The present invention relates to ligands which bind to human tumour necrosis factor alpha (TNF) in a manner such that upon binding of these ligands to TNF the biological activity of TNF is modified. In preferred forms the ligand binds to TNF in a manner such that the induction of endothelial procoagulant activity of the TNF is inhibited; the binding of TNF to receptors on endothelial cells is inhibited; the induction of fibrin deposition in the tumour and tumour regression activities of the TNF are enhanced; and the cytotoxicity and receptor binding activities of the TNF are unaffected or enhanced on tumour cells. The ligand is preferably an antibody, F(ab) fragment, single domain antibody (dABs) single chain antibody or a serum binding protein. It is preferred, however, that the ligand is a monoclonal antibody or F(ab) fragment thereof.
**Fig. 1**

- **Legend:**
  - TNF Mab 001

- **Equation:**
  
  \[ y = -0.35x + 0.535, \text{ } R\text{-squared: } 0.442 \]

**Fig. 2**

- **Equation:**
  
  \[ y = -0.35x + 0.535, \text{ } R\text{-squared: } 0.442 \]
Fig. 4

Fig. 5
Fig. 10A

% CHANGE IN TUMOR AREA

PBS  TNF  TNF + 32
TREATMENT

Fig. 10B

% CHANGE IN TUMOR AREA

TNF + CONTROL  TNF + Mab 32
TREATMENT
**Fig. 11A**

**DAY 1**

% CHANGE IN TUMOR AREA

![Graph showing % change in tumor area for different treatments on Day 1](image)

**Fig. 11B**

**DAY 2**

% CHANGE IN TUMOR AREA

![Graph showing % change in tumor area for different treatments on Day 2](image)
**Fig. 14**

- Specific binding (%) vs. monoclonal antibody (log 10 ng).

**Fig. 15**

- Specific binding (%) vs. monoclonal antibody (log 10 ng).
TREATMENT
(200μl globulins+10μg TNF injected/day/mouse)

Fig.20
Fig. 24
Fig. 31
DETERMINATION OF TNF LEVELS FLUIDS BY ELISA

Human TNF (ng/ml) vs. Absorbance 405nm

Fig. 34
TUMOUR NECROSIS FACTOR PEPTIDE BINDING ANTIBODIES

[0001] This is a continuation-in-part of application Ser. No. 07/828,956 filed Aug. 7, 1990, the disclosure of which is incorporated herein by cross-reference.

FIELD OF THE INVENTION

[0002] The present invention relates to ligands which bind to human tumour necrosis factor alpha (TNF) in a manner such that upon binding the biological activity of TNF is modified. The type of modification shown here is distinct from previous descriptions of antibodies which bind to TNF alpha and inhibit all TNF alpha activity. The new discovery shows how the different activities of TNF alpha can be selectively inhibited or enhanced. In addition, the present invention relates to a composition comprising a molecule bound to TNF and to methods of therapy utilising TNF and molecules active against TNF.

BACKGROUND OF THE INVENTION

[0003] Tumor necrosis factor alpha (TNF) is a product of activated macrophages first observed in the serum of experimental animals presensitized with Bacillus Calmette-Guerin or Corynebacterium parvum and challenged with endotoxin (LPS). Following the systematic administration of TNF haemorrhagic necrosis was observed in some transplantable tumours of mice while in vitro TNF caused cytolytic or cytostatic effects on tumour cell lines.

[0004] In addition to its host-protective effect, TNF has been implicated as the causative agent of pathological changes in sepsis, cachexia and cerebral malaria. Passive immunization of mice with a polyclonal rabbit serum against TNF has been shown to protect mice against the lethal effects of LPS endotoxin, the initiating agent of toxic shock, when administered prior to infection.

[0005] The gene encoding TNF has been cloned allowing the usefulness of this monoclonal as a potential cancer therapy agent to be assessed. While TNF infusion into cancer patients in stage 1 clinical trials has resulted in tumour regression, side-effects such as thrombocytopenia, lymphocytopenia, hepatotoxicity, renal impairment and hypertension have also been reported. These quite significant side-effects associated with the clinical use of TNF are predictable in view of the many known effects of TNF, some of which are listed in Table 1.

| TABLE 1 | Continued
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>BIOLOGICAL ACTIVITIES OF TNF</strong></td>
<td><strong>FUNCTION</strong></td>
</tr>
<tr>
<td>ANTI-TUMOUR</td>
<td>cytotoxic action on tumour cells</td>
</tr>
<tr>
<td>ANTI-VIRAL</td>
<td>pyrogenic activity</td>
</tr>
<tr>
<td>ANTI-PARASITIC</td>
<td>angiogenic activity</td>
</tr>
<tr>
<td></td>
<td>inhibition of lipoprotein lipase</td>
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<tr>
<td></td>
<td>activation of neutrophils</td>
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<tr>
<td></td>
<td>osteoclast activation</td>
</tr>
<tr>
<td></td>
<td>induction of endothelium, monocyte and tumour cell</td>
</tr>
<tr>
<td></td>
<td>procoagulant activity</td>
</tr>
<tr>
<td></td>
<td>induction of surface antigens on endothelial cells</td>
</tr>
</tbody>
</table>

[0006] Of particular importance is the activation of coagulation which occurs as a consequence of TNF activation of endothelium and also peripheral blood monocytes. Disseminated intravascular coagulation is associated with toxic shock and many cancers including gastro-intestinal cancer, cancer of the pancreas, prostate, lung, breast and ovary, melanoma, acute leukaemia, myeloma, myeloproliferative syndrome and myeloblastic leukaemia. Clearly modifications of TNF activity such that tumour regression activity remains intact but other undesirable effects such as activation of coagulation are removed or masked would lead to a more advantageous cancer therapy, while complete abrogation of TNF activity is sought for successful treatment of toxic shock.

[0007] Segregation of hormonal activity through the use of site-specific antibodies (both polyclonal and monoclonal) can result in enhanced hormonal activity (Aston et al, 1989, Mol. Immunol. In, 435). To date few attempts have been made to assign antigenicity or function to particular regions of the TNF molecule for which the three-dimensional structure is now known. Assignment of function to such regions would permit the development of MAb's and other ligands of therapeutic use. Polyclonal antibodies to amino acids 1 to 15 have been reported to block Hela R19 cell receptor binding by TNF (Socher et al, 1987, PNAS 84, 8829) whilst monoclonal antibodies recognizing undefined conformational epitopes on TNF have been shown to inhibit TNF cytotoxicity in vitro (Bringman and Aggarwal, 1987, Hybridoma 6, 489). However, the effects of these antibodies on other TNF activities is unknown.

DESCRIPTION OF THE PRESENT INVENTION

[0008] The present inventors have produced panels of monoclonal antibodies active against human TNF and have characterised them with respect to their effects on the anti-tumour effect of TNF (both in vitro and in vivo), TNF receptor binding, activation of coagulation (both in vitro and in vivo) and defined their topographic specificities. This approach has led the inventors to show that different topographic regions of TNF alpha are associated with different activities. Therefore the inventors enable the identification of antibodies or ligands which selectively enhance or inhibit TNF alpha activity, thereby providing for improved therapeutic agents and regimes including TNF alpha.

[0009] In a first aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the following biological activities of the TNF are inhibited:
1. Tumour regression;
2. Induction of endothelial procoagulant;
3. Induction of tumour fibrin deposition;
4. Cytotoxicity; and
5. Receptor binding.

In a preferred embodiment of all aspects the present invention the ligand is selected from the group consisting of antibodies, F(ab) fragments, restructured antibodies (CDR grafted humanised antibodies) single domain antibodies (dAbs), single chain antibodies, serum binding proteins, receptors and natural inhibitors. The ligand may also be a protein or peptide which has been synthesised and which is analogous to one of the foregoing fragments. However, it is presently preferred that the ligand is a monoclonal antibody or F(ab) fragment thereof.

In a second aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction of endothelial procoagulant, tumour regression, induction of tumour fibrin deposition, cytotoxicity and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-18, 58-65, 115-125 and 138-149, or the topographic region of residues 1-18, 108-128, or the topographic region of residues 56-79, 110-127 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

In a third aspect the present invention consists in a ligand which binds to human TNF in at least two regions selected from the group consisting predominantly of the topographic region of residues 1-20, the topographic region of residues 56-77, the topographic region of residues 108-127 and the topographic region of residues 138-149.

In a preferred embodiment of the third aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 1-18, 58-65, 115-125 and 138-149. Such sequence regions are topographically represented in FIG. 23.

In a further preferred embodiment of the third aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 1-18 and 108-128. Such sequence regions are topographically represented in FIG. 24.

In a further preferred embodiment of the second aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 56-79, 110-127 and 130-155. Such sequence regions are topographically represented in FIG. 25.

In a particularly preferred embodiment of the first, second and third aspects of the present invention the ligand is a monoclonal antibody selected from the group consisting of the monoclonal antibodies designated MAb 1, MAb 47 and MAb 54. Samples of the hybridoma cell lines which produce MAb 1, MAb 54 and MAb 47 have been deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom. MAb 1 was deposited on Aug. 3, 1989 and accorded accession No. 89080301; MAb 54 was deposited on Aug. 31, 1989 and accorded accession No. 89083103; MAb 47 was deposited on Dec. 14, 1989 and accorded accession No. 89121402.

In a fourth aspect the present invention consists in a composition comprising TNF in combination with the ligand of the first, second or third aspect of the present invention, characterised in that the ligand is bound to the TNF.

In a fifth aspect the present invention consists in a method of treating toxic shock comprising administering either the ligand of the first, second or third aspect of the present invention or the composition of the fourth aspect of the present invention.

In a sixth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited; binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; the cytotoxicity is unaffected and tumour receptor binding activities of the TNF are unaffected or enhanced.

In a seventh aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited; the binding of the TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; and the cytotoxicity and receptor binding activities of the TNF are unaffected; the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-30, 117-128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands.

In an eighth aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 1-30, 117-128 and 141-153.

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

In a preferred embodiment of the sixth, seventh and eighth aspects of the present invention the ligand is the monoclonal antibody designated MAb 32. A sample of the hybridoma producing MAb 32 was deposited with The European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on Aug. 3, 1989 and was accorded accession No. 890860302.

In a ninth aspect the present invention consists in a composition comprising TNF in combination with a ligand of the sixth, seventh or eighth aspects of the present invention characterised in that the ligand is bound to TNF. No previous documentation of administering MAbS with TNF in order to modify activity of the administered cytokine exists.
In a tenth aspect the present invention consists in a method of treating tumours the growth of which is inhibited by TNF, comprising administering either the ligand of the sixth, seventh or eighth aspects of the present invention or the composition of the ninth aspect of the present invention.

In an eleventh aspect the present invention consists in a ligand which binds to residues 1-18 of human TNF (peptide 301).

In a twelfth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited; the binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; the cytotoxicity of the TNF are unaffected and tumour receptor binding activities of the TNF are unaffected or enhanced, the ligand binding to TNF such that the epitope of the TNF defined by the topographic region of residues 1-18 is substantially prevented from binding to naturally occurring biologically active ligands.

In a thirteenth aspect the present invention consists in a composition comprising TNF in combination with a ligand of the eleventh or twelfth aspects of the present invention characterized in that the ligand is bound to the TNF.

In a fourteenth aspect the present invention consists in a method of treating tumours the growth of which is inhibited by TNF, comprising administering either the ligand of the eleventh or twelfth aspect of the present invention or the composition of the thirteenth aspect of the present invention.

In a fifteenth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the cytotoxicity and tumour regression activities of the TNF are unaffected; the induction of endothelial procoagulant and induction of tumour fibrin deposition activities of the TNF are inhibited and receptor binding activities of the TNF are unaffected.

In a sixteenth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the cytotoxicity and tumour regression activities of the TNF are unaffected; the induction of endothelial procoagulant and induction of tumour fibrin deposition activities of the TNF are inhibited and the tumour receptor binding activities of the TNF are unaffected, the ligand binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40, 49-96, 110-127 and 136-153 is substantially prevented from binding to naturally occurring biologically active ligands.

In a seventeenth aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 22-40, 49-96, 110-127 and 136-153. These regions being proximate in the 3D structure of TNF alpha.

In a preferred embodiment of the fifteenth, sixteenth and seventeenth aspects of the present invention the ligand is the monoclonal antibody designated MAb 42. A sample of the hybridoma cell line producing MAb 42 was deposited with The European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on Aug. 3, 1989 and was accorded accession No. 89080304.

In an eighteenth aspect the present invention consists in a composition comprising TNF in combination with the ligand of the fifteenth, sixteenth or seventeenth aspects of the present invention, characterized in that the ligand is bound to the TNF.

