TNF $\alpha$  molecule has been substituted by at least one peptide known to contain an immunodominant T cell epitope or a truncated form of said molecule containing an immunodominant epitope and one or both flanking regions of the human TNF $\alpha$ -molecule comprising at least one TNF $\alpha$  B cell epitope, wherein said modified TNF $\alpha$  molecule is substantially free from TNF $\alpha$  activity.

3. A modified human TNF $\alpha$  molecule according to claim 2, wherein the modified TNF $\alpha$  molecule, when tested in the L929 bioassay, is substantially free from TNF $\alpha$  activity, and wherein antibodies raised against the modified TNF $\alpha$  molecule in a suitable host significantly inhibit the activity of native TNF $\alpha$  in the L929 bioassay, and/or wherein said antibodies significantly inhibit the binding

of wild-type human TNF $\alpha$  to the 55 kD TNF $\alpha$  receptor 1 (TNF $\alpha$ -R55) or the to the 75 kD TNF $\alpha$  receptor (TNF $\alpha$ -R75).

4. A modified human TNF $\alpha$  molecule capable of raising neutralizing antibodies towards wild-type human TNF $\alpha$  following administration of said modified TNF $\alpha$  molecule to a human host, wherein at least one peptide fragment of the human TNF $\alpha$  molecule has been substituted by at least one peptide known to contain an immunodominant T cell epitope or a truncated form of said molecule containing an immunodominant epitope and one or both flanking regions of the human TNF $\alpha$ -molecule comprising at least one TNF $\alpha$  B cell epitope, wherein the substitution has been made in regions of the TNF $\alpha$  molecule so as to essentially preserve the  $\beta$ -sheet structure of the B and G strands.

5. Modified human TNF $\alpha$  molecule according to claims 1-4, wherein the substitution has been made in regions of the TNF $\alpha$  molecule which involves the strands of the front  $\beta$ -sheets and/or the connecting loops so as to essentially preserve the  $\beta$ -sheet structure of any of the strands of the back  $\beta$ -sheet.

6. Modified human TNF $\alpha$  molecule according to claims 1-4, wherein the substitution has been made in regions of the TNF $\alpha$  molecule which involve a segment of the D strand of the back  $\beta$ -sheet.

7. Modified human TNF $\alpha$  molecule according to claims 1-4, wherein the substitution comprises at least a segment of the H strand of the front  $\beta$ -sheet and of the connecting loop to the I strand, preferably amino acids 132 to 146.

8. Modified human TNF $\alpha$  molecule according to claims 1-4, wherein the substitution comprises segments of the H and I strands and the entire connecting loop, preferably amino acids 132 to 152

9. Modified human TNF $\alpha$  molecule according to claims 1-4, wherein the substitution comprises a segment of the D strand, at least a segment of the E strand and the entire connecting loop, preferably amino acids 65 to 79 or 64 to 84.
- 5 84.
10. Modified human TNF $\alpha$  molecule according to claims 1-4, wherein the substitution comprises the entire C' and C strands and a segment of the D strand, preferably amino acids 40 to 60.
- 10 11. Modified human TNF $\alpha$  molecule according to claims 1-4, wherein the substitution comprises at least a segment of the E strand and of the front  $\beta$ -sheet of one or both of the connecting loops, preferably amino acids 76 to 90.
12. Modified TNF $\alpha$  according to claims 1-4, having the amino acid sequence shown in SEQ ID NO:8.
- 15 13. Modified TNF $\alpha$  according to claims 1-4, having the amino acid sequence shown in SEQ ID NO:10.
14. Modified TNF $\alpha$  molecule according to claims 1-4, having the amino acid sequence shown in SEQ ID NO:4 or SEQ ID NO:16.
- 20 15. Modified TNF $\alpha$  according to claims 1-4, having the amino acid sequence shown in SEQ ID NO:20.
16. Modified TNF $\alpha$  according to claims 1-4, having the amino acid sequence shown in SEQ ID NO:14.
- 25 17. Modified human TNF $\alpha$  molecule according to any of claims 1-11, wherein the inserted T cell epitope is promiscuous and known to be immunogenic in a majority of human HLA class II types.

18. Modified human TNF $\alpha$  molecule according to claim 17, wherein the epitope is derived from Tetanus toxoid, preferably epitope P2 and/or P30.
19. Dimers, oligomers or multimers of the modified human  
5 TNF $\alpha$  molecule according to any one of claims 1-18.
20. An isolated DNA molecule that codes for a modified TNF $\alpha$  molecule according to any one of claims 1-18.
21. A vector which comprises the isolated DNA molecule according to claim 20.
- 10 22. An expression vector which comprises the isolated DNA molecule according to claim 20 operatively linked to an expression control sequence.
23. A host, which is transformed with the expression vector of claim 22.
- 15 24. A host according to claim 23 which is selected from strains of bacteria, yeast, or other fungi and insect, mammalian or avian cell lines.
25. A method of producing a modified human TNF $\alpha$  molecule according to any one of claims 1-18, which comprises  
20 growing the host cells of claim 23 under suitable conditions permitting production of the modified TNF $\alpha$  and recovering the modified TNF $\alpha$  so produced.
26. A modified human TNF $\alpha$  molecule according to any of claims 1-18 in the form of a fusion protein with an adjuvant molecule, preferably an immunologically active adjuvant, such as GM-CSF, HSP70 or interleukin.  
25
27. A vaccine against TNF $\alpha$ , comprising an immunogenic amount of one or more modified human TNF $\alpha$  molecules according to any of claims 1-18 and optionally a pharmaceu-

tically acceptable adjuvant, such as aluminium phosphate, aluminium hydroxide, calcium phosphate, muramyl dipeptide or iscom.

28. A vaccine according to claim 27 for the prevention  
5 or treatment of diseases promoted by TNF $\alpha$  release or activity such as chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel diseases, including Crohn's disease and Colitis Ulcerosa, and cancer, disseminated sclerosis, diabetes, psoriasis, osteoporosis  
10 and asthma.

29. A vaccine against TNF $\alpha$  comprising isolated DNA which codes for the modified human TNF $\alpha$  molecule according to any one of claims 1-18 inserted in a suitable expression vector.

15 30. A vaccine according to claim 29 containing a construct comprising a non-infectious non-integrating DNA sequence encoding a modified TNF $\alpha$  molecule according to any of claims 1-18 operatively linked to a promoter sequence which can control the expression of said DNA sequence in humans, in an amount sufficient that uptake of  
20 said construct occurs, and sufficient expression occurs to induce a neutralizing antibody response against TNF $\alpha$ .

31. A vaccine according to claim 29, comprising a viral  
25 expression vector, such as a retroviral expression vector.

32. A vaccine according to any one of claims 27-31 for oral or parenteral, e.g. subcutaneous, intramuscular or intradermal administration.

33. The use of antibodies raised by administering a vaccine  
30 according to any one of claims 27-32, preferably monoclonal antibodies.

34. Diagnostic use of antibodies according to claim 33.
35. A method of testing human body fluids for the presence of TNF $\alpha$  which comprises contacting a composition containing modified TNF $\alpha$  according to any one of claims 1-18 with a sample of human body fluid and determining whether said antibodies bind to TNF $\alpha$  in said sample.
36. A diagnostic method for TNF $\alpha$ -related diseases employing an in vitro immunoassay to detect TNF $\alpha$  in human body fluids.
37. The method of claim 35 or 36 which comprises the use of a sandwich assay, ELISA assay or equivalent assay, which can be unamplified or amplified, e.g. using avidin/biotin technology.
38. A method for the treatment or prevention of diseases, the pathophysiology of which is at least partially due to TNF $\alpha$  release or activity comprising administering to an animal, including a human being, an effective amount of at least one modified TNF $\alpha$  molecule according to claim 1-18 optionally in combination with a suitable adjuvant or carrier molecule.
39. Use of a modified TNF $\alpha$  molecule for the preparation of a medicament for the treatment or prevention of diseases the pathophysiology of which is at least partially due to TNF $\alpha$  release or activity.

Crystal structure of TNF

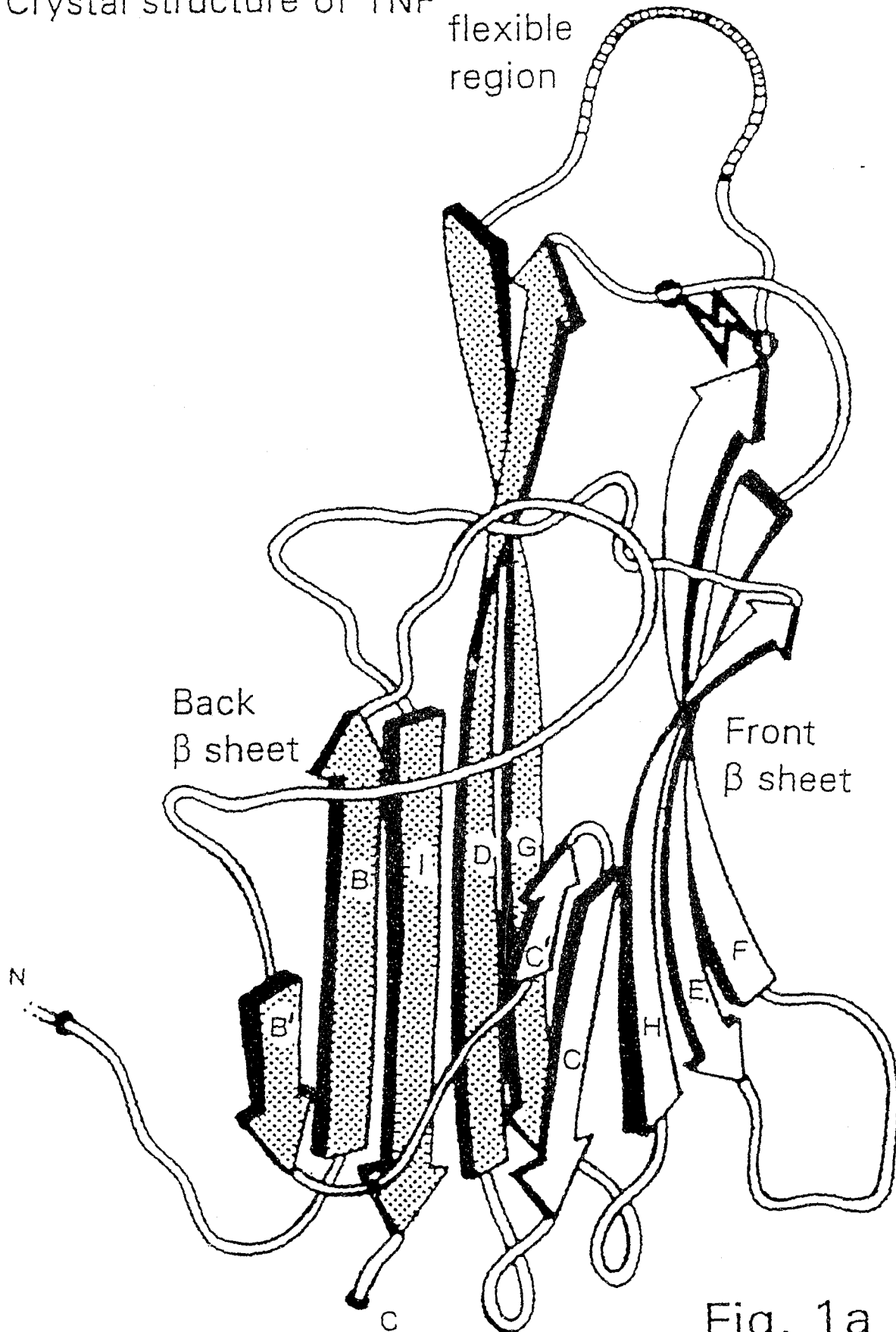


Fig. 1a

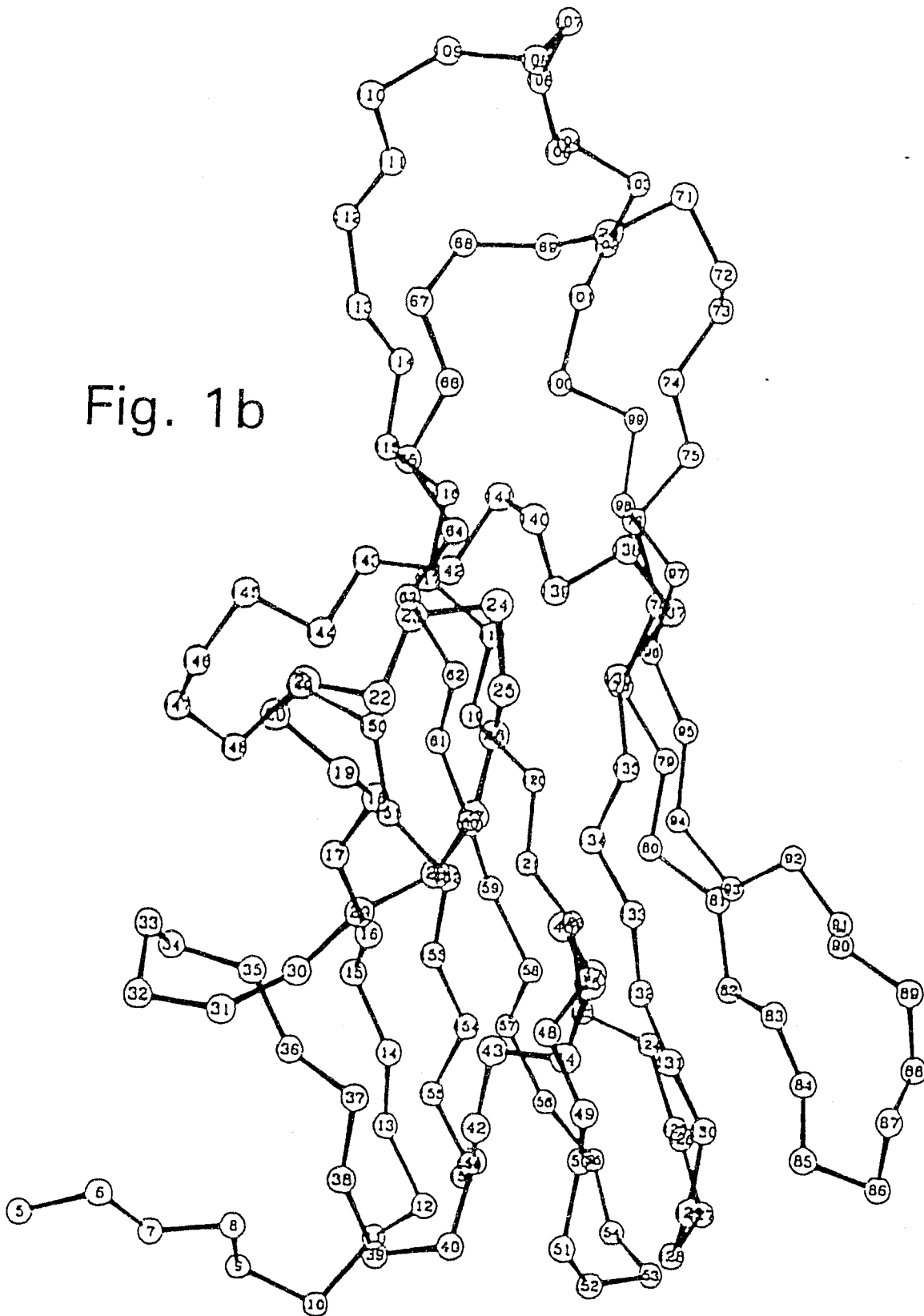


Fig. 1b



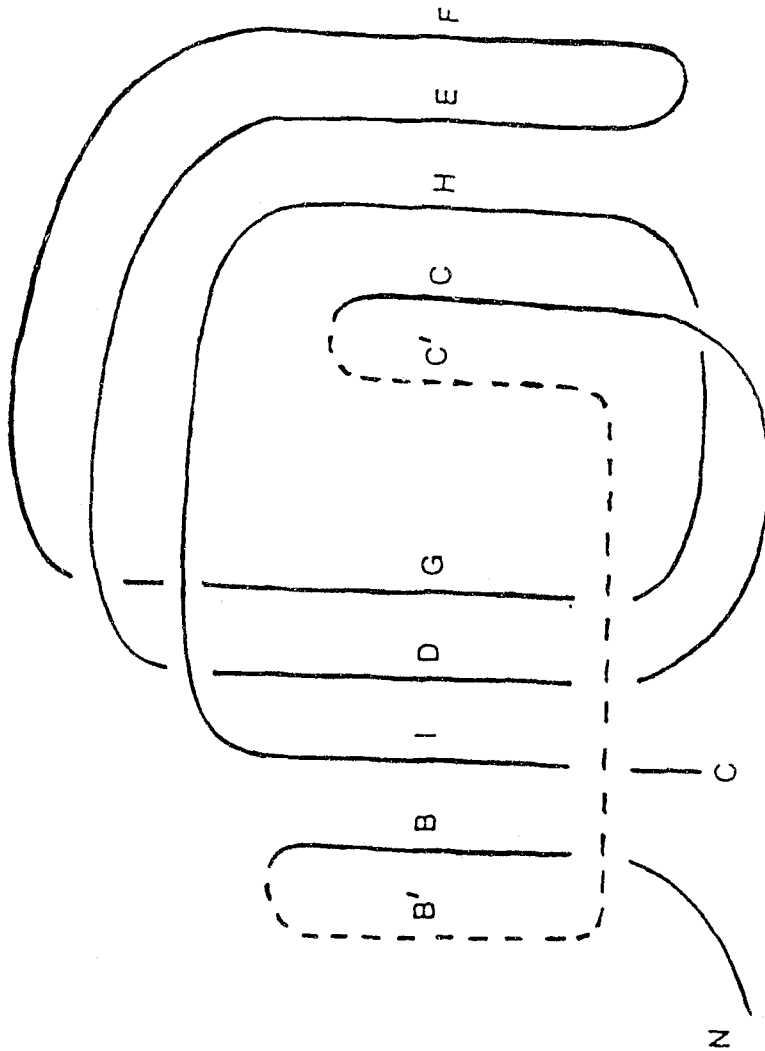
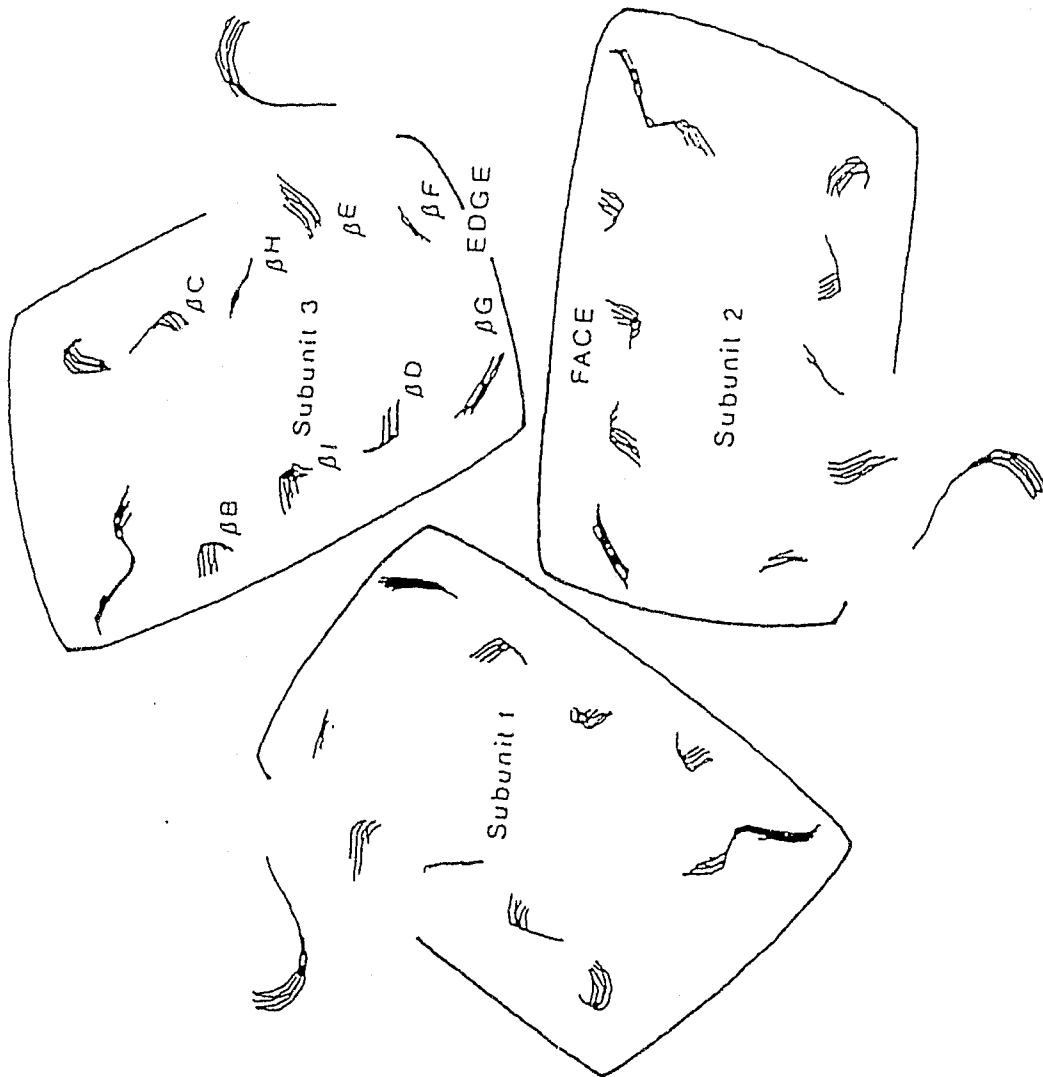


Fig. 1C

Fig. 2



Amino acid sequence for human TNF $\alpha$  8

Accession code: Swissprot P01375

-76 MSTESMIRDV ELAEEALPKK TGGPQGSRRC LFLSLESELI VAGATTLECL  
-26 LHEGVIGPQR EEFPROLSLI SPLAQA  
1 VRSSSRTPSD KPVAVVAVNP QAEGQLQWLN RRANALLANG VELRDNQLVV  
51 PSEGLYLIYS QVLFKGGQCP STHVLLTHTI SRIAVSYQTK VNLLSAIKSP  
101 CQRETPEGAE AKPWYEPIYL GGVFQLEKGD RLSAEINRPD YLDFAESGQV  
151 YFGIIAL

Conflicting sequence F->S at position -14. Disulphide bond between Cys69-101. Signal anchor sequence -41 to -21 (underlined). Myristylation on Lys-58/-57.

Fig. 3b

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## Fig. 3a

1 cacaccctga caagctgcc a ggcaggttct cttoctctca catactgacc  
 51 caccggtcca cctctctctc cctggaaagg acaccatgag cactgaaagc  
 101 atgatccggg acgtggagct ggccgaggag gcgctcccca agaagacagg  
 151 ggggccccag ggtccaggc ggtgcttggt cctcagcctc ttctccttcc  
 201 tgatcgtggc aggcgccacc acgctcttct gectgctgca ctttggagtg  
  
 251 atcggccccc agaggaaga gtcccccagg gacctctctc taatcagccc  
 301 tctggcccag gcagtcagat catcttctcg aaccccaggt gacaagcctg  
 351 tagcccatgt tgtagcaaac cctcaagctg aggggcagct ccagtggctg  
 401 aaccgcggg ccaatgccct cctggccaat ggcgtggagc tgagagataa  
 451 ccagctggtg gtgccatcag agggcctgta cctcatctac tcccaggtcc  
  
 501 tcttcaaggg ccaaggetgc cctccacc c atgtgctcct caccacacc  
 551 atcagccgca tcgccgtctc ctaccagacc aaggtcaacc tcctctctgc  
 601 catcaagagc cctgcccaga gggagacccc agagggggct gaggccaagc  
 651 cctggtatga gccatctat ctgggagggg tcttccagct ggagaagggt  
 701 gaccgactca gcgctgagat caatcggccc gactatctcg accttgccga  
  
 751 gtctgggcag gtctactttg ggatcattgc cctgtgagga ggacgaacat  
 801 ccaaccttcc caaacgcctc cctgccccca atccctttat taccctctcc  
 851 ttcagacacc ctcaacctct tctggctcaa aaagagaatt gggggcttag  
 901 ggtcgggaacc caagcttaga actttaagca acaagaccac cacttcgaaa  
 951 cctgggatc agaatgtgt ggcctgcaca gtgaagtgt ggcaaccact  
  
 1001 aagaattcaa actggggcct ccagaactca ctggggccta cagctttgat  
 1051 ccctgacatc tggaatctgg agaccagga gcctttggtt ctggccagaa  
 1101 tgetgcagga cttgagaaga cctcacctag aaattgacac aagtggacct  
 1151 taggccttcc tctctccaga tgtttccaga cttccttgag acacggagcc  
 1201 cagccctccc catggagcca gctccctcta tttatgtttg cacttgtgat  
  
 1251 tatttattat ttattatta tttatttatt tacagatgaa tgtatttatt  
 1301 tgggagaccg gggtatcctg ggggaccnaa tgtaggagct gcottggctc  
 1351 agacatgttt tccgtgaaaa cggaggctga acaataggct gtteccatgt  
 1401 ageccccctgg cctctgtgcc ttcttttgat tatgtttttt aaaatattat  
 1451 ctgattaagt tgtctaaaca atgctgattt ggtgaccaac tgtcactcat  
  
 1501 tgetgaggcc tctgetcccc agggagtgtg gtetgtaatc ggcctactat  
 1551 tcagtggcga gaaataaagg ttgcttagga aagaa