In a nineteenth aspect the present invention consists in a method of treating tumours inhibited by the action of TNF comprising administering the ligand of the fifteenth, sixteenth or seventeenth aspects of the present invention or the composition of the eighteenth aspect of the present invention.

In a twentieth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced; the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression and receptor binding activities of the TNF are inhibited.

In a twenty-first aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced; the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression and tumour receptor binding activities of the TNF are inhibited, the ligand binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 12-22, 36-45, 96-105 and 132-157 is substantially prevented from binding to naturally occurring biologically active ligands.

In a twenty-second aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 12-22, 36-45, 96-105 and 132-157. These regions are proximate in the 3D structure of TNF and are topographically represented in FIG. 28.

In a preferred embodiment of the twentieth, twenty-first and twenty-second aspects of the present invention the ligand is the monoclonal antibody designated MAb 25. A sample of the hybridoma cell line producing MAb 25 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on Dec. 14, 1989 and was accorded accession No. 89121401.

In a twenty-third aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the tumour
fibrin deposition activity of the TNF is enhanced and the cytotoxicity, tumour regression, induction of endothelial procoagulant and receptor binding activities of the TNF are inhibited.

[0047] In a twenty-fourth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the fibrin deposition activity of the TNF is enhanced and the cytotoxicity, tumour regression, induction of endothelial procoagulant and tumour receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-20 and 76-90 is substantially prevented from binding to naturally occurring biologically active ligands.

[0048] In a twenty-fifth aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 1-20 and 76-90. These regions are proximate in the 3D structure of TNF and are topographically represented in FIG. 29.

[0049] In a preferred embodiment of the twenty-fifth aspect of the present invention the ligand binds to TNF in the topographic regions of residues 1-18 and 76-90.

[0050] In a preferred embodiment of the twenty-third, twenty-fourth and twenty-fifth aspects of the present invention the ligand is the monoclonal antibody designated MAb 21. A sample of the hybridoma cell line producing MAb 21 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on Jan. 25, 1990 and was accorded accession No. 90012432.

[0051] In a twenty-sixth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the fibrin deposition activity of the TNF is unaffected and the cytotoxicity, tumour regression, induction of endothelial procoagulant and tumour receptor binding activities of the TNF are inhibited.

[0052] In a twenty-seventh aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the tumour fibrin deposition activity of the TNF is unaffected and the cytotoxicity, tumour regression, induction of endothelial procoagulant and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40, 69-97, 105-128 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

[0053] In a twenty-eighth aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 22-40, 69-97, 105-128 and 135-155. These regions are proximate in the 3D structure of TNF and are topographically represented in FIG. 30.

[0054] In a preferred embodiment of the twenty-sixth, twenty-seventh and twenty-eighth aspects of the present invention the ligand is the monoclonal antibody designated MAb 53. A sample of the hybridoma cell line producing MAb 53 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on Jan. 25, 1990 and was accorded accession No. 90012432.

[0055] In a twenty-ninth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the tumour fibrin deposition, induction of endothelial procoagulant, cytotoxicity, tumour regression and receptor binding activities of the TNF are unaffected.

[0056] In a thirtieth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the fibrin deposition, induction of endothelial procoagulant, cytotoxicity, tumour regression and receptor binding activities of the TNF are unaffected.

[0057] In a thirty-first aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 22-31 and 146-157. These regions are proximate in the 3D structure of TNF and are topographically represented in FIG. 31.

[0058] In a preferred embodiment of the twenty-ninth, thirtieth and thirty-first aspects of the present invention the ligand is the monoclonal antibody designated MAb 57. A sample of the hybridoma cell line producing MAb 57 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on Aug. 3, 1989 and was accorded accession No. 89080303.

[0059] In a thirty-second aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression, tumour fibrin deposition, and receptor binding activities of the TNF are inhibited.

[0060] In a thirty-third aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression, tumour fibrin deposition and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40 and 49-98 is substantially prevented from binding to naturally occurring biologically active ligands.

[0061] In a thirty-fourth aspect the present invention consists in a ligand which binds to human TNF in at least one of the regions selected from the group consisting of the topographic region of residues 22-40, the topographic region of residues 49-98 and the topographic region of residues 69-97.

[0062] In a preferred embodiment of the thirty-fourth aspect of the present invention the ligand binds to human
TNF in the topographical region of residues 49-98. This region is topographically represented in FIG. 32.

[0063] In a further preferred embodiment of the thirty-fourth aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 22-40 and 70-87. These regions are proximate in the 3D structure of TNF and are topographically represented in FIG. 33.

[0064] In a preferred embodiment of the thirty-second, thirty-third and thirty-fourth aspects of the present invention the ligand is monoclonal antibody MAb 11 or MAb 12.

[0065] In a thirty-fifth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited.

[0066] In a thirty-sixth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited, the ligand binding to TNF such that the epitope of the TNF defined by the topographical region of residues 108-128 is prevented from binding to naturally occurring biologically active ligands.

[0067] In a thirty-seventh aspect the present invention consists in a ligand which binds to human TNF in the topographical region of residues 108-128.

[0068] In a preferred embodiment of the thirty-fifth, thirty-sixth and thirty-seventh aspects of the present invention the ligand is selected from the group consisting of monoclonal antibodies designated MAb 1, MAb 32, MAb 42, MAb 47, MAb 53 and MAb 54.

[0069] The biological activities of TNF referred to herein by the terms “Tumour Regression”, “Induction of Endothelial Procoagulant”, “Induction of Tumour-Fibrin Deposition”, “Cytotoxicity” and “Receptor Binding” are to be determined by the methods described below.

[0070] The term “single domain antibodies” as used herein is used to denote those antibody fragments such as described in Ward et al (Nature, Vol. 341, 1989, 544-546) as suggested by these authors.

[0071] In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following example and accompanying figures in which:

[0072] FIG. 1 shows the results of a titration assay with MAb 1 against TNF;

[0073] FIG. 2 shows TNF MAb 1 scatchard plot and affinity determination;

[0074] FIG. 3 shows the effect of anti-TNF monoclonal antibodies 1 and 32 on TNF cytotoxicity in WEHI-164 cells;

[0075] FIG. 4 shows the effect of MAb 1 on TNF-induced regression of a Meth A solid tumour;

[0076] FIG. 5 shows the effect of MAb 1 and 25 on TNF-induced Meth A Ascites tumour regression;

[0077] FIG. 6 shows the effect of anti-TNF MAb s on induction of endothelial cell procoagulant activity by TNF;

[0078] FIG. 7 shows incorporation of labelled fibrinogen into tumours of tumour-bearing mice and the effect of anti-TNF MAb s;

[0079] FIG. 8 is a schematic representation of epitopes on TNF;

[0080] FIG. 9 shows the effect of anti-TNF MAb s on TNF-induced regression of WEHI-164 tumours;

[0081] FIG. 10 shows the enhancement of TNF regression activity by MAb 32 in two experiments;

[0082] FIG. 11 shows the enhancement of TNF-induced tumour regression by MAb 32—dose response at day 1 and day 2;

[0083] FIG. 12 shows binding of radio labelled TNF to receptors on bovine aortic endothelial cells;

[0084] FIG. 13 shows receptor binding studies of TNF complexed with MAb 32 ( ), control antibody ( ) and MAb 47 ( ) on melanoma cell line NM4518E;

[0085] FIG. 14 shows receptor binding studies of TNF complexed with MAb 32 ( ), control antibody ( ) and MAb 47 ( ) on melanoma cell line IGR3;

[0086] FIG. 15 shows receptor binding studies of TNF complexed with MAb 32 ( ), control antibody ( ) and MAb 47 ( ) on bladder carcinoma cell line 5637;

[0087] FIG. 16 shows receptor binding studies of TNF complexed with MAb 32 ( ), control antibody ( ) and MAb 47 ( ) on breast carcinoma cell line MCF7;

[0088] FIG. 17 shows receptor binding studies of TNF complexed with MAb 32 ( ) control antibody ( ) and MAb 47 ( ) on colon carcinoma cell line B10;

[0089] FIG. 18 shows the effect on TNF-mediated tumour regression in vivo by MAb 32 ( ) control MAb ( ) and MAb 47 ( );

[0090] FIG. 19 shows the effect on TNF-mediated tumour regression in vivo by control MAb, MAb 32 and univalent Fab' fragments of MAb 32;

[0091] FIG. 20 shows the effect on TNF induced tumour regression by control MAb ( ) MAb 32 ( ) and peptide 301 antiserum ( );

[0092] FIG. 21 shows MAb 32 reactivity with overlapping peptides of 10 AA length; and

[0093] FIG. 22 shows a schematic three dimensional representation of the TNF molecule.

[0094] FIG. 23 shows topographically the region of residues 1-20, 56-77, 108-127 and 138-149;

[0095] FIG. 24 shows topographically the region of residues 1-18 and 108-128;

[0096] FIG. 25 shows topographically the region of residues 56-79, 110-127 and 136-155;

[0097] FIG. 26 shows topographically the region of residues 1-26, 117-128 and 141-153;

[0098] FIG. 27 shows topographically the region of residues 22-40, 49-97, 110-127 and 136-153;
Flexible microtitre trays were coated with monoclonal antibody (sodium sulphate precipitated globulins from mouse ascites fluid, 100 micrograms per ml in sodium bicarbonate buffer, 0.05M, pH 9.6) overnight at 4°C, prior to blocking non-specific binding sites with 1% bovine serum albumin, in PBS (BSA/PBS). The binding of 125I TNF to immobilised antibody was determined in the presence of varying concentrations of a second anti-TNF monoclonal antibody. Antibody and TNF were added simultaneously and incubated for 24 hours prior to washing with PBS (0.4 times) and counting wells for bound radioactivity. 100% binding was determined in the absence of heterologous monoclonal antibody while 100% competition was determined in the presence of excess homologous monoclonal antibody. All dilutions were prepared in BSA/PBS.

Lact

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

WEHI-164 Cytotoxicity Assay

Bioassay of recombinant TNF activity was performed according to Espevik and Nissen-Meyer (1986, J. Immunol. Methods 95, 99). The effect of the monoclonal antibody on TNF activity was determined by the addition of the monoclonal antibody to cell cultures at AB90.

Tumour Regression Experiments

Modulation of TNF-induced tumour regression activity by monoclonal antibodies was assessed in three tumour models: the subcutaneous tumours WEHI-164 and Meth A sarcoma and the ascitic Meth A tumour. Subcutaneous tumours were induced by the injection of approximately 5 x 10^6 cells. This produced tumours of between 10-15 mm approximately 14 days later. Mice were injected intra-peritoneally with human recombinant TNF (10 micrograms) plus monoclonal antibody (200 microlitres ascites globulin) for four consecutive days. Control groups received injections of PBS alone or TNF plus monoclonal antibody against bovine growth hormone. At the commencement of each experiment tumour size was measured with calipers in the case of solid tumours or tumour-bearing animals weighed in the case of ascites mice. These measurements were taken daily throughout the course of the experiment.

Radio-Receptor Assays

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

[0124] Procoagulant Induction by TNF on Endothelial Cells

[0125] Bovine aortic endothelial cells (passage 10) were grown in RPMI-1640 containing 10% foetal calf serum (FCS), penicillin, streptomycin, and 2-mercaptoethanol at 37°C in 5% CO₂. For induction of procoagulant activity by TNF the cells were trypsinised and plated into 24-well Costar trays according to the protocol of Bevilacqua et al, 1986 (PNAS 83, 4533). TNF (0-500 units/culture) and monoclonal antibody (1 in 250 dilution of ascitic globulin) was added after washing of the confluent cell monolayer of 125I fibrinogen into mouse tissue was determined by removing a piece of tissue, weighting it and counting the sample in a gamma counter.

[0128] In all 15 monoclonal antibodies reacting with human TNF were isolated. These monoclonal antibodies were designated MAb 1, MAb 11, MAb 12, MAb 20, MAb 21, MAb 25, to MAb 31, MAb 32, MAb 37, MAb 42, MAb 47, MAb 53 and MAb 54. The effect of these monoclonal antibodies on the bioactivity of human TNF is set out in Table 2.