# Location of inserted epitopes

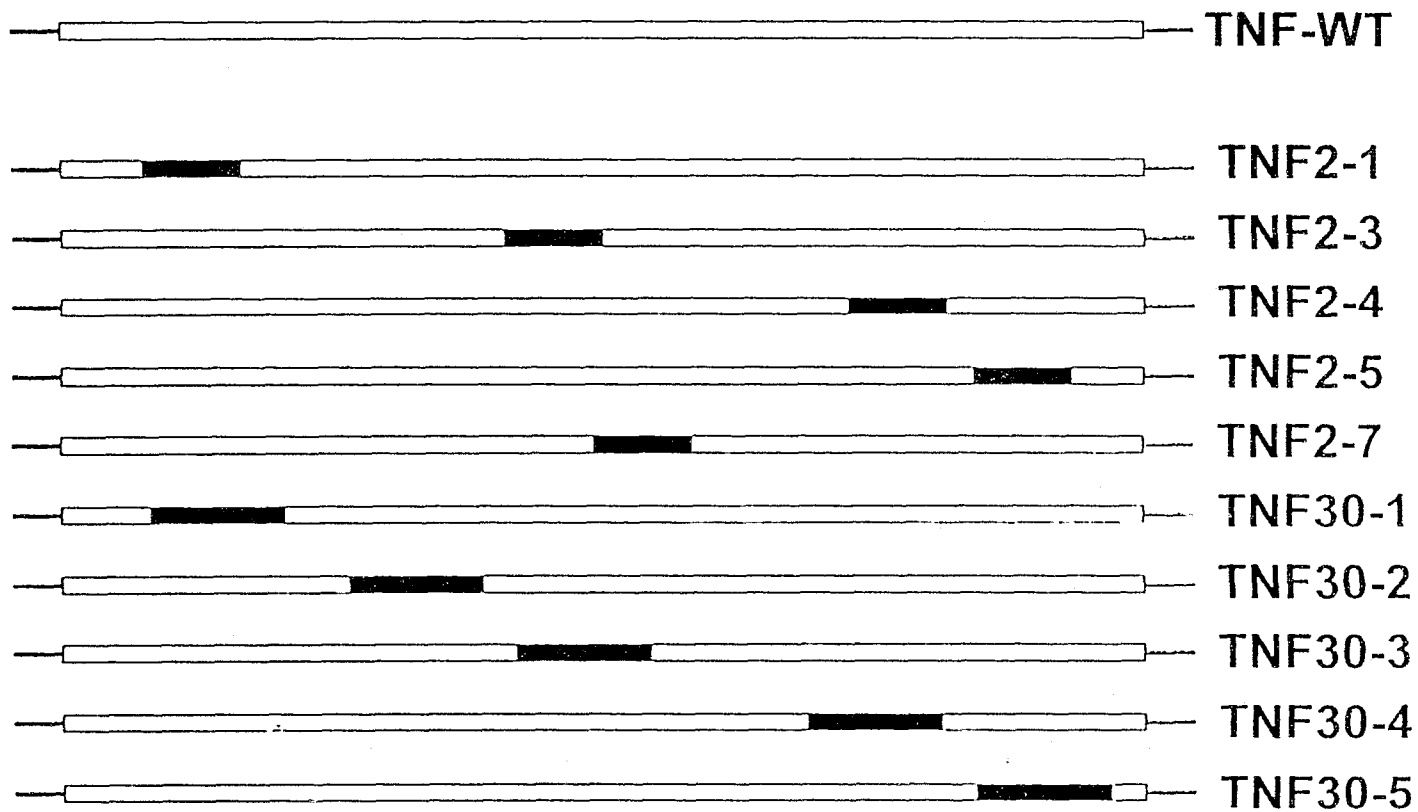


Fig. 4a

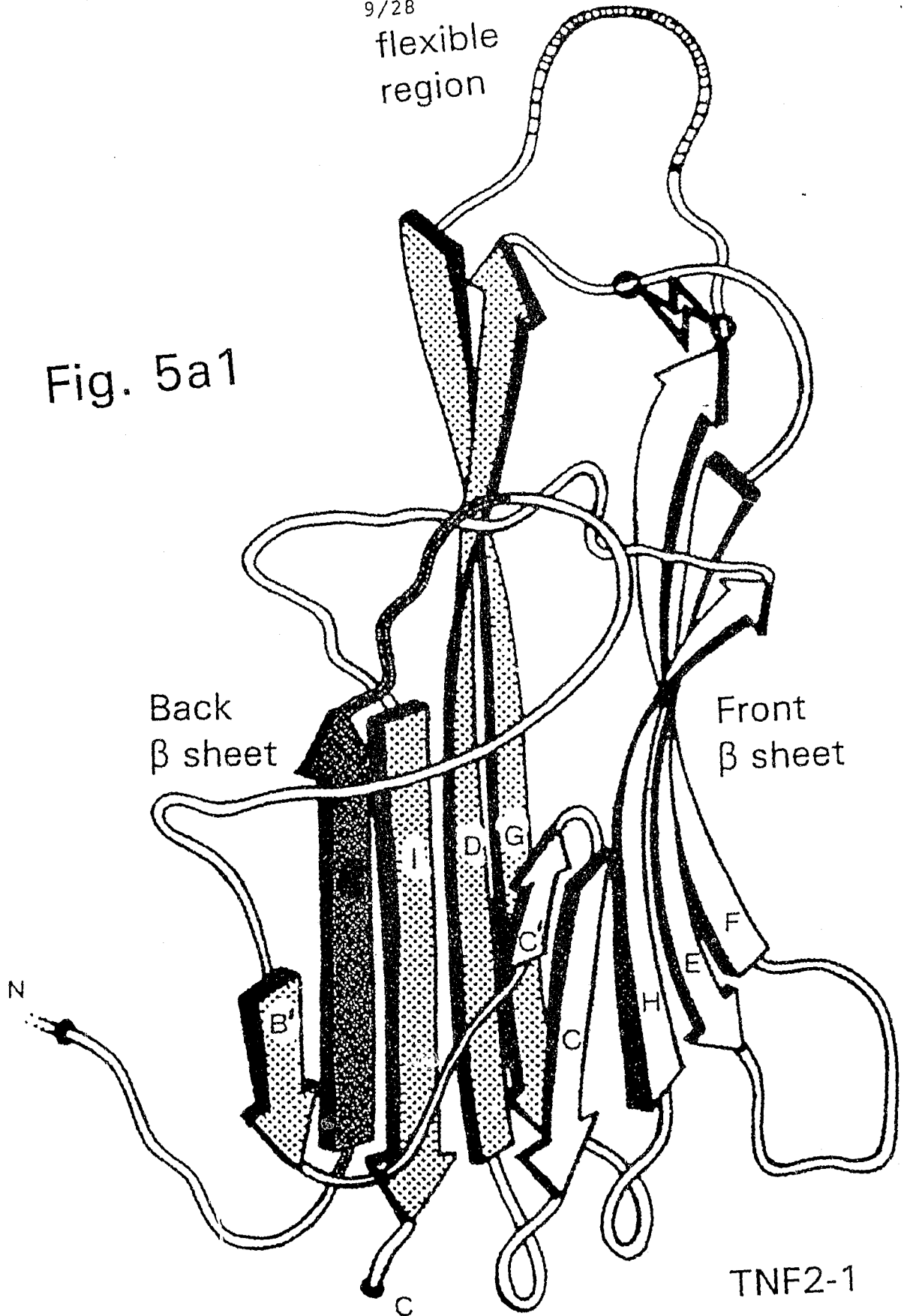
#	1	11	21	31	41	51	61	71
WT	VRSSSRTPSD	KPVAHVVANP	QAEGQLQWLN	RRANALLANG	VELRDNQLVV	PSEGLYLIYS	QVLFKQGQCP	STHVLLTHTI
2-1	-----Q	YIKANSKFIG	ITEL-----	-----	-----	-----	-----	-----
2-3	-----	-----	-----	-----	-----	-----	-----	-----
2-4	-----	-----	-----	-----	-----	-----	-----	-----
2-5	-----	-----	-----	-----	-----	-----	-----	-----
2-7	-----	-----	-----	-----	-----	-----	-----	-----
30-1	-----F	NNFTVSFWLR	VPKVSASHLE	-----	-----	-----	-----	-----
30-2	-----	-----	-----	-----	-----	-----	-----	-----
30-3	-----	-----	-----	-----	-----	-----	-----	-----
30-4	-----	-----	-----	-----	-----	-----	-----	-----
30-5	-----	-----	-----	-----	-----	-----	-----	-----

#	81	91	101	111	121	131	141	151
WT	SRIAVSYQTK	VNLLSAIKSP	CQRETPEGAE	AKPWYEPIYL	GGVFQLEKGD	RLSAEINRPD	YLDFAESGQV	YFGIIAL
2-1	-----	-----	-----	-----	-----	-----	-----	-----
2-3	-----	-----	-----	-----	-----	-----	-----	-----
2-4	-----	-----	-----	-----	-----	-----	-----	-----
2-5	-----	-----	-----	-----	-----	-----	-----	-----
2-7	NSKFIGITEL	-----	-----	-----	-----	-----	-----	-----
30-1	-----	-----	-----	-----	-----	-----	-----	-----
30-2	-----	-----	-----	-----	-----	-----	-----	-----
30-3	SHLE-----	-----	-----	-----	-----	-----	-----	-----
30-4	-----	-----	-----	-----	-----	-----	-----	-----
30-5	-----	-----	-----	-----	-----	-----	-----	-----

Fig. 4b

9/28  
flexible  
region

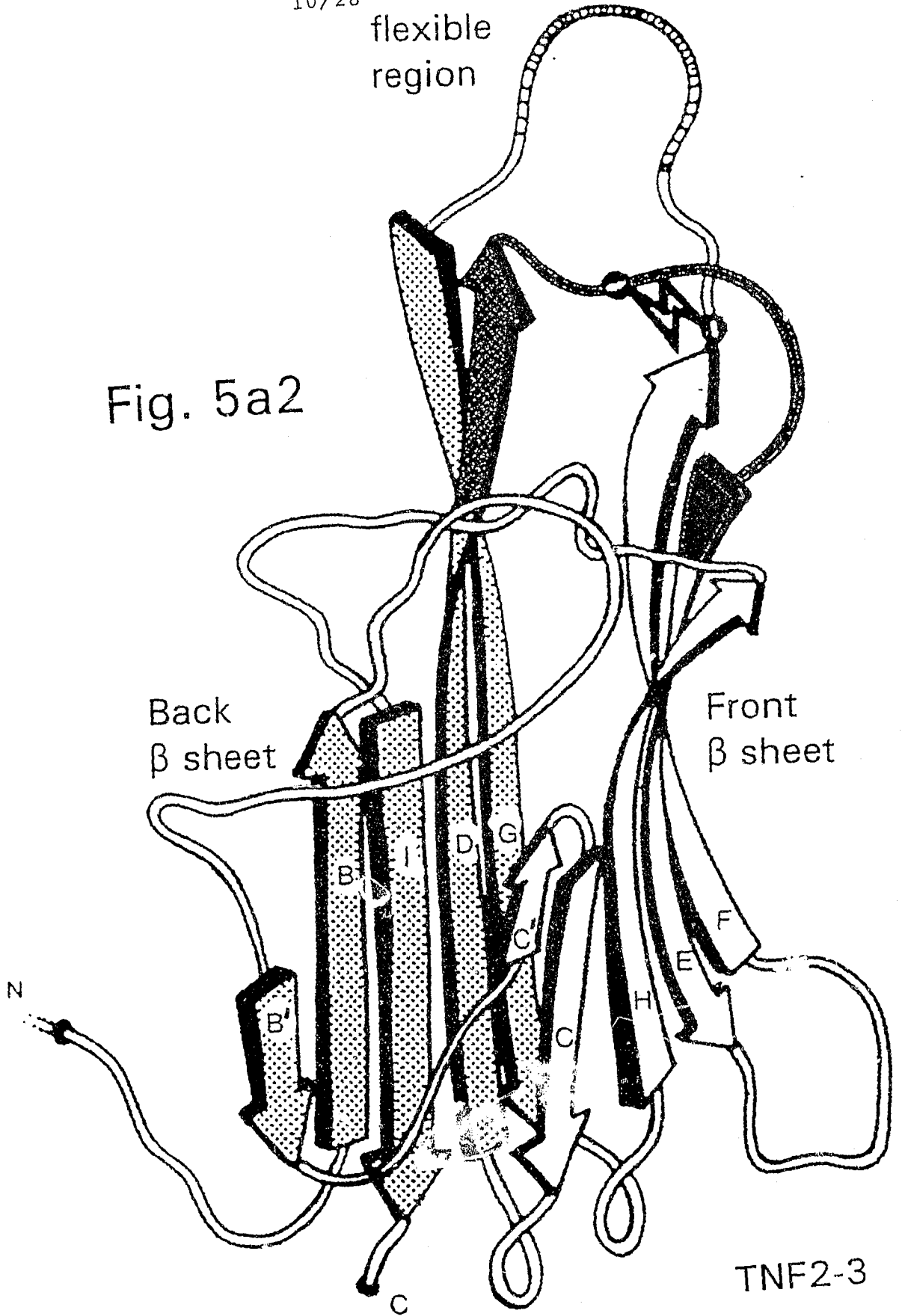
Fig. 5a1



10/28

flexible  
region

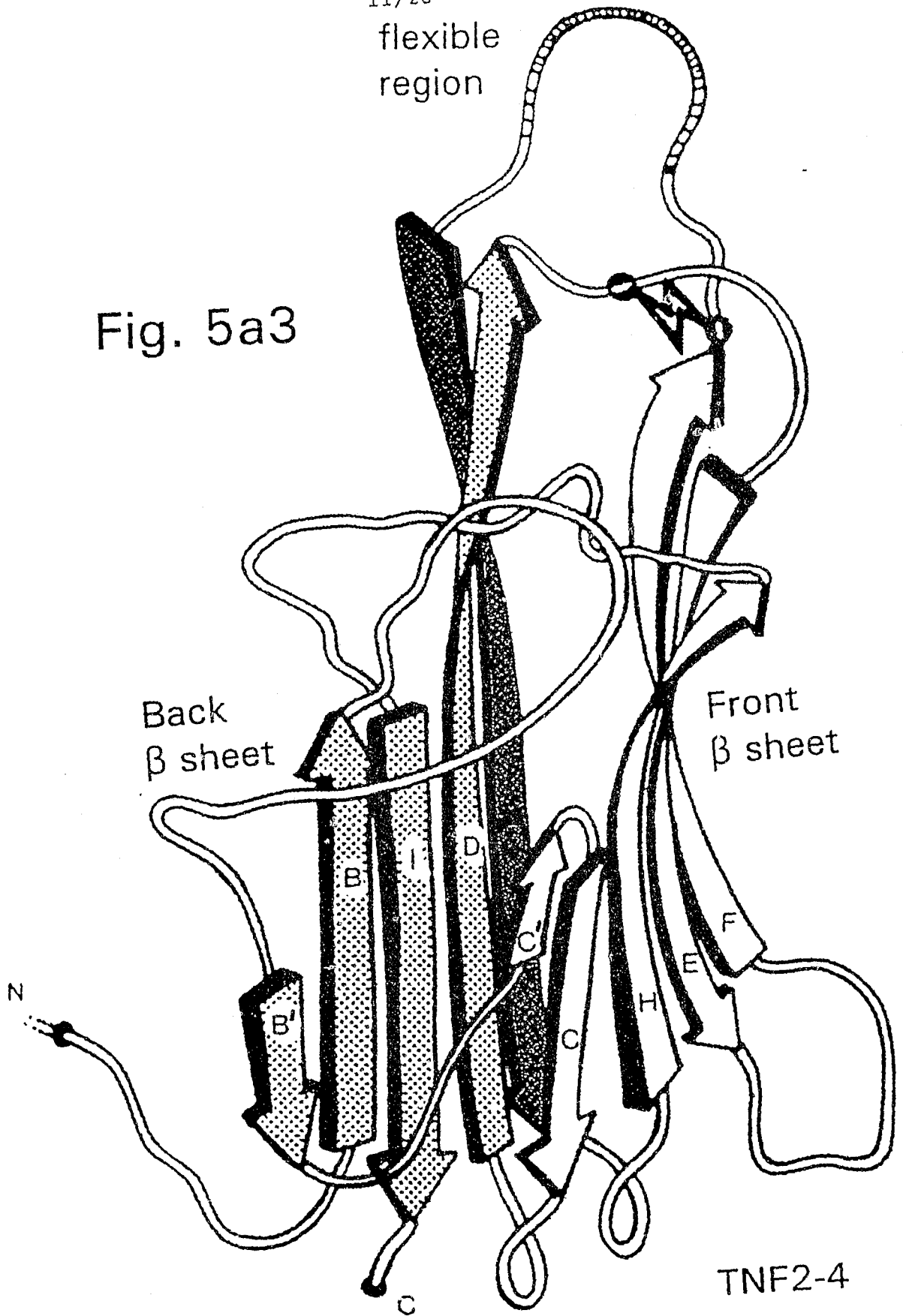
Fig. 5a2





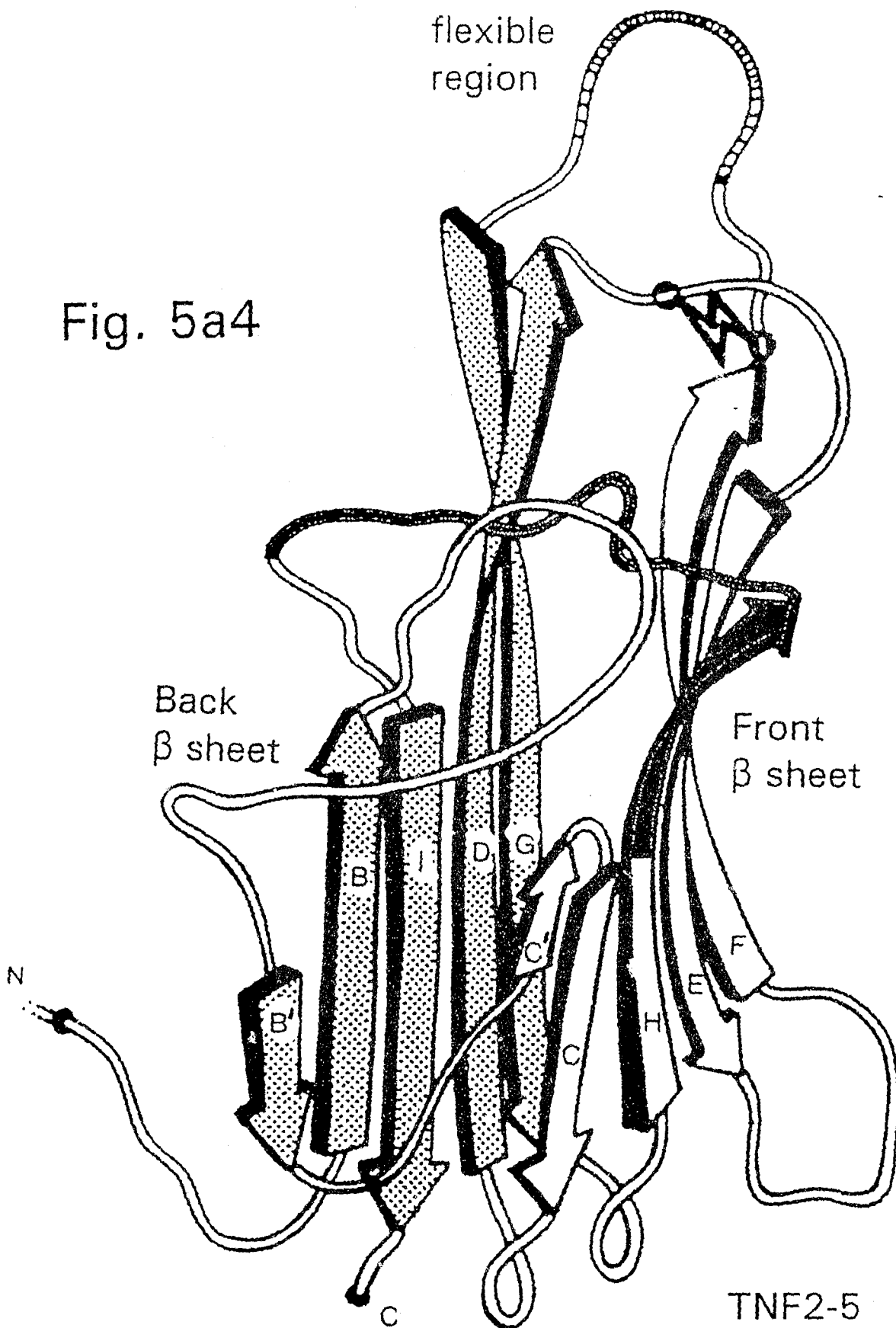
11/28  
flexible  
region

Fig. 5a3



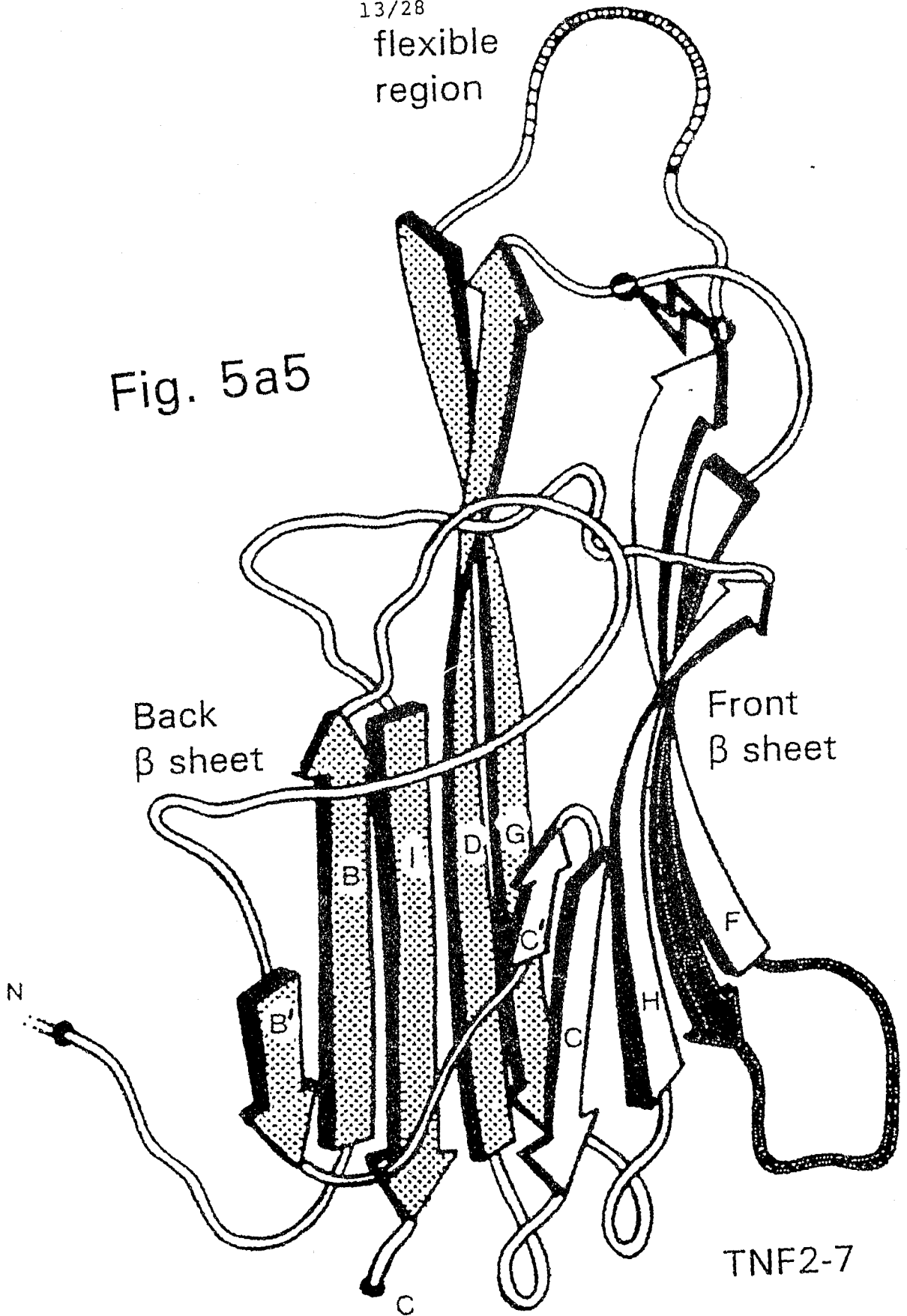
12/28  
flexible  
region

Fig. 5a4



13/28  
flexible  