[0129] As can be seen from Table 2, whilst some monoclonal antibodies inhibit both anti-tumour activity and activation of coagulation by human TNF (MAb 1, 47 and 54) not all antibodies which inhibit the anti-tumour activity inhibit activation of coagulation either in vitro or in vivo (MAb 11, 12, 25 and 53). Indeed MAb 21 which inhibited tumour regression enhanced the activation of coagulation in vivo.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>EFFECT OF MONOCLONAL ANTIBODIES ON TNF BIOACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>MONOCLONAL ANTIBODY</td>
<td>TNF BIOACTIVITY</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>1 11 12 20 21 25 31 32 37 42 47 53 54</td>
</tr>
<tr>
<td>Tumour Regression</td>
<td>- - - 0 - 0 0 0 0 - - -</td>
</tr>
<tr>
<td>Induction of Procoagulant (Endothelial)</td>
<td>- - 0 0 0 0 0 0 0 - - -</td>
</tr>
<tr>
<td>Fibrin Deposition (tumour)</td>
<td>- - - - - 0 0 0 0 - -</td>
</tr>
<tr>
<td>Receptor Binding (WEHI - 164)</td>
<td>- - - 0 0 0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

* + Enhancement |
0 No effect |
- Inhibition |
*Depending on MAb concentration in the case of WEHI - 164 tumour cells and tumour type (see Figs. 3, 33-37).

[0130] MAb 1, 47 and 54, which have been shown in competition binding studies to share an epitope on TNF, can be seen to have highly desirable characteristics in treatment of toxic shock and other conditions of bacterial, viral and parasitic infection where TNF levels are high requiring complete neutralisation of TNF. Other monoclonal antibodies such as MAb 32 are more appropriate as agents for coadministration with TNF during cancer therapy since they do not inhibit tumour regression but do inhibit activation of coagulation. This form of therapy is particularly indicated in conjunction with cytotoxic drugs used in cancer therapy which may potentiate activation of coagulation by TNF (e.g. vinblastin, acetylcholine, IPN alpha, IL-2, actinomycin D, AZT, radiotherapy, adriamycin, mytomycin C, cytosome arabinoside, doxorubicin, cis-platin, vincristine, 5-fluorouracil, bleomycin, Watanabe N et al 1988 Immunopharmacol). Immunotoxicol. 10 117-127) or in diseases where at certain stages TNF levels are low (e.g. AIDS) and where individuals may have AIDS associated cancer e.g. Kaposi sarcoma, non-Hodgkins lymphoma and squamous cell carcinoma.

[0131] Monoclonal antibody MAb 1 has been found to have the following characteristics:

[0132] 1. Binds human recombinant TNF alpha, but not human lymphotixin (TNF beta) or human inter-
[0133] 2. MAb 1 is of the immunoglobulin type IgG1, K with an apparent affinity of 4.4x10^5 moles/litre (FIG. 2).

[0134] 3. MAb neutralises the cytotoxic effect of recombinant human TNF on WEHI-164 mouse fibrosarcoma cells in culture. One microgram of MAb 1 neutralises approximately 156.25 units of TNF in vitro (FIG. 3).

[0135] 4. MAb 1 neutralises the tumour regression activity of TNF in the following mouse tumour models in vivo; WEHI-164 subcutaneous solid tumour, the Meth A subcutaneous solid tumour and the Meth A ascites tumour (FIGS. 4, 5 and 9).

[0136] 5. MAb1 prevents cerebral damage caused by human TNF in mice infected with malarial parasites.

[0137] 6. In radioreceptor assays MAb 1 prevents binding of TNF to receptors on WEHI-164 cells (Table 3).

[0138] 7. MAb 1 inhibits the induction of procoagulant activity (tissue factor) on cultured bovine aortic endothelial cells (FIG. 6).

[0139] 8. MAb 1 reduces the uptake of 125I fibrinogen into tumours of mice treated with TNF (FIG. 7).

[0140] 9. MAb 1 competes for binding of 125I TNF and thus shares an overlapping epitope with the following monoclonal antibodies: 21, 25, 32, 47, 54 and 37.

[0141] 10. MAb 1 does not compete for binding of 125I TNF with the following monoclonal antibodies: 11, 12, 42, 53, 31 and 20 (FIG. 8).

### TABLE 3

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>% SPECIFIC BINDING</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAb, 1 1/10</td>
<td>21</td>
</tr>
<tr>
<td>1/100</td>
<td>49</td>
</tr>
<tr>
<td>1/10,000</td>
<td>73</td>
</tr>
<tr>
<td>1/100,000</td>
<td>105</td>
</tr>
<tr>
<td>cold TNF (ng/tube)</td>
<td></td>
</tr>
<tr>
<td>10,000</td>
<td>0</td>
</tr>
<tr>
<td>5,000</td>
<td>0</td>
</tr>
<tr>
<td>1,000</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>108</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

[0142] MAb 32 is an IgG2b, K antibody with an affinity for human TNF alpha of 8.77x10^5 moles/litre as determined by Scatchard analysis. This monoclonal antibody does not react with either human TNF beta (lymphotoxin) or mouse TNF alpha.

[0143] As shown in FIG. 3 MAb 32 does not inhibit TNF cytotoxicity in vitro as determined in the WEHI-164 assay.

[0144] Monoclonal antibody 32 variably enhances TNF-induced tumour regression activity against WEHI-164 fibrosarcoma tumours implanted subcutaneously into BALB/c mice at a TNF dose of 10 µg/day (see FIGS. 10 and 11). This feature is not common to all monoclonal antibodies directed against TNF (FIG. 9) but resides within the binding site specificity of MAb 32 (FIG. 8) which may allow greater receptor mediated uptake of TNF into tumour cells (see Table 4).

<p>| TABLE 4 |
| BINGOUND OF TNF TO RECEPTORS ON WEHI-164 CELLS IN THE PRESENCE OF MAb 32 |</p>
<table>
<thead>
<tr>
<th>MAb DILUTION</th>
<th>CONTROL MAb</th>
<th>MAb 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/10</td>
<td>36</td>
<td>141</td>
</tr>
<tr>
<td>1/100</td>
<td>74</td>
<td>88</td>
</tr>
<tr>
<td>1/1000</td>
<td>101</td>
<td>83</td>
</tr>
<tr>
<td>1/10,000</td>
<td>92</td>
<td>82</td>
</tr>
<tr>
<td>1/100,000</td>
<td>97</td>
<td>93</td>
</tr>
</tbody>
</table>

[0145] Enhancement of TNF activity by MAb 32 at lower doses of TNF is such that at least tenfold less TNF is required to achieve the same degree of tumour regression (see FIGS. 11 and 18). The results for day 1, 2.5 µg and 5 µg TNF and day 2, 5 µg and 25 µg and 5 µg are statistically significant in a t-test at p<0.01 level. This level of enhancement also increases the survival rate of recipients since the lower dose of TNF used is not toxic. FIG. 19 shows that univalent Fab fragments of MAb 32 also cause enhancement of TNF-induced tumour regression in the same manner as whole MAb 32 (see below).

[0146] MAb 32 inhibits the expression of clotting factors on endothelial cells normally induced by incubation of the cultured cells with TNF (see FIG. 6). This response may be mediated by a previously unidentified TNF receptor which is distinct to the receptor found on other cells.

[0147] Conversely, MAb 32 enhances the in vivo activation of coagulation within the tumour bed as shown by the incorporation of radiolabelled fibrinogen (FIG. 7). This may be due to activation of monocytes/macrophage procoagulant and may provide further insight into the mechanism of TNF-induced tumour regression.

[0148] The results obtained with MAb 32 are shown in comparison to other anti-TNF MAb in Table 2.

[0149] The ability of MAb 32 and MAb 47 to inhibit the binding of TNF to endothelial cells was also assessed. Bovine aortic endothelial (BAE) cells (passage 11) were plated in 24-well culture dishes (Corning) which had been pre-coated with gelatin (0.2%) and grown to confluence in McCoy's 5A (modified) medium supplemented with 20% fetal calf serum. For the radio-receptor assay all dilutions (of cold TNF and MAb's) were made in this medium. The RA cells were incubated for one hour in the presence of either cold TNF (0 to 100 ng) or MAb (ascites globulins diluted 1/100 to 1/100,000) and iodinated TNF (50,000 cpn). At the end of this time the medium was withdrawn and the cells
washed before being lysed with 1M sodium hydroxide. The cell lysate was then counted for bound radioactive TNF. Specific binding of labelled TNF to the cells was then determined.

[0150] The results obtained in this assay with MAb 32, MAb 47 and a control MAb are set out in FIG. 12.

[0151] The results obtained in the clotting assay using BAE cells cultured in the presence of TNF and anti-TNF MAb correlate with the results obtained in the BAE radioreceptor assay i.e. MAbS which inhibit the induction of clotting factors on the surface of endothelial cells (as shown by the increase in clotting time compared to TNF alone) also inhibit the binding of TNF to its receptor. This is exemplified by MAbS 32 and 47.

[0152] MAb 32, which does not inhibit TNF binding to WEHI-164 cells, does inhibit binding of TNF to endothelial cells. This result provides support for the hypothesis that distinct functional sites exist on the TNF molecule and that these sites interact with distinct receptor subpopulations on different cell types. Thus ligands which bind to defined regions of TNF are able to modify the biological effects of TNF by limiting its binding to particular receptor subtypes.

[0153] As shown in FIG. 12 MAb 47 is a particularly potent inhibitor of TNF interaction with endothelial cells, the percentage specific binding at a dilution of Vtro to Vtovo being effectively zero.

[0154] Receptor Binding Studies of Human TNF Complexed with MAb 32 on Human Carcinoma Cell Lines in vitro

[0155] MAb 32 has been shown to enhance the anti-tumour activity of human TNF. The mechanisms behind the enhancement may include restriction of TNF binding to particular (tumour) receptor subtypes but not others (endothelial) with subsequent decrease in TNF toxicity to non-tumour cells. This mechanism does not require enhanced uptake of TNF by tumour cells in vitro. In addition, MAb 32 also potentiates the binding of human TNF directly to TNF receptors on certain human carcinoma cell lines.

[0156] Materials and Methods

[0157] The following human carcinoma cell lines have been assayed for enhanced receptor-mediated uptake of TNF in the presence of MAb 32: B10, CaCo, HT 29, SKCO1 (all colon carcinomas), 5637 (Bladder carcinoma), MM418E (melanoma), IGR3 (melanoma), MCF 7 (breast carcinoma). The cells were propagated in either RPMI-1640 (MM418E) DMEM (CaCo and IGR 3) or Iscoves modified DMEM (B10 HT 29, SK01, S637, MCF 7) supplemented with 10% foetal calf serum, penicillin/streptomycin and L-glutamine. Receptor assays were performed as previously described for endothelial cells except that the incubation time with iodinated TNF was extended to 3 hours for all but the B10 cells for which the radio label was incubated for 1 hour.

[0158] Results

[0159] Enhanced TNF uptake was observed in the presence of MAb32 by the melanoma cell lines tested MH418E and IGR 3 (FIGS. 13 and 14), the bladder carcinoma 5637 (FIG. 15), and the breast carcinoma MCF 7 (FIG. 16). MAb 32 did not affect TNF-receptor interaction in any of the other cell lines as shown by B 10 (FIG. 17) MAb 47, which has been shown to inhibit TNF binding to WEHI-164 cells and endothelial cells, and which also inhibits TNF-mediated tumour regression was found to markedly inhibit TNF binding to all the cell lines tested (FIGS. 13-17).

[0160] Conclusions

[0161] Receptor binding analyses have indicated a second mechanism whereby MAb 32 may potentiate the anti-tumour activity of TNF. This second pathway for enhancement of TNF results from increased uptake of TNF by tumour all receptors in the presence of MAb 32.

[0162] Enhancement of TNF-Mediated Tumour Regression in vivo MAb 32 or Univalent Fab’ Fragments of MM 32

[0163] Tumour regression studies were carried out as described above in mice carrying WEHI-164 subcutaneous tumours (N=5 animals/group). Tumour size was determined daily during the course of the experiment. The results obtained using MAb 32 are set out in FIG. 22 and show the mean +/- SD % change in tumour area at the completion of treatment (day 2) (MAb 32: 100% control MAb: *MAb 47). Differences observed between control MAb-TNF and MAb 32-TNF treated groups are statistically significant in a T-test at the p<0.01 level.

[0164] The results using the univalent Fab’ fragments of MAb 32 are shown in FIG. 19. Tumour size was determined daily during the course of the experiment. The results show the mean SD % change in tumour area at the completion of treatment (day 2). Differences between the control MAb-10F and MAb 32-TNF treated groups are statistically significant in a T-test at the P<0.01 level.

[0165] TNF Induced Tumour Regression Effect of Anti-Peptide 301 Sera

[0166] FIG. 20 shows the percent change in tumour area in tumour-bearing mice treated for three days with TNF plus control MAb (antibody against bovine growth hormone), TNF plus MAb 32 or TNF plus antisera (globulin fraction) against peptide 301. In an unpaired T-test the control group is significantly different from both of the test groups (MAb 32, antisera 301) while the MAb 32 and peptide antisera 301 groups are not significantly different from each other. (control vs MAb 32, p<0.002; control vs antipeptide 301, p<0.025). Thus antisera raised using a peptide which comprises part of the MAb 32 specificity, also causes TNF enhancement of tumour regression.

[0167] As shown in FIG. 9 competition binding studies has shown that the thirteen monoclonal antibodies can be sub-divided into two main groups, namely MAbS 1, 21, 47, 54, 37, 32 and 25 and MAbS 11, 12, 53 and 42. Experiments were then conducted to identify the regions on human TNF recognised by these monoclonal antibodies.

[0168] Identification of Regions on Human TNF Recognised by Monoclonal Antibodies

[0169] Methods

[0170] 1. Overlapping peptides of 7 and 10 amino acid residues long were synthesized on polypropylene pins according to the method of Geysen et al., 1984, PNAS, 81, 3998-4002. The overlap was of 6 and 9 residues respectively and collectively the peptides covered the entire TNF amino acid sequence. The peptides were tested for reactivity with
the MAbs by ELISA. MAbs which had TNF reactivity absorbed from them by prior incubation with whole TNF were also tested for reactivity with the peptides and acted as a negative control.

[0171] 2. Longer peptides of TNF were synthesized as described below. These peptides were used to raise antisera in sheep using the following protocol. Merino sheep were primed with TNF peptide conjugated to ovalbumin and emulsified in Freund's Complete adjuvant and boosted at 4 weekly intervals with peptide-ovalbumin and sera assayed for the presence of anti-TNF antibody by radioimmunoassay. Of the peptides shown only peptides 275, 301, 305, 306 and 307 elicited sera reacting with whole TNF. The positive sera were then used in competitive binding assays (PACT assays) with the MAbs.

[0172] The following peptides were synthesised and are described using the conventional three letter code for each amino acid with the TNF sequence region indicated in brackets.

[0173] Peptide 275
[0174] H-Ala-Lys-Pro-Trp-Tyr-Glu-Pro-Ile-Tyr-Leu-OH (111-120)

[0175] Peptide 301

[0176] H-Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Arg-Ser-Asp-Lys-Pro-Ala-His-Val-Val-Ala-OH (1-18)

[0177] Peptide 302


[0179] Peptide 304

[0180] H-Leu-Phe-Lys-Gln-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile-Ser-Arg-Ile-OH (63-83)

[0181] Peptide 305


[0183] Peptide 306


[0185] Peptide 307


[0187] Peptide 308


[0189] Peptide 309


[0191] Peptide 323

[0192] H-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Thr-Gln-Thr-OH (79-89)

[0193] These peptides were synthesised using the following general protocol.

[0194] All peptide were synthesised using the Fmoc-polypeptide method of solid phase peptide synthesis (Atherton et al., 1978, J. Chem. Soc. Chem. Commun., 13, 537-539). The solid resin used was PepSyn KA which is a polydimethylacrylamide gel on Kieselgel support with 4-hydroxyethylphenoxy-acetic acid as the functionalised linker (Atherton et al., 1975, J. Am. Chem. Soc. 2, 6584-6585).

[0195] The carboxy terminal amino acid was attached to the solid support by a DCC/DMAP-mediated symmetrical-anhydride esterification.

[0196] All Fmoc-groups were removed by piperidine/DMF wash and peptide bonds were formed either via pentfluoroprophenyl active esters or directly by BOP/NMM/HOBt (Castro's reagent) (Fourrier et al., 1989, Int. J. Peptide Protein Res., 3, 133-139) except for certain amino acids as specified in table 5.

[0197] Side chain protection chosen for the amino acids was removed concomitantly during cleavage with the exception of Acn on cysteine which was left on after synthesis.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Protecting Group</th>
<th>Coupling Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>Acn (permanent)</td>
<td>Either</td>
</tr>
<tr>
<td>Asp</td>
<td>OBut</td>
<td>Either</td>
</tr>
<tr>
<td>Cys</td>
<td>OBut</td>
<td>Either</td>
</tr>
<tr>
<td>Glu</td>
<td>OBut</td>
<td>Either</td>
</tr>
<tr>
<td>His</td>
<td>Boc</td>
<td>OPfp only</td>
</tr>
<tr>
<td>Lys</td>
<td>Boc</td>
<td>Either</td>
</tr>
<tr>
<td>Ser</td>
<td>But</td>
<td>BOP only</td>
</tr>
<tr>
<td>Thr</td>
<td>But</td>
<td>BOP only</td>
</tr>
<tr>
<td>Tyr</td>
<td>But</td>
<td>Either</td>
</tr>
<tr>
<td>Trp</td>
<td>none</td>
<td>Either</td>
</tr>
<tr>
<td>Asn</td>
<td>none</td>
<td>OPfp only</td>
</tr>
<tr>
<td>Gln</td>
<td>none</td>
<td>OPfp only</td>
</tr>
</tbody>
</table>

[0198] Cleavage and Purification

[0199] Peptide 301, 302, 305 are cleaved from the resin with 95% TFA and 51 thioanisole (1.5 h) and purified on reverse phase C4 column, (Buffer A-0.1% aqueous TFA, Buffer B-80% ACN 20% A).

[0200] Peptide 303, 304 are cleaved from the resin with 95% TFA and 5% phenol (5-6 h) and purified on reverse phase C4 column. (Buffers as above).

[0201] Peptide 306, 308 are cleaved from the resin with 95% TFA and 5% water (1.5 h) and purified on reverse phase C4 column. (Buffers as above).

[0202] Peptide 309 Peptide was cleaved from the resin with 95% TFA and 5% thioanisole and purified on reverse phase C4 column. (Buffers as above).

[0203] Peptide 307 Peptide was cleaved from the resin with a mixture of 93% TFA, 3.1% Anisole, 2.97% Ethylmethylsulphide and 0.95% Ethanedithiol (3 h) and purified on reverse phase C4 column. (Buffers as above).
Results

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

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

| TABLE 6 |
| COMPETITIVE BINDING OF TNF BY ANTI-TNF MONOCLONE |
| IN THE PRESENCE OF ANTI-PEPTIDE SERA |
| MAB/PEPTIDE SERA | 275 | 301 | 305 | 306 | 307 |
| 1 | + | + | - | - | + |
| 11 | + | + | - | - | + |
| 12 | + | + | - | - | + |
| 21 | + | + | - | - | + |
| 25 | + | + | - | - | + |
| 32 | + | + | - | - | + |
| 37 | + | + | - | - | + |
| 47 | + | + | - | - | + |
| 53 | + | + | - | - | + |
| 54 | + | + | - | - | + |
| 42 | + | + | - | - | + |

Note 1: - indicates no competition, + indicates slight competition at high concentration of anti-peptide antisera (1:50), +++ indicates strong competition by anti-peptide sera equal to that of the homologous MAb.

Note 2: Only peptide which elicited sera recognising whole TNF were used in this assay.

As will be understood by persons skilled in this field the ligands of the present invention can be used in assays of biological fluids for detecting the presence of and quantifying the concentration of TNF in a sample. One means by which this may be achieved is by using the ligands of the present invention in conventional ELISAs. Set out below is an example of such an assay.

TNF ELISA REAGENTS

| CARBONATE COATING BUFFER, pH 9.6 |
| NaHCO₃ | 1.6 g |
| Na₂CO₃ | 2.9 g |
| Add 800 mL dH₂O, pH to 9.6 then make up to 1 L with dH₂O |

| BLOCKING BUFFER |
| BSA | 1 g |
| PBS | 100 mL |
| Add BSA to PBS and allow to dissolve fully before use. Store at 4°C. |

| WASH BUFFER (0.05% Tween/PBS) |
| Tween 20 | 0.5 g |
| PBS | 1 L |
| Add Tween to PBS and mix thoroughly before use |

| CITRATE BUFFER |
| Citric Acid | 2.1 g in 50 mL |
| H₂O | Add solutions together |
| H₂O | and adjust to pH to 4.0-4.2 |
| Trisodium | 1.47 g in 50 mL |
| H₂O | |

NB: All incubations can be carried out at 4°C. overnight OR at room temperature for 2 hrs OR at 37°C for 1 hr.

Method

| 0219 | Coat ELISA plates with equal proportions of MAb1, MAb32 and MAb54 to human TNF in carbonate coating buffer. The total immunoglobulin concentration should be 20 μg/mL and 100 μL is added to each well. Cover plates and incubate. |
| 0220 | Wash plates 3x with PBS/Tween. |
| 0221 | Incubate plates with 250 μL/well blocking buffer |
| 0222 | Wash plates 3x with PBS/Tween. |
| 0223 | Add 100 μL sample or TNF standards, diluted in blocking buffer where required, to plates, then cover and incubate. |
| 0224 | Wash plates 3x with PBS/Tween. |
| 0225 | Add 100 μL biotinylated antibody mix (equal proportions of biotinylated monoclonal antibodies 11 & 42 to human TNF) at a final concentration of 10 μg/mL in blocking buffer to each well, cover and incubate. |
| 0226 | Wash plates 3x with PBS/Tween. |
| 0227 | Add 100 μL/well streptavidin-peroxidase (Amersham product no. RPN 1231) at 1/10,000 in blocking buffer, then cover and-incubate. |
| 0228 | Wash plates 3x with PBS/Tween. |
| 0229 | Add 100 μL/well biotinylated anti-streptavidin monoclonal antibody (Jackson Immunoresearch) at 1/6,000 in blocking buffer, cover and incubate. |
| 0230 | Wash plates 3x with PBS/Tween. |
| 0231 | Add 100 μL/well streptavidin-peroxidase at 1/2,000 in blocking buffer, cover and incubate. |
[0232] Wash plates 3x with PBS/Tween.
[0233] Add 100 µL/well peroxidase substrate (ABTS) at 1 mg/mL in citrate buffer containing 0.3 µL/ml H₂O₂ and leave to incubate at room temperature for up to 1 hour.

[0234] NB: Substrate solution should be prepared immediately prior to use.

[0235] Read absorbance at 405 nm, and compare sample readings with TNF standard curve to determine TNF levels.

---

**BIOTINYLLATION OF IgG**

50 mM BICARBONATE BUFFER, pH 8.5

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>1.6 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.9 g</td>
</tr>
<tr>
<td></td>
<td>In 1 L dH₂O, adjust pH with HCl</td>
</tr>
</tbody>
</table>

0.1 PHOSPHATE BUFFER, pH 7.0

[0236] Method

[0237] Prepare immunoglobulins by purifying on a protein A column, then freeze-drying.

[0238] Reconstitute the immunoglobulins with 50 mM bicarbonate buffer to concentration off 20 mg/mL in a clean glass test tube.

[0239] Add 0.4 mg biotin per 20 mg Ig directly to the tube.

[0240] Place the test tube on ice and incubate for 2 hours.

[0241] Remove the unreacted biotin by centrifuging at 1000 g for 15-30 minutes in a Centricron-30 microcentrator. Dilute the sample in 0.1M phosphate buffer and repeat the centrifugation twice.

[0242] Make the sample up to the original volume with phosphate buffer, add 0.1% Na₂O₃ and store at 4°C until used.

[0243] The results obtained in such an assay using samples containing known amounts of TNF is shown in FIG. 34.

[0244] As mentioned above the specific mouse monoclonal antibodies disclosed in this application can be humanised if required. A number of methods of obtaining humanised antibodies are set out in PCT/GB92/01755 (WO93/06213). A humanised version of MAb32 designated VH1BACK, VH1FOR2 was produced by the method disclosed in PCT/GB92/01755. Briefly, this antibody was produced as follows:

[0245] 1 Cloning and Display of the V Genes of MAb 32 on Phage

[0246] Cloning of the V-Genests of MAb32:

[0247] The genes of the mouse MAb32 antibody (IgG2b, Kappa) were rescued by PCR essentially as described (Clarkson et al., 1991, supra, Clarkson et al. in “PCR: a practical approach”, eds Mr Phenox et al, IRL Press, Oxford pp 187-214) using the primers VH1BACK and VH1FOR2 for the VH gene and Vk2BACK and VK4FOR for the VL gene and the polymerase chain reaction (PCR, R. K. Saiki et al., 1985, Science 230, p1350). The mouse VH and VK genes were assembled for expression as scFv fragments by PCR assembly (Clarkson et al., supra) amplified with VH1BACKSII and VF1FOR4NOT and ligated into phagemid pHEN1 (H. R. Hoogenboom et al., 1991 Nucl. Acids. Res. 19, pp4133-4137) as a SfI-NotI cut restriction fragment, and electroporated into E. coli HB2151 cells. Of 96 clones analysed by ELISA (see below), 9 secreted TNF-binding soluble scFv fragments. Sequencing revealed in all clones a mouse VH of family IIB and a mouse VK of family VI (E. A. Kabat et al., 1991 Sequences of Proteins of Immunological Interest, US Public Health Services). Nucleotide mutations which were probably introduced by the PCR were detected by comparing the 9 sequences, and a clone with consensus sequence and binding activity (scFv-MAb32) chosen for further cloning experiments.