region

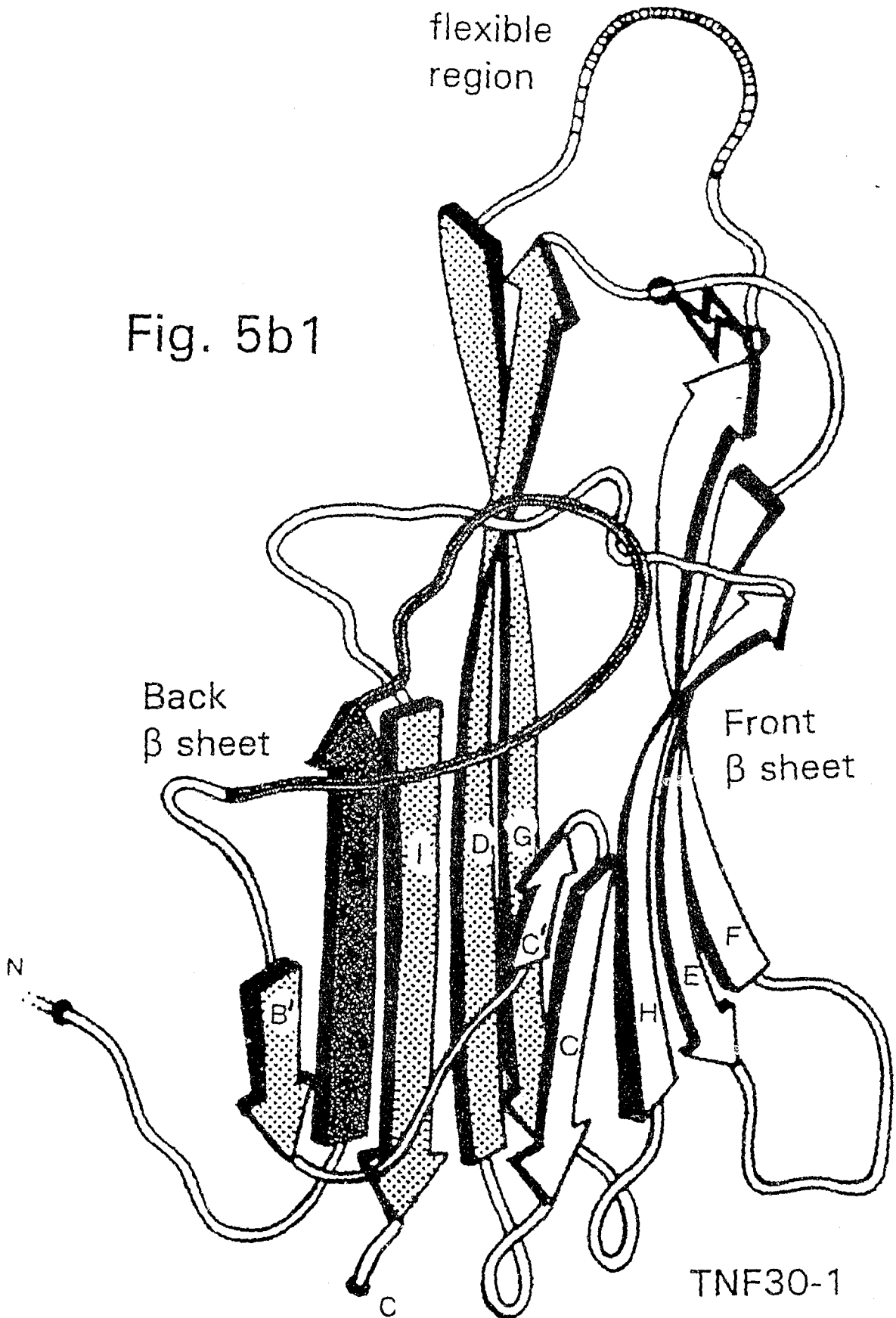
Fig. 5a5



TNF2-7

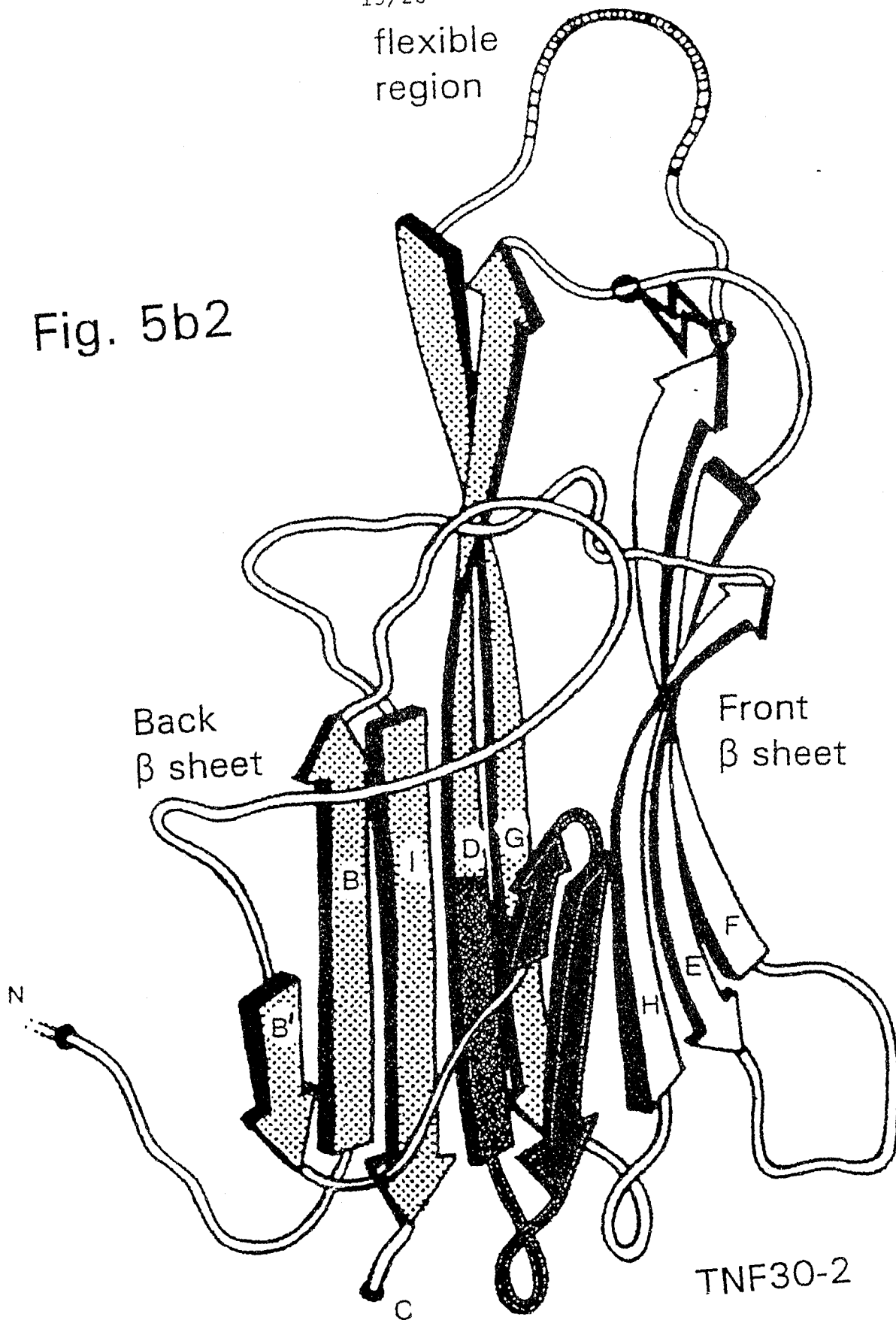
14/28  
flexible  
region

Fig. 5b1



15/28  
flexible  
region

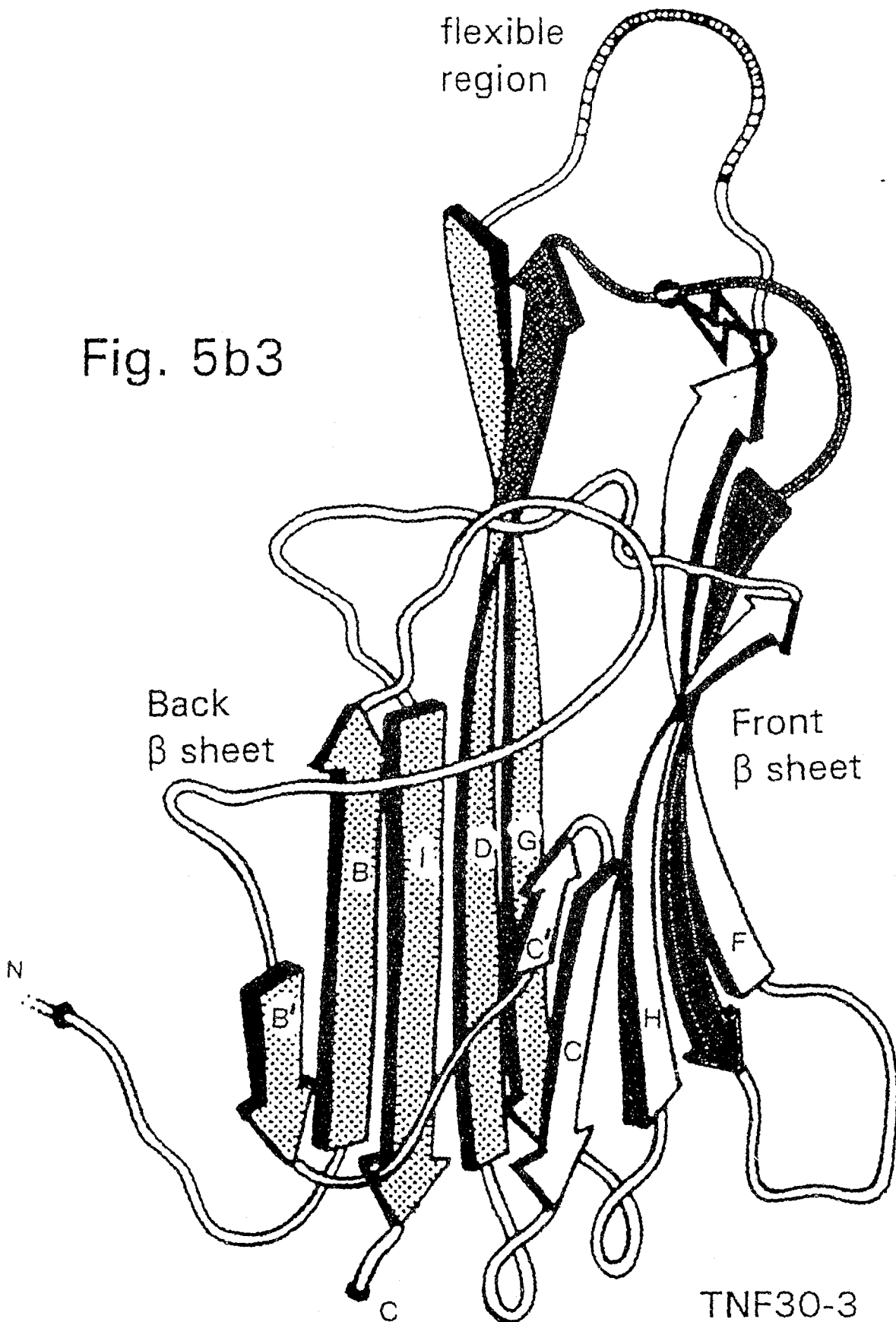
Fig. 5b2



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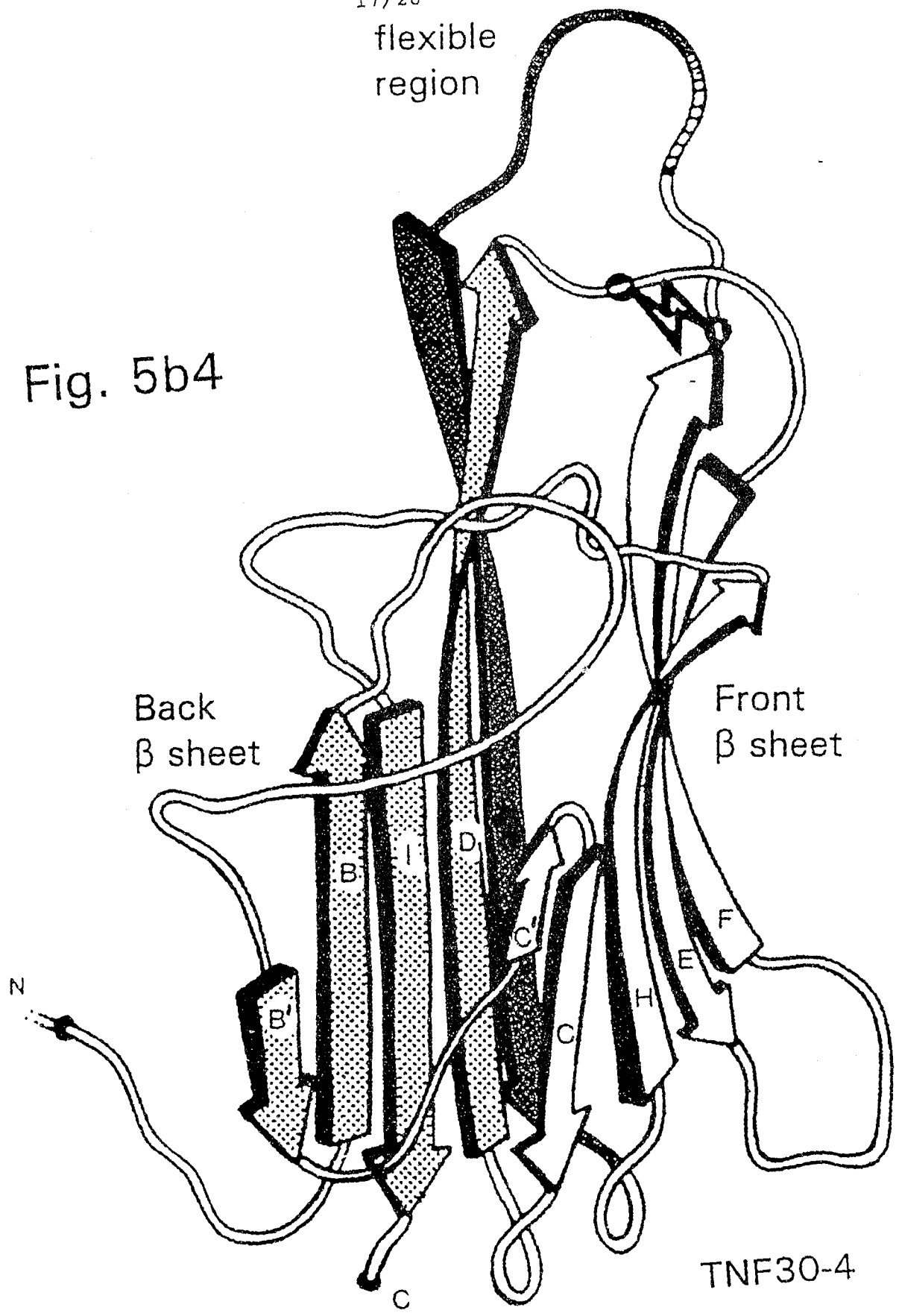
flexible  
region