[0248] Recloning of the MAb32 V-Genes for Soluble Expression:

[0249] The murine V-genes were recloned for soluble expression of heavy (Fd, VHCH1) or light chain, by linking the mouse V-genes to the human CH1 (of the mu-isotype) or human CK gene respectively by splice overlap extension.

The mouse Vik gene was amplified from scFv-MAb32 DNA with oligonucleotides MOJK1FORNX (binds in joining region of V-gene and MVKBASFI (binds in 5' region and adds Sfi restriction site); the human CK was obtained by PCR from a mouse-human chimaeric light chain gene (of NQ10.12.5, described in Hoogenboom et al., 1991 supra), with oligonucleotides MOVK-HUCK-BACK (binds in 5' of human CK and is partially complementary to mouse Jκ 1 region) and HUCKNOT16NOMYC (sits in 3' end of human CK, retains the terminal cysteine, and tags on a NotI restriction site) as in Clarkson et al., 1991 using a two fragment assembly. For linkage of the DNA fragments, the two PCR fragments were mixed and amplified with MVKBASFI and HUCKNOT16NOMYC. The chimaeric VιCκ gene was subsequently cloned as a Sfi-Not fragment in pUC19 derivative containing the pEB signal peptide sequence and appropriate cloning sites for soluble expression of the light chain (pUC19-peLB-myc). Similarly, the mouse VH7 gene (amplified from scFv-MAB32 with LMB3 and VH1FOR2) was combined by splicing by overlap extension PCR with the human u-CH1 domain (amplified from human IgM-derived cDNA (Marks et al., 1991, supra WO 92/01847) with Mo-VH-Ku-CH1 and HCM1FON0, and cloned as Sfi-Not fragment into a pUC19-peLB-myc for soluble expression of a tagged chain.

[0250] Display of the MAb32 Antibody on Phage:

[0251] The chimaeric light chain was displayed on phage Fd by reamplification of the mouse/human chimaeric chain with HUCK/CYSNOT and MVK/BAAPA and cloning into Fd-tet-DOG1 as an ApaLI-Not fragment. Cells harbouring A plasmid with the heavy Fd chain gene were grown in 2xTY containing AMP-GLU (1%) to logarithmic phase (OD600 of 0.5) and infected with a 20-fold excess of light-chain displaying phage. After 45 min at 37°C without shaking and 45 min at 37°C with shaking in the 2xTY, ampicillin (100 µg/ml). Glucose it medium, a sample was diluted into 50-fold volume of prewarmed (37°C) 2xTY, ampicillin (100 µg/ml) and tetracyclin (15 µg/ml), grown for 1 hr at 37°C and then overnight at 30°C (shaking). Phage particles collected from the supernatant of such culture displayed TNF-binding Fab fragments anchored through the light chain on their surface.
Similarly, the reversed configuration was made. The heavy chain VHCH1 fragment was cloned into fd-tet-DOG1 (after amplification of the Fd chain gene from the mouse/human chimera construct with VH1BACKAPA and HCM1FON0), and phage used to infect cells capable of producing soluble light chain. Phage particles collected from the supernatant of such culture displayed TNF-binding Fab fragments anchored through the heavy chain VHCH1 fragment on their surface.

Properties of MAb 32 Fragments Displayed on Phage:

The V-genes of the murine antibody MAb32 were cloned by amplifying the hybridoma V-genes, cloning the VH and Vk genes as scFv genes in phagemid pHEN1 as above. Antibody scFv fragments which bind to TNF were identified by ELISA. The Fab gene was recloned into pUC19-pelB-myc for soluble expression as a mouse VH linked to human mu-CH1, while the light chain was recloned with the human Ck domain in vector fd-tet-DOG1 as a fusion with g3p. When cells harbouring the heavy chain construct were infected with the fd-phage carrying the light chain, phage particles emerged which carried light chain-g3p associated with the fd heavy chain. Indeed, binding to TNF and the 301 peptide was retained, as judged by ELISA with phage displaying the mouse-human chimaeric Fab fragment. In the phage ELISA, the background signal of phage carrying the light chain only was a lightly higher than wild-type fd-tet-DOG1 phage, but always lower than the signal obtained with Fab-displaying phage. Similarly, TNF binding phage was made with the heavy chain VHCH1 fragment anchored on phage, and the light chain provided as a soluble fragment. Hence, MAb32 is functional in the dual combinatorial format in both display orientations.

2 Chain Shuffling by Epitope Imprinted Selection (EIS) Construction of One Chain-Libraries:

Kappa, lambda light chain and Mu-specific cDNA was made from the mRNA prepared from the peripheral blood lymphocytes from two healthy donors essentially as in Marks et al., 1991, supra. The first-strand cDNA synthesis was performed with oligonucleotides RCM1FO, HUCLYS and HUCKYS for Mu-specific, lambda and kappa libraries respectively. The VH-CH 1 repertoire was amplified from this cDNA with oligonucleotides HCM1FO and six family specific VHBACK primers (as in Marks et al., 1991, supra), reamplified with a NofI-tagged forward primer (HCM1FONO) and ApaLI tagged VHBACK primers (6 primers HuVH1BAAPA to HuVH6BAAPA). Similarly, the light chain repertoire were amplified with HUCLYS or HUCKYS forward primers and HUVA1BACK to HuVA6BACK or HuVki1BACK to HuVki6BACK back primers described in Marks et al., 1991, supra and PCT/GB91/01134 (WO 92/01047). In each case described in this section the lambda and kappa chain variable repertoire were amplified separately. The amplified repertoires were realigned with ApaLI and NofI tagged versions of these oligonucleotides (13 back primers HuV1.1BAAPA to Hu6BAAPA or HuVki1BAAPA to HuVki6BAAPA and two forward primers HuCLCYSNOT and HuCKCYSNOT, respectively). All three repertoires were cloned into vector fd-tet-DOG1 as ApaLI-NofI fragments, and electroporated into E.coli MC1061 cells, to obtain libraries of 1.0 x 10^7 clones for IgM-derived VHCH1. The presence of insert was checked and the frequency of inserts in the library found to be higher than 95% in all three cases.

Selecting a Human VL Using the Mouse VH Domain as Docking Chain:

In a first chain shuffling experiment, the mouse VH (linked to the human CH1 domain), expressed from pUC19-pelB-myc, was paired a Fab fragment with a library of 10^7 different human V\(\gamma\)/C\(\alpha\) domains. Phage displaying the antibody fragments were subjected to rounds of panning on TNF-coated tubes. By following the titre of the eluted phage, the extent of selection was monitored. After 4 rounds (with a 100-fold increase in the titre of eluted phage), 24 out of 28 individual clones were found to be binding to TNF in an ELISA with phage expressing Fab fragments (all with the mouse VH-human CH1). Phage only displaying the selected human V\(\gamma\)/C\(\alpha\) domains gave a background similar to phage displaying only the chimeraic mouse Vk-humank Ck. Sixteen clones taken after the first round of selection were found to be negative.

Only three different BstNI fingerprints were found amongst the 24 binders, with one pattern dominating (21/24). Light chains V\(\gamma\)2A, V\(\gamma\)4 and V\(\gamma\)1 were found with frequencies of 21/24, 2/24 and 1/24 respectively. Sequencing revealed that all three light chains are derived from the same germline gene, a human V\(\gamma\)2-1-1. Clone V\(\gamma\)4 has 1, clone V\(\gamma\)1.1 has 2 and clone V\(\gamma\)2A 7 amino-acid residue differences from the germline. However, clone V\(\gamma\)2A uses a framework-1 region which more closely resembled the germline sequence of a related V\(\gamma\)1, humV1117, and therefore may be the result of a cross-over. The germline character of the clones was also noted in the CDR3 sequence, with minimal variation in sequence and no length variation between the three clones. Apparently, only a very limited number of genes with very similar sequences fix the stringent requirements (being compatible with the mouse VH and forming an antigen-binding pair).

Selecting a Human VH Using the Selected Human VL Domains as Docking Chains:

Three selected V\(\gamma\) genes were recloned in pUC19-pelB-myc for soluble expression as V\(\gamma\)/C\(\alpha\) chains. E.coli cells harbouring the three light chain plasmids were mixed, infected with a phage library of human VHCH1 genes, expressed from the fd-tet-DOC1 library described earlier and the library subjected to rounds of panning on TNF-coated Immuno tubes. Clones were picked after 5 rounds, when the titre of eluted phage increased 100-fold. Fifteen out of 20 clones analysed by BstNI fingerprint of the DNA insert used one of two patterns (with approximately the same frequency). The 15 clones when combining their heavy chain VHCH1 fragments with the V\(\gamma\)2A light chain gave stronger phage ELISA signals than when combined with the V\(\gamma\)4 or V\(\gamma\)1 domain light chain. Background signals obtained with phage displaying the heavy chain VHCH1 fragment only were similar to the signal of the murine VH-human CH1.

Sequencing revealed that the two patterns could be assigned to three unique human VH sequences (clones VHP1/2/3, with clone VHP1 having a BstNI fingerprint which is nearly identical to that of clone VHP2). Like the selected light chain genes, the selected heavy chain genes
are derived from the same germline VH gene (germline DP-51 from the VH3 family, Tomlinson et al., J. Mol. Biol. 227, pp776-798 1992), with minimal residue differences. The selected human V-genes were aligned to their closest germline homologue; identical residues in the selected genes are represented by hyphens. Framework 4 of the VH genes was truncated at 4th residue. Clone VHI P1 was most likely a cross-over between DP-51 and a related germline, DP-47. All three selected VH-genes had relatively short CDR3 loops (8, 9 and 10 residues), but shared little homology in this sequence.

[0262] Specificity of Binding of the Selected V-Gene Pairs:

[0263] Aspecificity ELISA with MAb32 and soluble scFv fragments on a number of antigens showed that MAb32, its scFv-derivative and three of the humanised TNF-binding (as scFv-fragments) bind specifically to TNF. No significant binding was obtained to ELISA plates coated with keyhole limpet haemocyanin, ovalbumin, cytochrome c, bovine serum albumin, human thyroglobulin, or 2-phenylazol-5-one-BSA or to plastic only. Fully humanized clones were obtained which bound to both peptide 301 and TNF.

[0264] In addition, to show that the human scFv fragments compete with the original antibody for binding to TNF, the binding of the scFv constructs in a competition ELISA with the Fab fragment derived by proteolytic cleavage of MA b32 was analysed. Single chain Fv fragments were incubated on a TNF-coated surface with increasing amounts of the Fab fragment and the amount of bound scFv detected in ELISA. Each of the scFv fragments competed with the Fab MA b32 for binding to TNF, including both the original scFv-MA b32 and the humanised scFv fragments.

[0265] Thus the fine specificity of MA b32 for peptide 301 of TNF was retained through the humanisation process.

[0266] Affinity of Binding of the Selected V Gene Pairs:

[0267] MA b32 and purified, monomeric forms of the recombinant mouse scFv-MA b32 and the human scFv antibodies VHP1-VH2, VHP2-VH2, and VHP3-VH2, were subjected to competition ELISA for the determination of the relative affinity for TNF. Antibodies were incubated on a TNF-coated surface in the presence of increasing amounts of soluble TNF. All the clones showed a roughly similar decrease in the ELISA signal over the same range of increasing TNF concentrations (with an IC50 in the 10 nM to 100 nM range).

[0268] MA b32 and VHP3-VH2 fragments were also analysed for binding properties using the Pharmacia BIACore. TNF was indirectly immobilised on the surface, and the binding of antibody monitored. On the TNF surface, the Fab fragment from MA b32 by proteolytic cleavage and the scFv MA b32 showed very similar fast off rates (approximately 10^3 s^-1). The human VHP3-VH2 antibody has an off rate in the same range as the original scFv-MA b32. On rates for antibody protein interactions were in the range seen for the interaction between other proteins and their receptors, and cover a 100 fold range between 10^6 and 10^8 M^-1 s^-1 (Mason D. W. and Williams, A. F., 1986, Kinetics of Antibody Reactions and the Analysis of Cell Surface Antigens, Blackwell, Oxford; Pecht, L., 1992 in Sela, M. (ed), Dynamic Aspects of Antibody Function, Academic Press Inc., New York, Vol. 6 pp 1-68). Assuming the on rates of the antibody TNF interactions are typical of antibody protein interactions, the off rate derived by the BIACore analysis is consistent with the affinity indicated by the competition ELISA (Km=10^-7 to 10^-8 M).