Fig. 5b3



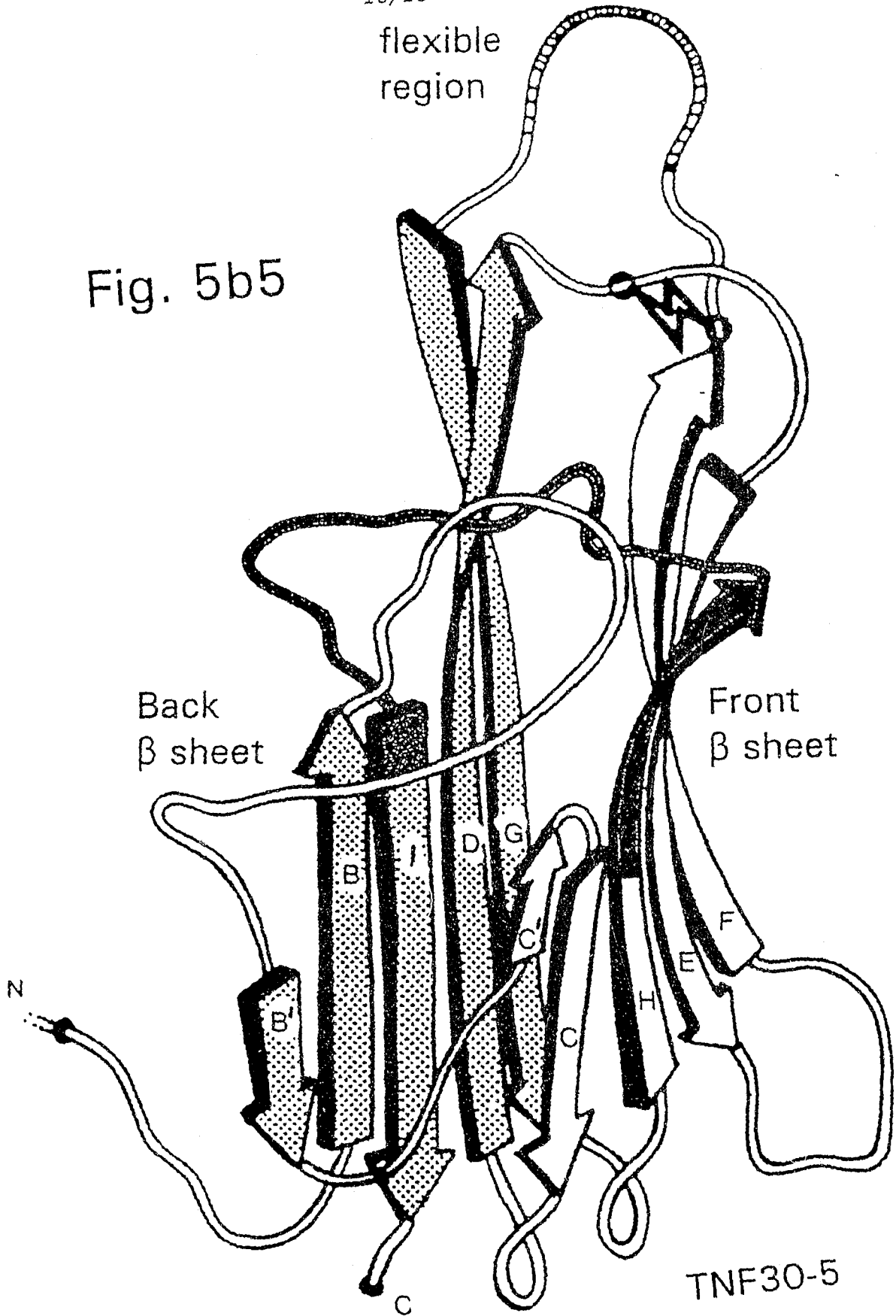
17/28  
flexible  
region

Fig. 5b4



18/28  
flexible  
region

Fig. 5b5





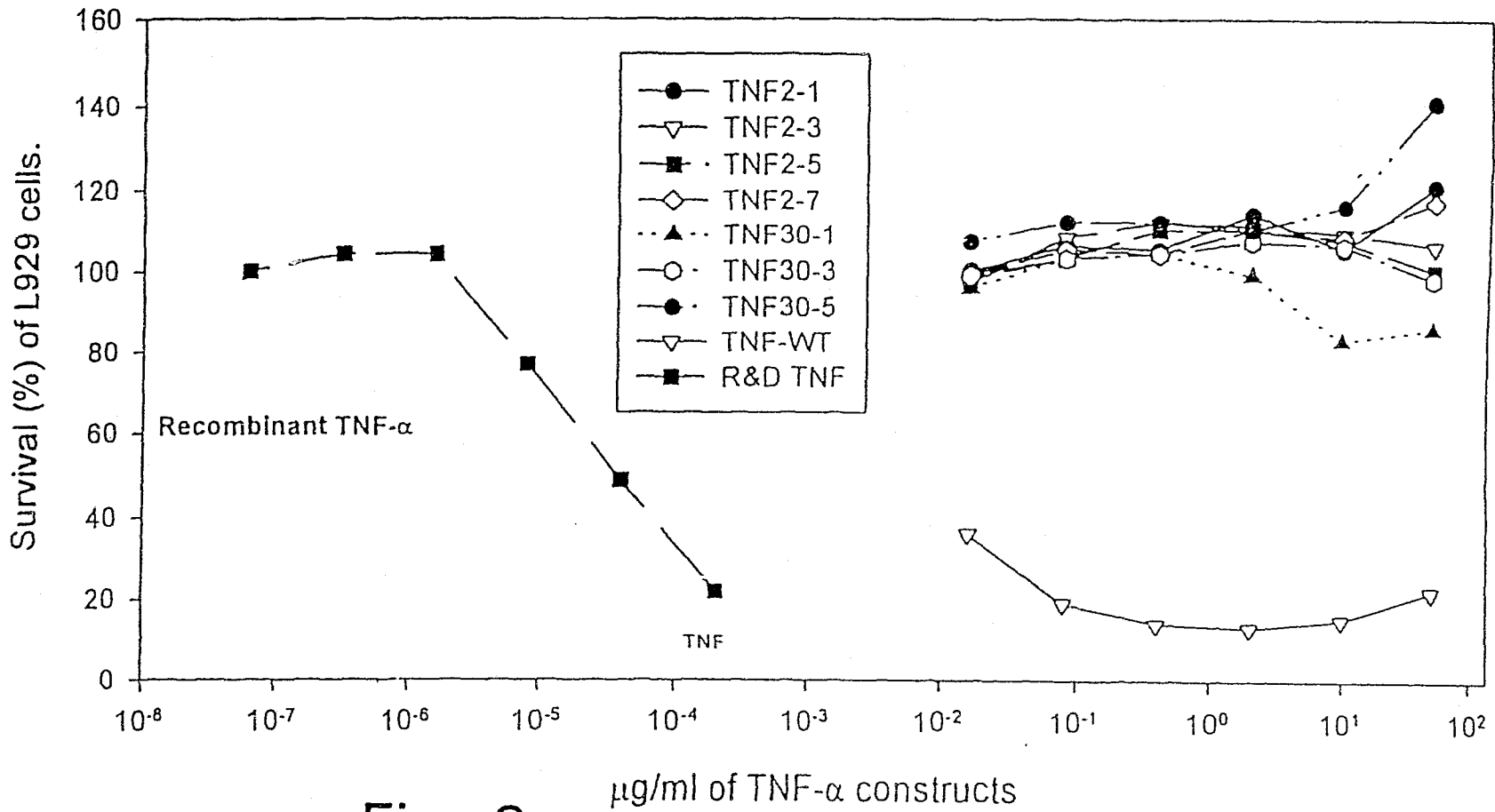


Fig. 6

The anti human TNF $\alpha$  antibody response in rabbits.

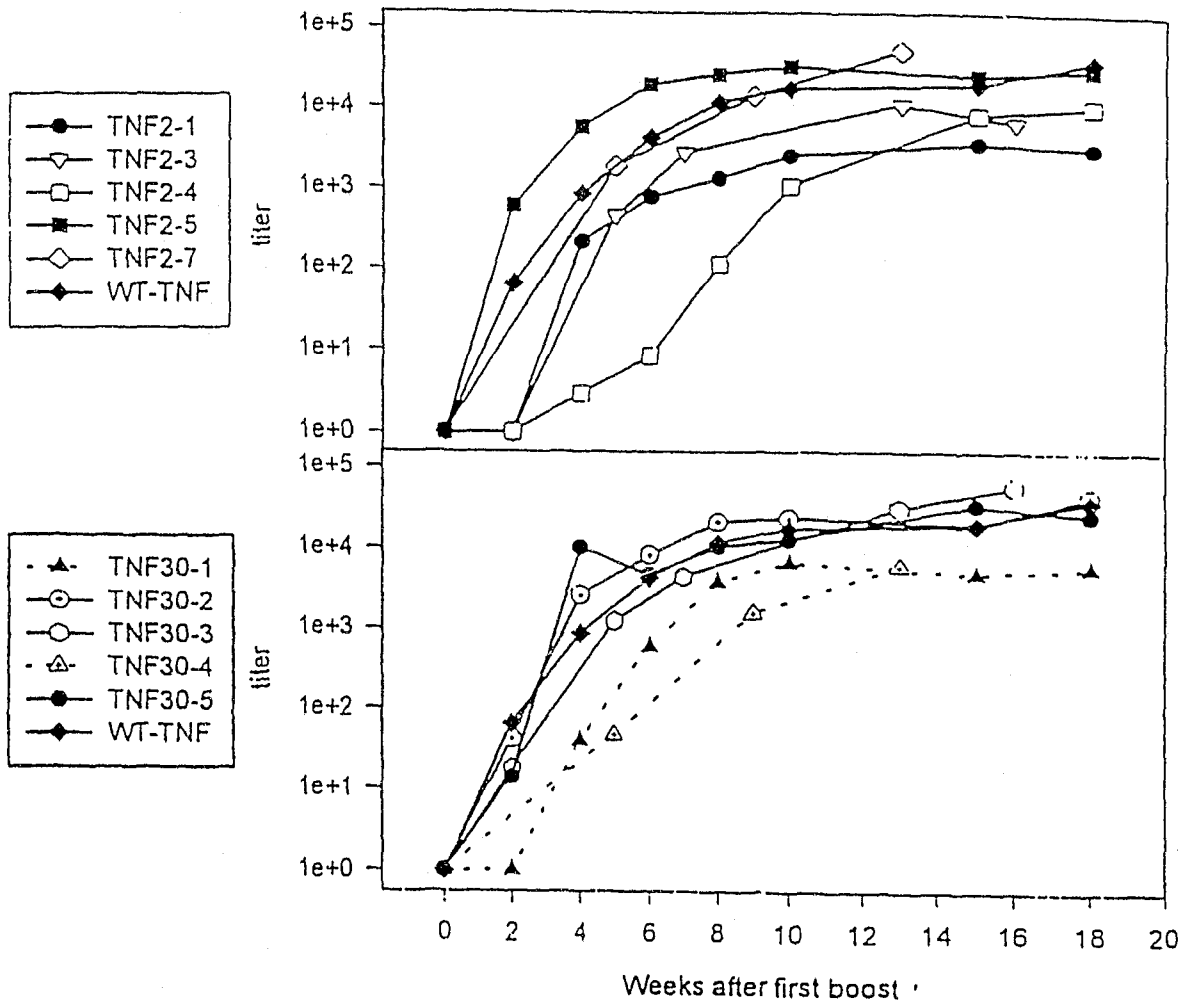


Fig. 7

The ability of P2/P30 modified human TNF $\alpha$  molecules to induce neutralizing antibodies as measured in the L929 cell assay.

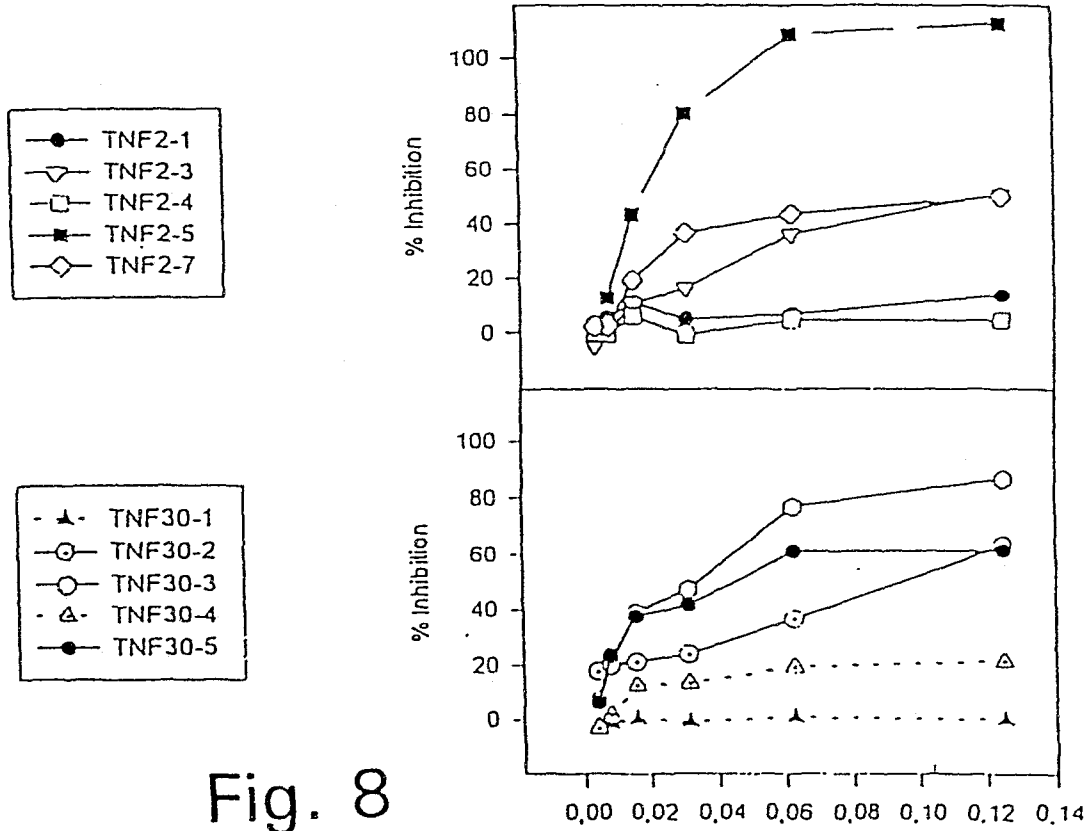


Fig. 8

The ability of P2/P30 modified human TNF $\alpha$  molecules to induce neutralizing antibodies as measured in the receptor assay