[0269] Thus, these determinations are consistent with scFvMA b32 and the humanised scFv clone VHP3-VH2 having a similar affinity and thus with the retention of affinity, as well as specificity, through epitope imprinted selection.

[0270] Conclusion

[0271] We have shown that a mouse antibody can be rebuit into a human antibody with the same specificity by the process of epitope imprinted selection (EIS).

[0272] A library of human light chains were shuffled with a mouse VH domain, binding combinations selected and then used in a second shuffle as " docking domains" for a library of human VH genes. Completely human antibodies were isolated from such "genuine" human library. The antibodies were shown to bind retain binding specificity. Alternatively, the mouse VL was used as docking chain for selecting human VH partners. Such VH domains can be used to find human VL genes, or alternatively, can be combined with human VL domains selected with the mouse VH domain. Indeed, binding activity was obtained by combining two independently selected V-genes, pointing towards potential additivity of the EIS procedure.

[0273] The EIS approach may serve to humanise antibodies more rapidly than by CDR-grafting (Riechmann et al., 1988, supra), as this method requires very often a detailed knowledge of the 3-D structure of the antibody. However, the EIS method can be extended to for example antibody repertoires obtained by phage selection from immunised rodents. Following immunisation with antigen, a repertoire of V-genes with high affinity and specificity may be selected and then used in an epitope imprinted selection (see example 4) to generate a range of human antibodies of high affinity and enriched for the desired specificity.

Enhancement of TNF-Induced Tumour Regression by Antibody VHP3-VH2, the Human Equivalent of MA b32

[0274] BALB/c mice were inoculated with WEHI-164 tumour cells as described above. After development of subcutaneous tumours the mice were treated daily with TNF (1 or 10 µg) alone or in combination with purified P3A2 (50 µg) by intraperitoneal injection. Tumour size was measured throughout the course of the treatment period.

[0275] Results are shown in FIG. 35.

[0276] VHP3-VH2 enhanced the anti-tumour activity of TNF at both the 1 and 10 µg levels.

[0277] Conclusions

[0278] Mapping of the regions recognised by each of the MAbs has indicated that MAbs in group I (MAbs 1, 21, 47, 54, 37, 32 and 25) as shown on the schematic diagram bind TNF in-the region of residues 1-18 with the exception of MAbs 37 and 54, while MAbs in group II of the schematic diagram (MAbs 11, 12, 53 and 42) bind TNF in the region of residues 70-96 which encompasses a so-called pallendromic loop on the TNF 3-D structure. MAbs which inhibit the
induction of endothelial cell procoagulant activity (MAbs 1, 32, 42, 47, 54 and 53) all bind in the region of residues 108-128 which again contains a loop structure in the 3-D model and may indicate that this region interacts with TNF receptors which are found on endothelial cells but not tumour cells. MAb 32 which potentiates the in vivo tumour regression and anti-viral activity of TNF is the only antibody which binds all the loop regions associated with residues 1-26, 117-128, and 141-153 and hence binding of these regions is crucial for enhanced TNF bioactivity with concommitant reduction of toxicity for normal cells.

[0279] As is apparent from Table 2 MAb 1, 47 and 54 have the same effect on the bioactivity of TNF. From the results presented above it is noted that these three monoclonals bind to similar regions of the TNF molecule. Accordingly, it is believed that a ligand which binds to TNF in at least two regions selected from the group consisting predominately of the region of residues 1-20, the region of residues 56-77, the region of residues 108-128 and the region of residues 138-149 will effect the bioactivity of TNF in a manner similar to that of MAb 1, 47 and 54. Similarly, it is believed that a ligand which binds to TNF predominately in the regions of residues 1-20 and 76-90 will have the same effect on the bioactivity of TNF as MAb 21. A ligand which binds to TNF predominately in the regions of residues 22-40 and 69-97 will have the same effect on bioactivity of TNF as MAb 12. A ligand which binds to TNF predominately in the regions of residues 1-30, 117-128, and 141-153 would be expected to have the same effect on the bioactivity of TNF as MAb 32 and a ligand which binds to TNF predominately in the regions of residues 22-40, 49-97, 110-127 and 136-153 would be expected to have the same effect on the bioactivity of TNF as MAb 42. A ligand which binds to TNF predominately in the regions of residues 22-31 and 146-157 would be expected to have the same effect on the bioactivity of TNF as MAb 37 and a ligand which binds to TNF predominately in the regions of residues 22-40, 69-97, 105-128 and 135-155 would be expected to have the same effect on the bioactivity of TNF as MAb 53.

[0280] The present inventors have quite clearly shown that the bioactivity of TNF can be altered by the binding of a ligand to the TNF, and that the effect on the bioactivity is a function of the specificity of the ligand. For example, the binding of MAb 32 to TNF in the regions of residues 1-26, 117-128 and 141-153 results in the induction of endothelial procoagulant activity of the TNF and binding of TNF to receptors on endothelial cells being inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF being enhanced; the cytotoxicity being unaffected and the tumour receptor binding activities of the TNF being unaffected or enhanced. It is believed that this effect on the bioactivity of the TNF may be due to the prevention of the binding of the epitope of the TNF recognised by MAb 32 to naturally occurring biologically active ligands. Accordingly, it is believed that a similar effect to that produced by MAb 32 could also be produced by a ligand which binds to a region of TNF in a manner such that the epitope recognised by MAb 32 is prevented from binding to naturally occurring biologically active ligands. This prevention of binding may be due to steric hindrance or other mechanisms.

[0281] Accordingly, it is intended that the prevention of the binding of epitopes recognised by the various monoclonal antibodies described herein to naturally occurring biologically active ligands is within the scope of the present invention.
Peptide Synthesis Calculation Sheet:

Notebook name is "e305".
Notebook file is "e309.NBK".

Target Peptide: length = 22, Mw = 2507.037

NH2-His-Val-Leu-Leu-Thr-His-Thr-Ile-Arg-Ile-Ala-Val-Ser-Tyr-Gln-Thr-Lys-Val-Lys-Leu-Leu-COOH

Resin substitution = 0.100 meq/g
Resin quantity = 3.000 g
Excess amino acid = 4.000 x
Peptide Quantity = 0.300 mmoles 23-20-69
Theoretical Yield = 0.752 g 3.Y
Starting Resin: FMOC-Leu-PepSyn-KA

EXHIBIT 1
Peptide Synthesis Calculation Sheet:

Notebook name is "s323".
Notebook file is "s323.NBK".

Target Peptide: length = 11, MW = 1238.416

NH2-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-Gln-Thr-COOH

Resin substitution = 0.460 meq/g
Resin quantity = 4,000 g
Excess amino acid = 4,000 µmol

Peptide Quantity = 0.400 µmol
Theoretical Yield = 0.395 g

Starting Resin: FMOC-Thr-PepSyn-KA
In the application of:

Deborah A. RATHJEN and Roger ASTON

Serial No.: 09/364,039
Filing Date: July 30, 1999
For: TUMOR NECROSIS FACTOR BINDING LIGANDS

Examiner: J. Roark
Group Art Unit: 1644

DECLARATION OF DEBORAH A. RATHJEN
PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Deborah A. Rathjen, declare as follows:

1. I am an inventor of the above-referenced patent application, and am familiar with the contents thereof.

2. I currently reside at [INSERT]: 56 Kingfisher Circuit, Flagstaff Hill, South Australia, SA 5606.

3. I have reviewed the publication Socher et al. (1985) Proc. Natl. Acad. Sci. USA 82:4829-4833, cited in the specification of the above-listed application Serial No. 09/364,039 and submitted in the Supplemental IDS filed April 20, 2001. The cited publication lists the complete deduced sequence of mature recombimt human tumor necrosis factor α (HTNF-α) (page 4830, Figure 1A), which is a 157-residue protein of molecular weight approximately 17,000 kD. The source (page 4832, column 1, 1st full paragraph) cited for this sequence is Peanica et al. (1984) Nature 312:724-729 "Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin" (Appendix). The primary amino acid sequence listed...
in Socher et al. is the mature form of the protein, as distinguished from the precursor protein which has an open reading frame of 233 amino acids in length (Pennica et al.).

4. The hTNF-α amino acid sequence listed in Socher et al., agrees with the amino acids listed in the peptide sequences in the application specification Serial No. 09/364,039 on page 31 (line 16) through page 32 (line 7) for all residues, with the exception of amino acid residues 59 and 87, verifying that the numbered peptide sequences in application Serial No. 09/364,039, and therefore the hTNF-α numbering used throughout the specification, correspond to the numbering of the mature hTNF-α protein.

5. Regarding residues 59 and 87, Socher et al., lists these residues as Thr-59 and Tyr-87, while application Serial No. 09/364,039 lists these residues as Tyr-59 and Thr-87. In Pennica et al., both of these residues are shown as the amino acid Tyr (page 725, Figure 1; page 728, Figure 4). One of ordinary skill in the art would realize that the Tyr→Thr switches at position 59 in Socher et al., and position 87 of application Serial No. 09/364,039 are typographical errors, as the earlier Pennica et al., (cited in Socher et al.) is in agreement with sequences of the human TNF-α precursor (additional 76 residues at N-terminus) deposited in publicly accessible databases, for example, 1985 (pir accession number QWHUN) and 1986 (SWISS-PROT accession number P01375) (see Appendix) and references cited therein.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

19 May 2001
Deborah A. Mannon

Docket No. 273402004221301
pa-590032

Serial No. 09/364,039
APPENDIX
Human tumour necrosis factor: precursor structure, expression and homology to lymphotixin

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**Manuscript received 20 April and accepted 5 September 1983

Human tumour necrosis factor (TNF) has about 30% homology in its amino acid sequence with lymphotixin, a lymphokine that shows similar biological properties. Recombinant tumour necrosis factor can be obtained by expression of its complementary DNA in Escherichia coli and induces the haemorrhagic necrosis of transplanted methylcholanthrene-induced sarcomas syngeneic mice.

**Molecular structure.** TNF has been associated with in vivo and in vitro killing of tumour cells. This activity was observed originally in the sera of mice and rabbits injected with mycobacterium leprae strain bacillus Calmette-Guérin (BCG) or other immunostimulatory agents, and subsequently in the sera of mice treated with endotoxin. Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplantable tumours in mice. TNF-like activity has also been detected in the media of BCG-endotoxin-induced monocyte cultures (reviewed in ref. 2) and mononuclear-stimulated peripheral blood lymphocytes.

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[Further references and details on the role of TNF in tumor necrosis and its relationship to other biological factors and processes.]
**Fig. 1** TNF cDNA sequences and predicted amino acid sequence. Schematic representation of the complete cDNA structure is shown. Line, untranslated sequence; bars, coding sequence; white portion, sequence encoding the signal peptide; shaded regions code for mature TNF. The black box on the 5' end of clone 144-4 indicates that this clone was obtained by specific hybridization with cDNA sequence and deduced amino acid sequence of human TNF cDNA. Numbers below each line refer to amino acid positions and numbers below each line refer to nucleotide positions. The 26 amino acids labelled 1 represent the first amino acids of matured TNF. The 76 amino acids preceding this position are indicated by lower case lettering. Sequence underlined indicates the poly(A) addition recognition site.

**Method**

Total RNA was extracted from HL-60 cells by the method of Chirgwin et al. (1979). Double-stranded cDNA was prepared by oligo(dT) priming using 7 S g mRNA and fractionated on a 3 M NaCl-polyacrylamide gel. An oligo(dT)-18 probe, prepared by digoxigenin labelling, was used for visualisation of cDNA clones. Hybridization was performed with digoxigenin-labeled probes under conditions described (Chirgwin et al., 1979). Plaque screening, DNA-DNA hybridization, and Southern blotting of total and cDNA were performed. cDNA sequencing was performed by the dideoxynucleotide chain termination procedure (Sanger et al., 1977).

Table I: Human TNF production -  summary of cell populations and cell lines

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Inducing agents</th>
<th>Cytotoxic activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>None</td>
<td>&lt;8</td>
</tr>
<tr>
<td>PBLs</td>
<td>LPS</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>BCG</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>BCG/LPS</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>BCG/LPS/Reagents</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>BCG/LPS/Reagents</td>
<td>200</td>
</tr>
<tr>
<td>PBLs (adherent cells)</td>
<td>None</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td>BCG/LPS</td>
<td>150</td>
</tr>
<tr>
<td>PBLs (non-adherent cells)</td>
<td>None</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>BCG/LPS</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>BCG/LPS/Reagents</td>
<td>350</td>
</tr>
<tr>
<td>L-60</td>
<td>None</td>
<td>350</td>
</tr>
<tr>
<td>U-937</td>
<td>None</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>350</td>
</tr>
</tbody>
</table>

PBLs were obtained from peripheral blood mononuclear cells (PBLs) by Ficoll-Hypaque centrifugation. Separation of PBLs into adherent and non-adherent populations was performed as described previously. HL-60 (CCL 143) and U-937 cell lines (CRL 993) were obtained from the American Type Culture Collection. Cells were suspended at 5 x 10⁵ cells/ml in RPMI 1640 media containing 10% fetal bovine serum. Cultures were induced with one or more of the following agents: 2 x 10⁵ organisms per ml of BCG (Cohn strain), 20 μg/ml Subramanian thymidine lipopolysaccharide (LPS, Sigma), 1 μg/ml lipopolysaccharide endotoxin B (SER, Sigma), 1 μg/ml Sendai virus nucleoprotein, and 10 μg/ml ATP (P-L Biochemicals). Cell-free supernatants were collected 24 h after induction except for the BCG/LPS and BCG/LPS/Reagents treatments; for these two inductions a 24 h BCG stimulation was followed by an additional 24 h treatment with LPS and LPS/Reagents, respectively. Samples were analysed for cytotoxic activity on mouse L929 fibroblasts as described previously. The activities shown represent TNF-specific or lymphokine-specific units as determined after antibody neutralisation at 4°C for 4 h before assay. The units indicated were obtained from one representative clone in the case of the PBLs and from a single experiment when cell lines were used. Rabbit anti-human TNF antiserum was prepared against partially purified TNF from PBLs (I. Barlow, unpublished results). Rabbit anti-human lymphokine antiserum was prepared against partially purified TNF from RPMI 1640 lymphokine cell line. Here we identify a cell line with monococyte-like characteristics providing a source for human TNF and its messenger RNA. CDNA clones were isolated that encode a polypeptide related structurally to lymphokinin. This cDNA was engineered to direct the synthesis of a relative molecular mass (Mr) 17,000 protein in E. coli with the immunological characteristics as well as in vitro and in vivo biological properties of the natural human TNF. A human TNF-producing cell line

We isolated PBLs by Ficoll-Hypaque density centrifugation and fractionated them into adherent mononuclear and non-adherent lymphocytic fractions. After stimulation with BCG and endotoxin (lipopolysaccharide, LPS), we detected an activity cytokotoxic to murine L-929 cells in the culture media of unfractionated mononuclear cells and monocytes (Table I). No cytotoxic activity was produced by the non-adherent cells following the same BCG/LPS induction procedure. The failure of rabbit anti-human lymphokinin antibodies to neutralize the cytotoxic activity demonstrates its difference from lymphokinin. Moreover, the results of previous in vivo studies using BCG/LPS induction procedures demonstrate that the activity can probably be attributed to TNF. Antibodies raised against partially purified PBL-produced TNF completely neutralized this activity (Table I).
Yields of adherent cells from peripheral blood were low and levels of TNF produced were variable and donor-dependent. Therefore, the use of alternative induction schemes for the production of TNF from total PBMC (Table 1). An increase in cytotoxic activity was observed when the PBMC were co-stimulated with phorbol ester (B, desacetylphorbol 12β-myristate 13-acetate MA). However, antibody neutralization experiments demonstrated that a significant portion of the measured activity was lym- 

FpDNA eDNA clone identification

The identification of NF-cDNA was by a human cDNA clone from the HL-60 cell lineage. The primer 5'-GGC-ACCC-TCG-CAAG-CTG-TAG-GCTG-3' was used to amplify the cDNA sequence from the HL-60 cell line. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence of the amplified product matched the published sequence of the human TNF gene. The sequence was also compared to the sequence of the bovine TNF gene and was found to be identical. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplify the TNF gene from the human genome. The amplified product was cloned into the pGEM-T easy vector and sequenced as described previously. The sequence was then used to design a primer set that was used to amplifier
Table 2. Necrosis of Meth A sarcoma in vivo.

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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>T19, kp</td>
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</table>

(RBAL/c57BL/6J, female mice were injected intrastradially with 1×10⁶ BALB/c Meth A sarcoma cells. Ten days later, the tumours (0.7 cm in diameter) were injected intradermally (Li, 1×10⁶ U), intraperitoneally (p, 5×10⁶ U) or intramuscularly (i.m., 5×10⁶ U) with TNF in a total volume of 0.1 ml PBS. At 24 h after TNF treatment, the tumours were excised, sectioned and scored for necrotic/necrotic response by visual and histological examination as described previously. In the maximum response (+++), 90-100% of the tumour mass is markedly necrotic after 24 h; ++ denotes a moderate response, that is, 25-50% of tumour necrosis; +, a minimal response of 5-25%; and −, no necrosis. Necrotic TNF was purified from HL-60 culture supernatants as described elsewhere. Recombinant TNF (rTNF) was purified from E. coli W3110/pTNFp with a purity of >99% and a specific activity of ~10⁵ units mg⁻¹ (J. Bergman, unpublished results).

Fig. 3. SDS-polyacrylamide gel electrophoresis of human TNF synthesized in E. coli. E. coli K-12 strain WH16, transformed with pTNFp or pH322, was grown in M9 medium containing 5 µg/ml tetracycline. Cells were collected, lysed in 1% SDS, 5% β-mercaptoethanol and precipitated with 10 volumes of cold acetone. Samples were electrophoresed on a 12.5% SDS-polyacrylamide slab gel using the buffer system of Maitz et al. and the gel stained with Coomassie brilliant blue. The left lane contains protein standards (x×10⁴); phosphorylase b (92,000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500) and lysozyme (14,500). Lanes 1, 2, 5, 7, 8, cell lysates of E. coli strain BY4741; lanes 3, 4, cell lysates of E. coli W3110/pTNFp; lane 6, partially purified human TNF isolated from the HL-60 cell line; lane 9, mixture of the E. coli strain W3110/pTNFp cell lysate and the HL-60-derived, purified TNF.

The absence of any potential N-glycosylation sites in the deduced amino acid sequence suggests that TNF is not a glycoprotein. These data suggest also that TNF may occur naturally in multicellular form, as the M, estimated previously for human TNF ranging from 34,000-140,000 (refs 6, 28). There are two cysteine residues (positions 69 and 101) in TNF which are likely to be involved in a single intramolecular disulfide bond. The CpG DNA clone A4-2 contains the entire coding region of mature TNF but lacks a complete signal peptide coding sequence and initiation codon. To obtain the missing sequence information, a specifically-prime-d cDNA library was prepared (see Fig. 4 legend) and screened with the 32P-labelled A14-2 cDNA insert. A cDNA clone (A16-4) was identified which contained an insert extending 337 bp further 5' than the 442-4 insert (Fig. 1). From the analysis of the TNF cDNA sequence, it seems that TNF is synthesized initially as a larger precursor (pre-TNF) and released by terminal proteases (Duffy et al.) to occur naturally as heterogeneous glycoprotein as a consequence of N-terminal and C-terminal proteolysis, respectively.

The presence of 76 residues is most probably involved in the secretion of TNF as it is not observed on the mature TNF peptide and contains an unusually long hydrophilic region of 26 amino acids (residues 46 to 20). Typically, signal peptides involved in protein secretion are only 20-30 amino acids long. However, a signal sequence for the Rosai sarcoma virus envelope glycoprotein is unusually long (63 residues) and contains many charged amino acids at its amino terminus, such as pre-TNF. It is interesting to note the presence of Arg-Arg and Lys-Lys dipeptides in the first 30 amino acids of the TNF pre-sequence, as pairs of basic amino acids often serve as cleavage sites for the release of physiologically-important peptides from precursor molecules. We used the 32P-labelled A14-2 cDNA insert to examine TNF gene structure and mRNA size. Results from Southern hybridizations indicate that only a single gene for TNF is present in the human genome. Northern hybridization analysis shows that a single mRNA species (~15S) is in size is synthesized in PMA-induced HL-60 cultures and BCG/LPS-treated macrophages isolated as described elsewhere. This provides additional evidence that the same cytokine is produced from both cell sources and suggests that the TNF cDNA sequence shown in Fig. 1 represents a nearly full-length copy of TNF mRNA. No hybridization was detected to mRNA isolated from uninduced cultures (data not shown).

TNF synthesis in E. coli

Proof that the cDNA described here encodes TNF requires the demonstration that it can direct the synthesis of a gene product with the properties of authentic human TNF. To allow characterization of the protein encoded by the cloned cDNA, we engineered the TNF cDNA sequence for direct expression in E. coli (Fig. 2). In the resulting expression plasmid, pTNFp, the TNF DNA sequence is under the transcriptional control of a 300-bp DNA fragment of the E. coli trp operon containing the trp promoter, and Shine-Dalgarno sequence of the trp leader peptide. Total extracts of E. coli K-12 strain WH16 transformed with pTNFp contained a prominent polypeptide with an apparent M, 17,000 (Fig. 3, lanes b, e). This protein is not visible in cells transformed with pH322 (lanes a, f), strongly suggesting that it represents the translational product of the TNF cDNA sequence. Furthermore, this protein co-migrates with authentic TNF (lane c) isolated from the HL-60 cell line (lane d), suggesting that no significant post-translational processing of TNF sequence occurs in the HL-60 cell line. This is unlike lymphocytes and y-interferon, both of which occur naturally as heterogeneous glycoproteins as a consequence of N-terminal and C-terminal proteolysis, respectively.
lymphokinin to determine whether similarities in their biological properties might be attributed to common structural features (Fig. 4). By introducing two gaps, the lymphokinin sequence can be aligned with the TNF sequence so that distinct homologies are apparent: we find 44 of the 157 TNF residues (28%) to be identical to corresponding lymphokinin amino acids with many of the remaining differences between the two polypeptides resulting from conservative amino acid changes. The nucleotide homology over this coding region is 46% (data not shown). Two particularly conserved regions occur at amino acids 35-66 and 110-133 (TNF numbering) where 50% of the residues (28 of 56) are identical for TNF and lymphokinin. The hydrophobic carboxy-terminal of the two molecules are also significantly conserved. It is probable that the conserved regions are crucial to the shared cytokine activities of TNF and lymphokinin, perhaps through interaction with a common receptor expressed on the surface of transformed cells. Support for this hypothesis is provided by the lack of cytokine activity in a truncated lymphokinin polypeptide lacking its last 16 amino acids.11

Lymphokinin has 18 more NH2-terminal amino acids than TNF (Fig. 4), suggesting that this region is not required for cytokine activity. In fact, a 148 residue lymphokinin, consisting of amino acids 24-171 of mature lymphokinin, and having similar cytokine effects on L-929 cells, has been isolated from the RPMI-1788 cell line.11,12 It is also interesting that amino acids 67-109 of TNF are unrelated to the corresponding region of lymphokinin: only two of 43 residues are identical. This region includes all of the amino acids spanned by the Cys-69-Cys 101 disulfide bridge of TNF. One possible role for this non-conserved region could be to position correctly the two surrounding homologous regions in a conformation essential for cytokine activity. Such positioning, which could be acquired by a TNF disulfide bond, may require a very different sequence of amino acids in lymphokinin, where no disulfide bond exists.

These apparently unrelated regions of TNF and lymphokinin might specify also as yet undiscovered differences in biological activity and/or target sites between the two molecules. The availability of efficient expression systems for TNF and lymphokinin,3,14 in combination with the techniques of site-directed mutagenesis,15 will make it possible to address questions of this type directly.

We thank Phil Hass for growing HL-60 cells; Dr. Lloyd Swedensky and Tim Bringeman for preparing TNF antisemur; Mark Vasser, Parkash Jharuani and Peter Ng for deoxynucleotidyl-clotide synthesis; Irene Figari and Rekha Shalby for assistance with the tumour necrosis assays; Roxanne Chang and the Genentech Biologics Group for performing in vivo TNF assays; and Dr. Richard Harkins for helpful suggestions. G.E.N. dedicates this work to his late J. L. Levenson.
Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing

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Institute for Immunology, Kyoto University Faculty of Medicine, Kyoto 606, Japan

A novel mammalian neuropeptide, the tachykinin substance K, is specified by a discrete genomic segment. Alternative RNA splicing generates two distinct mRNAs encoding the neuropeptide substance K alone or with substance K from a single preprotachykinin gene. Relative amounts of the mRNAs vary in different tissues, suggesting that the substance K-encoding sequence is regulated in a tissue-specific manner.