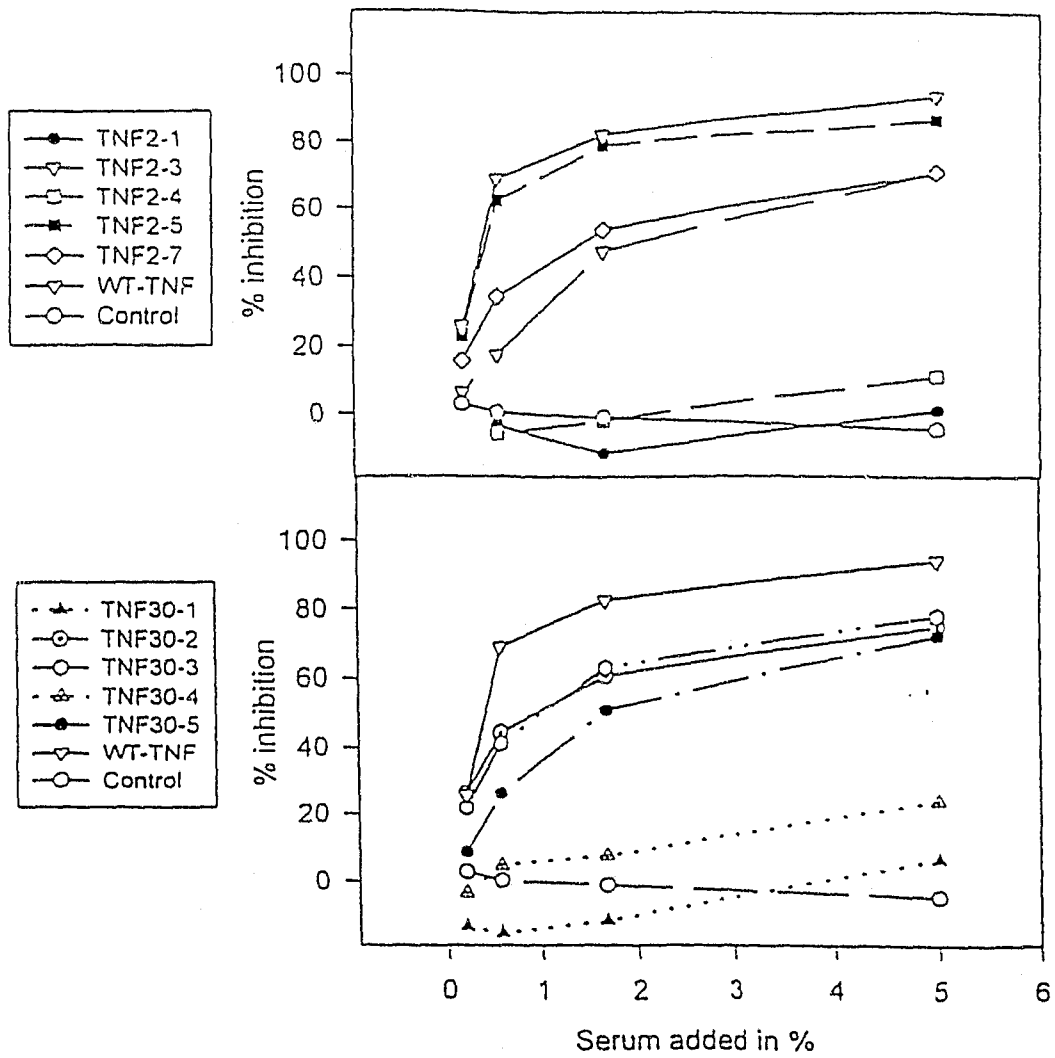


Fig. 9

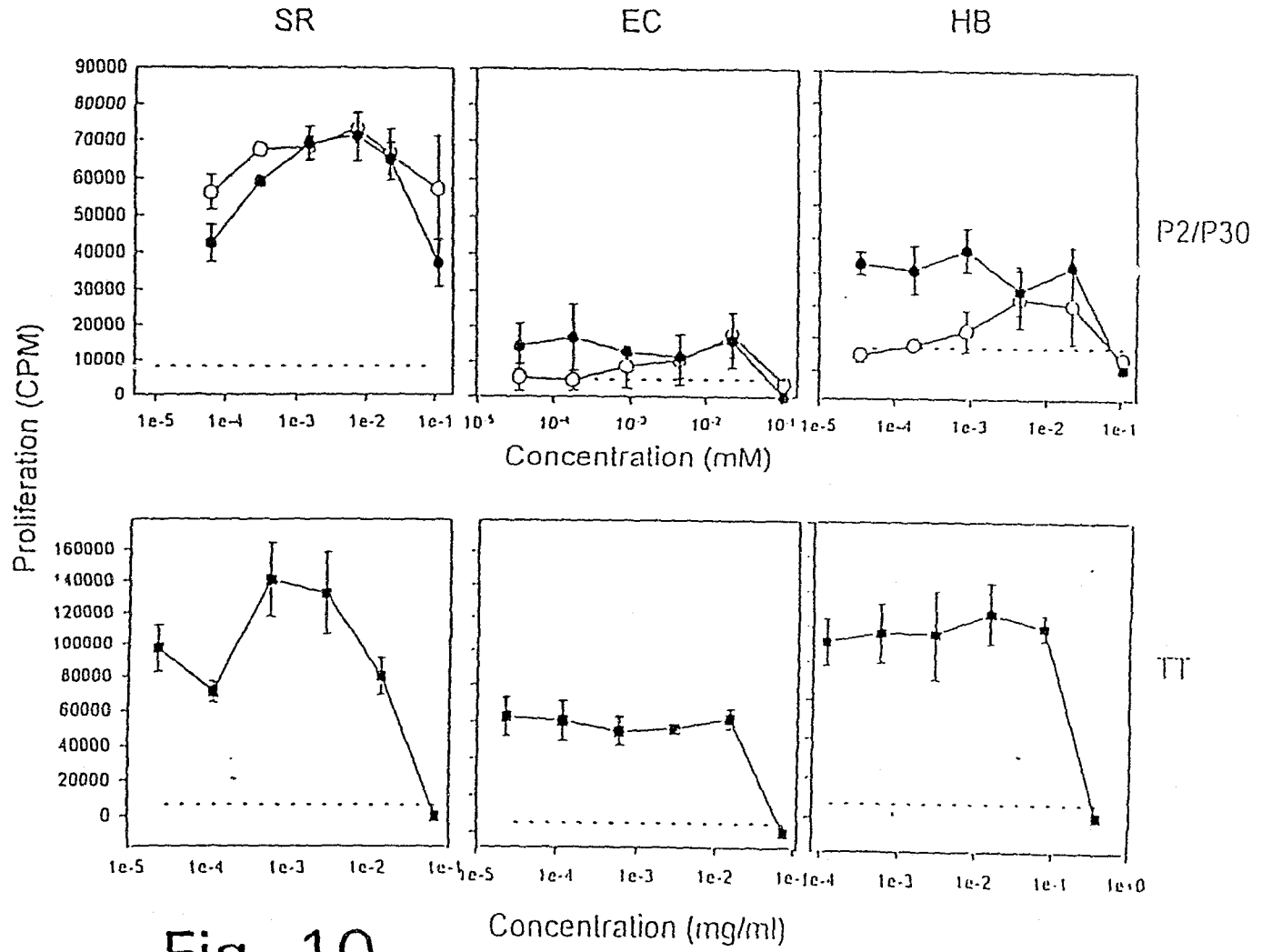


Fig. 10

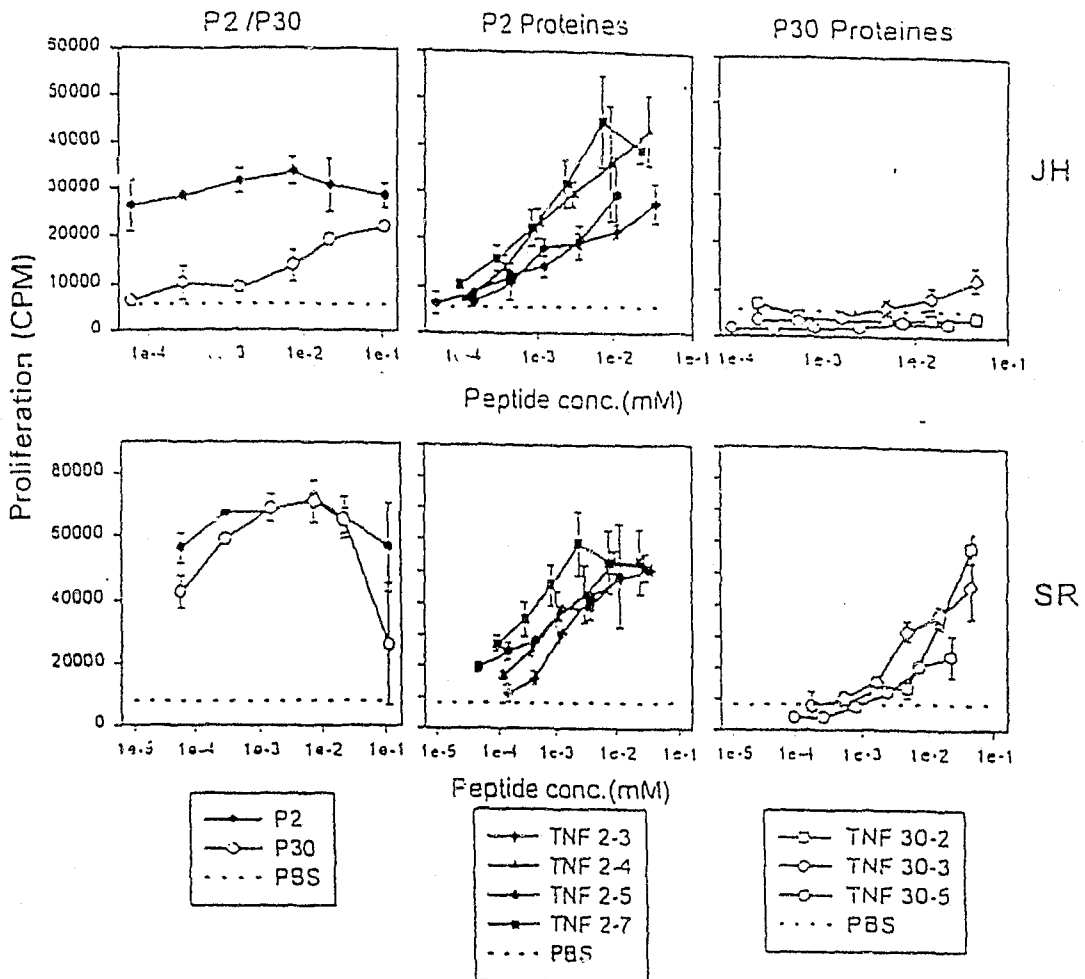


Fig. 11

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PBMC Assays

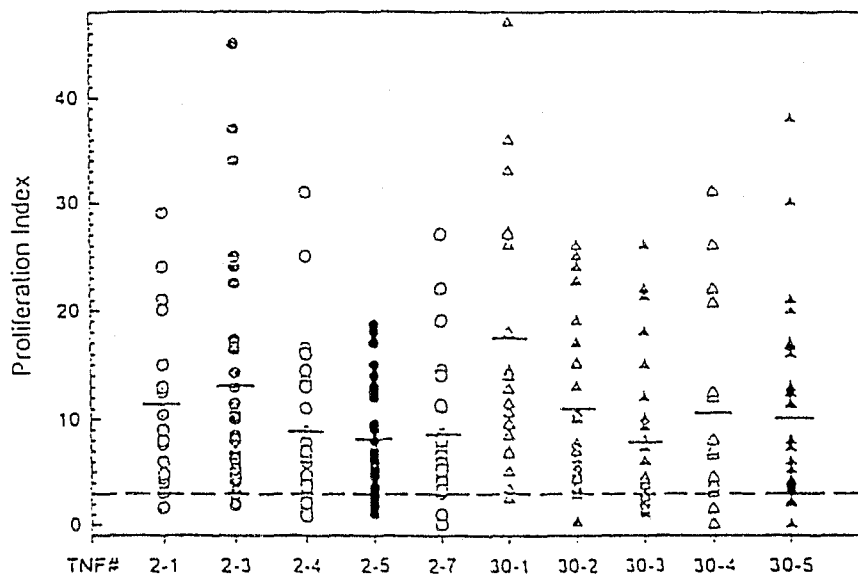


Fig. 12

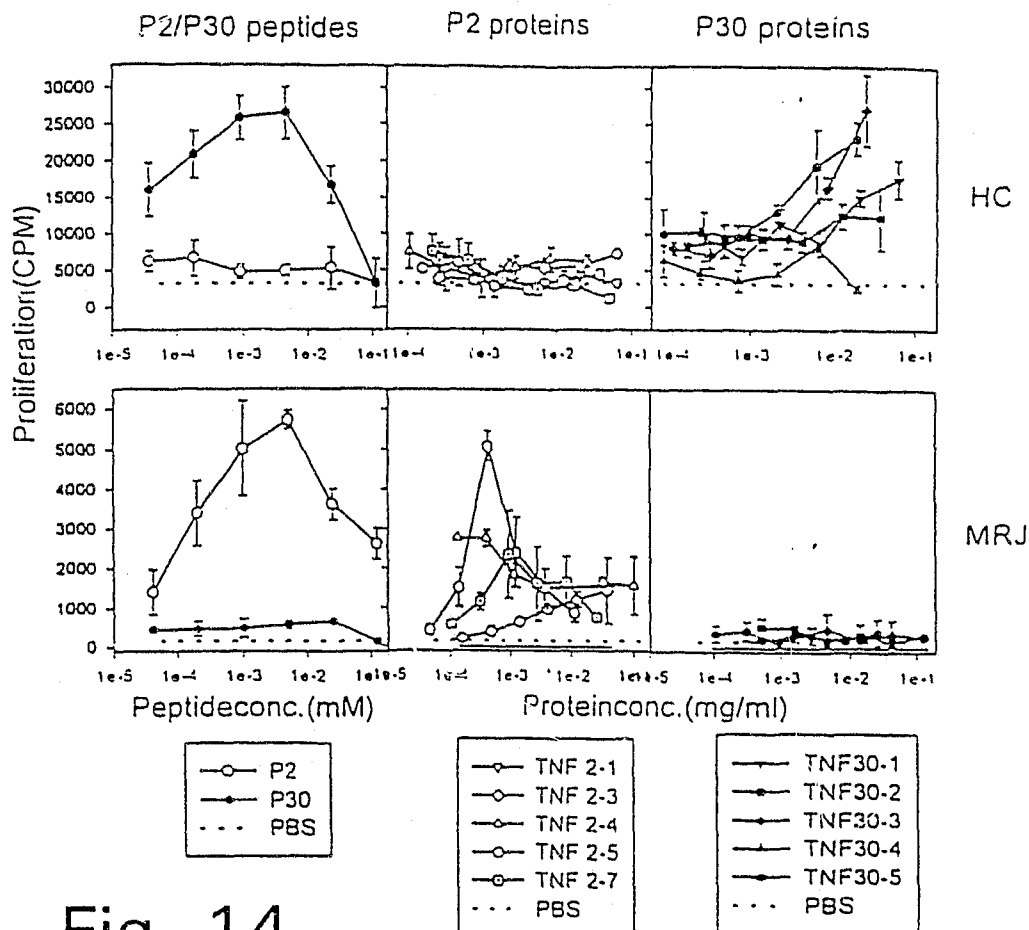


Fig. 14

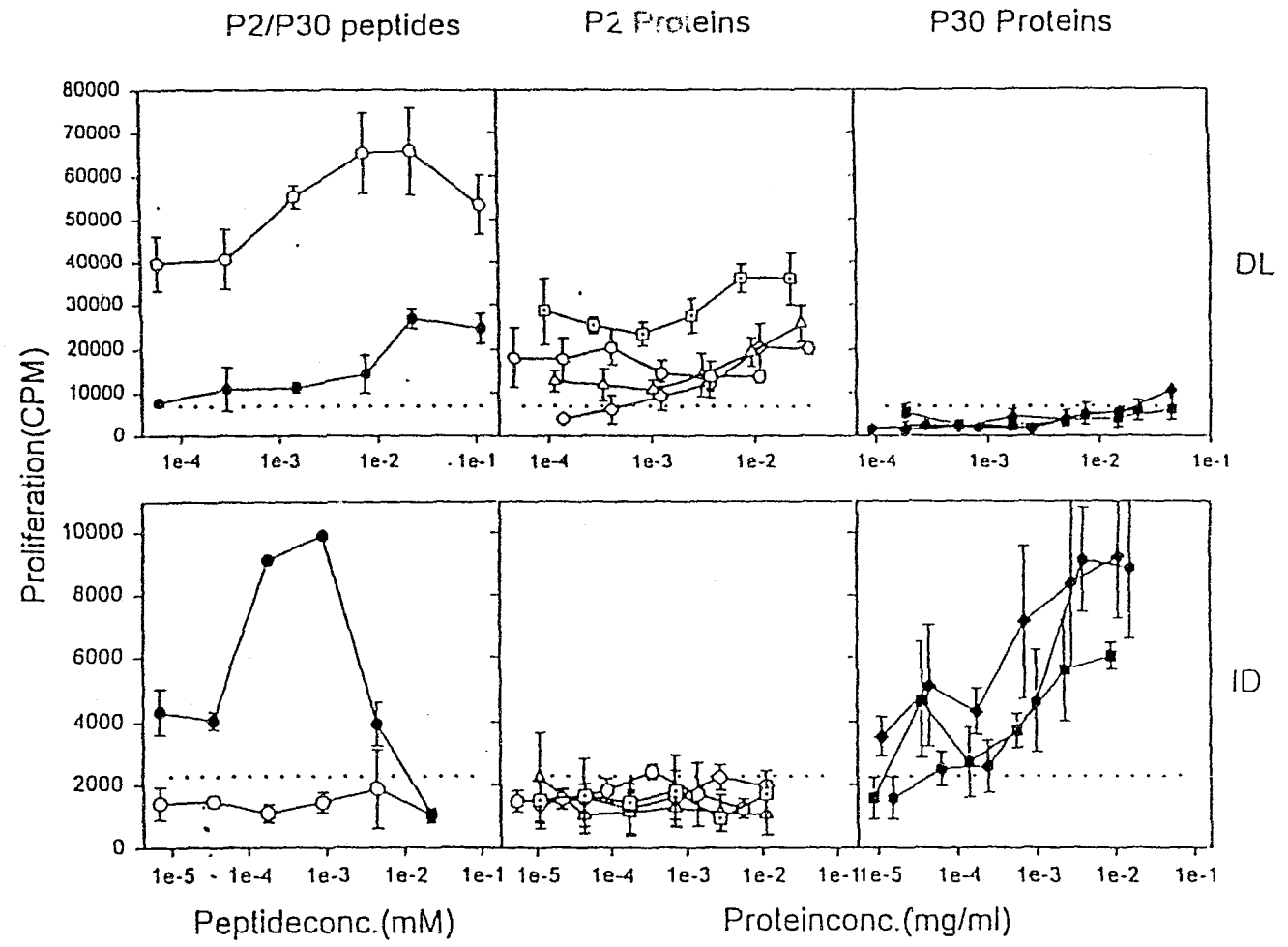
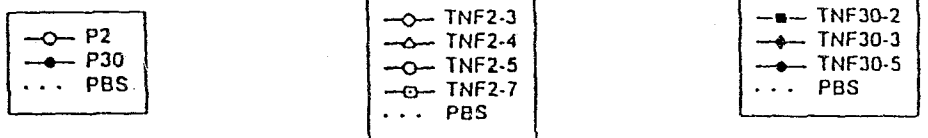


Fig. 13





The influence of flanking amino acids on the T cell recognition of P2 and P30

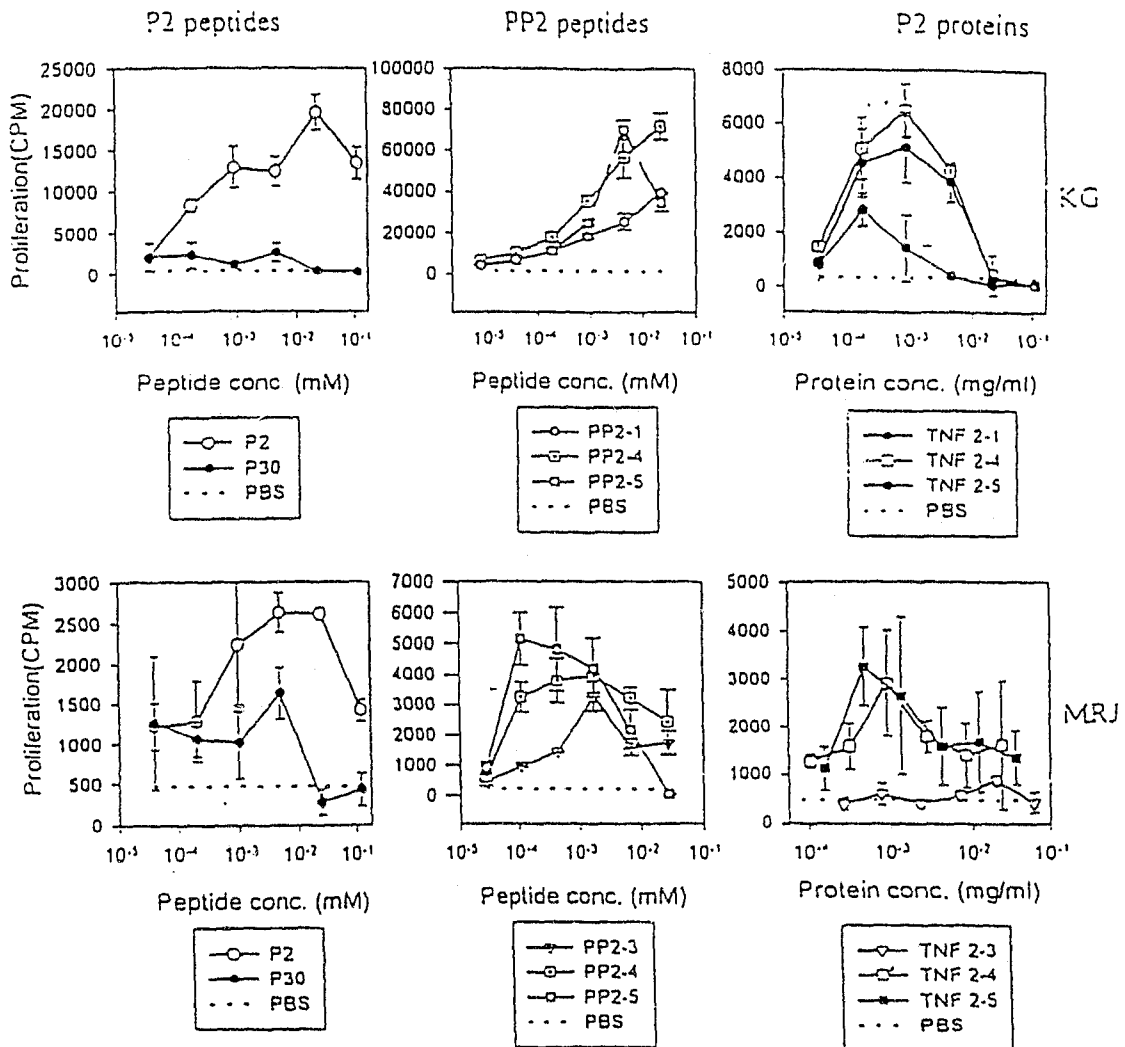
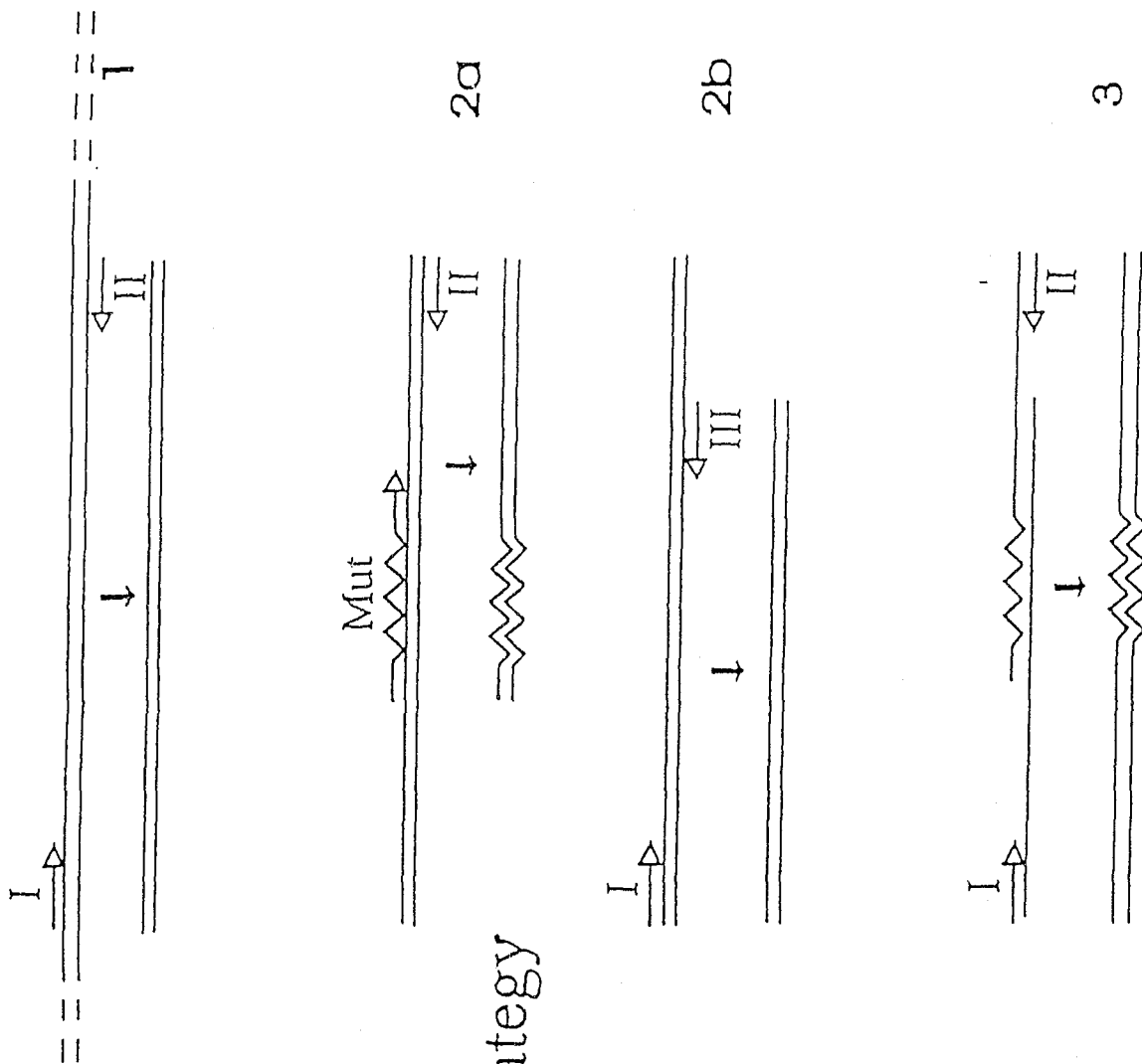


Fig. 15



Mutation Strategy

Fig. 16

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK 98/00157

A. CLASSIFICATION OF SUBJECT MATTER		
IPC6: C07K 14/525, C07K 19/00 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: C07K, A61K, C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, MEDLINE, BIOSIS, DBA, CA		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9505849 A1 (MOURITSEN & ELSNER A/S), 2 March 1995 (02.03.95), page 10, line 19 - line 22; page 6, line 29 - page 7 line 3 and the whole document --	1-37
A	EP 0251037 A2 (DAINIPPON PHARMACEUTICAL CO., LTD), 7 January 1988 (07.01.88), the claims --	1-37
A	WO 9007579 A1 (BASF AKTIENGESELLSCHAFT), 12 July 1990 (12.07.90), the claims --	1-37
A	EP 0619372 A1 (F. HOFFMAN-LA ROCHE & CO.), 12 October 1994 (12.10.94), the claims --	1-37
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "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 to the international filing date but later than the priority date claimed "I" 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 when the document is taken alone "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		
Date of the actual completion of the international search		Date of mailing of the international search report
31 July 1998		06-08-1998
Name and mailing address of the ISA Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00157

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

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

1.  Claims Nos.: 38-39  
because they relate to subject matter not required to be searched by this Authority, namely:  
Methods for treatment of the human or animal body by therapy, see PCT Rule 39(iv).
2.  Claims Nos.:  
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.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

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.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  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 claims Nos.:

Remark on Protest

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

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT  
Information on patent family members

International application No.  
PCT/DK 98/00157

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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