SUBSTANCE K is one of the best characterized neuropeptides in mammalian tissues; several lines of evidence suggest that it acts as a neurotransmitter or neuromodulator in primary sensory neurons. Substance K belongs to a family of related peptides, the tachykinins, and it is thought to be the only member of this family that is endogenously synthesized in mammals. Recently, we elucidated the entire primary structures of two types of bovine brain substances K precursors (α- and β-preprotachykinins) by determining their cloned cDNA sequences. β-Preprotachykinin (β-PPT) contains not only the substance K sequence but also a novel tachykinin sequence designated substance Kα, whereas α-preprotachykinin (α-PPT) lacks the latter sequence, containing only substance Kα. The decapeptide substance K has been found independently as neurokinin A, a gut-contracting peptide in porcine spinal cord. The chemically synthesized substance K peptide possesses biological activities characteristic of the tachykinins family, but is considerably more potent than substance P in mammalian organisms.

The two PPT mRNAs exhibit an interesting structural relationship. They have complete identity in their 5′ and 3′ sequences and differ only in the insertion/deletion of the coding sequence for the substance K region. The characteristic structural relationship poses intriguing questions about the gene organization for these two mRNAs and the regulation for the generation of the two biologically different mammalian tachykinins. Our present investigations thus concern the structural organization of the substance K genes and the distribution and regulation of the two PPT mRNAs in the nervous system and peripheral tissues. We report here that the sequence specifying the substance K region is encoded by a discrete genomic segment, and that both α- and β-PPT mRNAs arise from a single gene by alternative RNA splicing events. We also present evidence indicating the tissue-specific regulation of the PPT gene for the differential generation of the two PPT mRNAs.

PPT gene organization

Genomic clones containing the bovine preprotachykinin gene were isolated from a bovine genomic library by hybridization in situ with a bovine β-PPT cDNA probe, and all the isolated genomic DNA fragments were arranged into an approximately 36 kilobase-pair (kb) length of a continuous genomic DNA fragment (Fig. 1a; see Fig. 1 legend for experimental details of cloning). Nucleotide sequence analysis was performed on DNA fragments containing exons and their surrounding regions (Fig. 1b). Comparison of the genomic DNA sequence with the cDNA sequence enabled us to construct a structural organization of the bovine PPT gene (Fig. 1c). Introns A (403 base pairs, bp), B (192 bp), C (255 bp), D (653 bp), E (1076 bp) and F (3633 bp) all interrupt the protein-encoding region of the gene. The sequences at the exon-intron boundaries are consistent with the splice junction sequences observed for other genes. Exons 2–7 consist of 132, 97, 45, 24, 54 and 566 bp, each encoding the protein sequence corresponding to the signal peptide, substance Kα, two spacer sequences, substance Kβ, and the C-terminal sequence, respectively. It is remarkable that exon 4 precisely specifies the substance Kβ region minus 5′-PPT. Because blot-hybridization analysis of total cellular DNAs (data not shown) as well as the genomic cloning described above showed that no more than one PPT gene is present in the bovine genome, we conclude that both α- and β-PPT mRNAs are produced from a single gene as a consequence of alternative RNA splicing events.

The 5′ termini of the PPT mRNAs were identified by S1 nuclease mapping and primer extension analysis (Fig. 2). Both analyses revealed a length heterogeneity at the 5′ end of the PPT transcripts. The major 5′ termini of the PPT mRNAs mapped at 106–108, 110 and 111 bp upstream from the 5′ end of exon I (Fig. 1c). Several minor mRNA species starting further upstream were also observed and these 5′ termini mapped at roughly 130, 137, 146 and 156 bp upstream from the 5′ end of exon I. In support of these assignments, we found that three of the four cDNA clones isolated previously (clones pSP301, pSP302 and pSP305) contained the extreme 5′ sequences corresponding to the major 5′ ends, while the remaining one (clone pSP307) extended its 5′-terminus up to one of the minor 5′ ends. Based on the assignments of the 5′ termini of the PPT mRNAs, we conclude that the bovine PPT gene is 8.4 kb long.
REFERENCE 5 (residues 1 to 233)
TITLE Molecular cloning and expression of human tumor necrosis factor and comparison with mouse tumor necrosis factor
MEDLINE 86030296
REFERENCE 6 (residues 1 to 231)
TITLE Simultaneous production of natural human tumor necrosis factor-alpha, beta and interferon-alpha from BALL-1 cells stimulated by HIV
JOURNAL Lymphokine Res. 7 (2), 175-185 (1988)
MEDLINE 8801041
REFERENCE 7 (residues 1 to 231)
TITLE Myristylation of the tumor necrosis factor alpha precursor on specific lysine residues
MEDLINE 93018286
REFERENCE 8 (residues 1 to 233)
TITLE Dense Alu clustering and a potential new member of the NFkappaB family within a 90 kilobase HLA class III segment
REFERENCE 9 (residues 1 to 233)
AUTHORS D'Alciso, S. and Richardi, P.M.
TITLE A polymorphic variation in a putative regulation box of the TNF-alpha promoter region
MEDLINE 24102809
REFERENCE 10 (residues 1 to 233)
AUTHORS Takakura-Yamamoto, R., Yamamoto, S., Fukuda, S., and Kurimoto, M.
TITLE 0-glycosylated species of natural human tumor necrosis factor-alpha
MEDLINE 2620267
COMMENT Secreted from mitogen-activated macrophages within 4-24 hours after induction, TNF-alpha can cause cytolysis of certain tumor cell lines and have an antiproliferative effect on others without detriment to normal cells. It can also act synergistically with interferon gamma to kill certain transformed cell lines.

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181 tpegaeakwp yepniylggvf qlekgdrlsa einrpdyldf aeggyvfgsi ial

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NCBI | NLM | NIH
TUMOR NECROSIS FACTOR PRECURSOR (TNF-ALPHA) (CACHETIN)

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DEFINITION TUMOR NECROSIS FACTOR PRECURSOR (TNF-ALPHA) (CACHETIN).
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VERSION P01375 GI:135934
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sequence updated: Jul 21, 1986.
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KEYWORDS Cytokine; Cytotoxic; Transmembrane; Glycoprotein; Signal-anchor; Myristate; 3D-structure.
SOURCE human.
ORGANISM Homo sapiens
REFERENCE 1 (residues 1 to 233)
TITLE Tandem arrangement of genes coding for tumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta) in the human genome
MEDLINE 82717060
REMARK SEQUENCE FROM N.A.
REFERENCE 2 (residues 1 to 233)
TITLE Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin
MEDLINE 85086244
REMARK SEQUENCE FROM N.A.
REFERENCE 3 (residues 1 to 233)
AUTHORS Shirai, T., Yamaguchi, H., Ito, H., Todd, C.W. and Wallace, R.B.
TITLE Cloning and expression in Escherichia coli of the gene for human tumor necrosis factor
JOURNAL Nature 313 (6005), 803-806 (1985)
MEDLINE 85137898

REMARK SEQUENCE FROM N.A.
REFERENCE 4 (residues 1 to 233)
TITLE Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization
JOURNAL Nucleic Acids Res. 11 (17), 6361-6373 (1983)
MEDLINE 86016093

REMARK SEQUENCE FROM N.A.
REFERENCE 5 (residues 1 to 233)
TITLE Molecular cloning of the complementary DNA for human tumor necrosis factor
JOURNAL Science 228 (4686), 149-154 (1985)
MEDLINE 85341990

REMARK SEQUENCE FROM N.A.
REFERENCE 6 (residues 1 to 233)
TITLE Molecular cloning and expression of human tumor necrosis factor and comparison with mouse tumor necrosis factor
MEDLINE 86030296

REMARK SEQUENCE FROM N.A.
REFERENCE 7 (residues 1 to 233)
TITLE Danne Alu clustering and a potential new member of the NF kappa B family within a 90 kilobase HLA class III segment
MEDLINE 91272029

REMARK SEQUENCE FROM N.A.
REFERENCE 8 (residues 1 to 233)
AUTHORS Rowen, L., Madan, A., Qin, S., Shaffer, T., James, R., Ratcliffe, A., Abdell, N., Dickhoff, R., Loretz, C., Madan, A., Dorz, M., Young, J., Landy, D. and Hood, L.
TITLE Direct Submission
JOURNAL Submitted (??-OCT-1999) to the EMBL/GenBank/DDBJ databases

REMARK SEQUENCE FROM N.A.
REFERENCE 9 (residues 1 to 233)
AUTHORS Jones, E.Y., Stuart, D.I. and Walker, N.P.
TITLE Structure of tumour necrosis factor
JOURNAL Nature 338 (6212), 225-228 (1989)
MEDLINE 89154409

REMARK X-RAY CRYSTALLOGRAPHY (2.9 ANGSTROMS)
REFERENCE 10 (residues 1 to 233)
AUTHORS Jones, E.Y., Stuart, D.I. and Walker, N.P.
TITLE The structure of tumour necrosis factor--implications for biological function
MEDLINE 91193276

REMARK X-RAY CRYSTALLOGRAPHY (2.9 ANGSTROMS)
REFERENCE 11 (residues 1 to 233)
AUTHORS Eck, M.J. and Sprang, S.R.
TITLE The structure of tumor necrosis factor-alpha at 2.6 A resolution. Implications for receptor binding
JOURNAL J. Biol. Chem. 264 (129), 17595-17605 (1989)
MEDLINE 90008932

REMARK X-RAY CRYSTALLOGRAPHY (2.6 ANGSTROMS)
REFERENCE 12 (residues 1 to 233)
AUTHORS Reed, C., Fu, Z.Q., Wu, J., Xue, Y.H., Harrison, R.M., Chen, M.J. and
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**TITLE**
Crystal structure of TNF-alpha mutant R31D with greater affinity for receptor 1 compared with R2

**JOURNAL**
Protein Eng. 10 (10), 1101-1107 (1997)

**MEDLINE**
98147459

**REMARK**
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**REFERENCE**
13 (residues 1 to 233)

**AUTHORS**

**TITLE**
High resolution crystal structure of a human tumor necrosis factor-alpha mutant with low systemic toxicity

**JOURNAL**

**MEDLINE**
9811178

**REMARK**
X-RAY CRYSTALLOGRAPHY (1.8 ANGSTROMS) OF MUTANT N38.

**REFERENCE**
14 (residues 1 to 233)

**AUTHORS**
Van Ostade, X., Tavernier, J., Prange, T. and Fiers, W.

**TITLE**
Localization of the active site of human tumour necrosis factor (TNF) by mutational analysis

**JOURNAL**

**MEDLINE**
91101128

**REMARK**
MYRISTYLATION

**REFERENCE**
15 (residues 1 to 233)

**AUTHORS**

**TITLE**
Myristyl acylation of the tumor necrosis factor alpha precursor on specific lysine residues

**JOURNAL**

**MEDLINE**
91018820

**REMARK**
MYRISTYLATION

**COMMENT**
This SWISS-PROT entry is copyright. It is produced through a collaboration between the Swiss Institute of Bioinformatics and the EMBL outstation, the European Bioinformatics Institute. The original entry is available from http://www.expasy.ch/sprot and http://www.ebi.ac.uk/sprot

**[FUNCTION]**
TNF is mainly secreted by macrophages. It is a cytokine with a wide variety of functions. It can cause cytosis of certain tumor cell lines, it is implicated in the induction of cachexia. It is a poten fever causing fever by direct action or by stimulation of interleukin 1 secretion. It can stimulate cell proliferation and induce cell differentiation under certain conditions.

**[SUBUNIT]**
HOMOTTRIMER.

**[SUBCELLULAR LOCATION]**
TYPE II MEMBRANE PROTEIN. ALSO EXISTS AS AN EXTRACELLULAR SOLUBLE FORM.

**[PTM]**
The soluble form derives from the membrane form by proteolytic processing.

**[DISEASE]**
Cachexia accompanies a variety of diseases, including cancer and infection. It is characterized by general ill health and malnutrition.

**[SIMILARITY]**
Belongs to the tumor necrosis factor family.

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NCBI Sequence Viewer

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We claim:

1. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the in vivo tumour regression activity of the TNF is enhanced.

2. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the in vivo tumour regression activity of the TNF is enhanced; the ligand binding to the TNF such that the epitope of the TNF defined by the topographic region of residues 1-30, 117-128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands.

3. A ligand which binds to human TNF in the topographic regions of residues 1-30, 117-128 and 141-153.

4. A ligand as claimed in claim 3 in which the ligand binds to human TNF in the topographic regions of residues 1-26, 117-128 and 141-153.

5. A ligand as claimed in claim 1 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, single domain antibodies (dABs) restructured antibodies, single chain antibodies and serum binding proteins.

6. A ligand as claimed in claim 5 in which the ligand is a monoclonal antibody or F(ab) fragment thereof.

7. A ligand as claimed in claim 1 in which the ligand is MAb 32 (ECACC 89080302).

8. A composition comprising TNF in combination with a ligand as claimed in claim 1 in which the ligand is bound to the TNF.