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(54) Title: CHIMERIC ANTIBODIES WITH PART NEW WORLD PRIMATE BINDING REGIONS

(57) Abrégé/Abstract:
The present invention provides a chimeric antibody polypeptide comprising an antigen binding site, wherein the antigen binding site comprises a human variable domain having at least one New World Primate CDR.

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Chimeric antibodies with part New World primate binding regions

FIELD OF THE INVENTION

The present invention relates to engineered antibody polypeptides. More particularly, the present invention provides antibody polypeptides comprising an antigen binding site, wherein the antigen binding site comprises a human variable domain having at least one New World Primate CDR. In particular the present invention relates to antibody polypeptides directed against TNF-α.

BACKGROUND OF THE INVENTION

As the name implies, Tumor Necrosis Factor-α (TNF-α) was originally described as a molecule having anti-tumor properties, but the molecule was subsequently found to play key roles in other processes, including a prominent role in mediating inflammation and autoimmune disorders. TNF-α is a key proinflammatory cytokine in inflammatory conditions including, for example, rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis and other bowel disorders, psoriasis, toxic shock, graft versus host disease and multiple sclerosis. The pro-inflammatory actions of TNF-α result in tissue injury, such as inducing procoagulant activity on vascular endothelial cells (Pober, *et al.*, 1986, J. Immunol. 136:1680-1687), increasing the adherence of neutrophils and lymphocytes (Pober, *et al.*, 1987, J. Immunol. 138:3319-3324), and stimulating the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells (Camussi, *et al.*, 1987, J. Exp. Med. 166:1390-1404). TNF-α is synthesized as a 26 kD transmembrane precursor protein with an intracellular tail that is cleaved by a TNF-α-converting metalloproteinase enzyme and then secreted as a 17 kD soluble protein. The active form consists of a homotrimer of the 17 kD monomers which interacts with two different cell surface receptors, p55 TNFR1 and p75 TNFR2. There is also evidence that the cell surface bound precursor form of TNF-α can mediate some biological effects of the factor. Most cells express both p55 and p75 receptors which mediate different biological functions of the ligand. The p75 receptor is implicated in triggering lymphocyte proliferation, and the p55 receptor is implicated in TNF-mediated cytotoxicity, apoptosis, antiviral activity, fibroblast proliferation and NF-κB activation (see Locksley *et al.*, 2001, Cell 104: 487-501). The TNF receptors are members of a family of membrane proteins including the NGF receptor, Fas antigen, CD27, CD30, CD40, Ox40 and the receptor for the lymphotoxin α/β heterodimer. Binding of receptor by the homotrimer induces aggregation

of receptors into small clusters of two or three molecules of either p55 or p75. TNF-α is produced primarily by activated macrophages and T lymphocytes, but also by neutrophils, endothelial cells, keratinocytes and fibroblasts during acute inflammatory reactions. TNF-α is at the apex of the cascade of pro-inflammatory cytokines (Reviewed in Feldmann & Maini, 2001, Ann. Rev. Immunol. 19: 163-196). This cytokine induces the expression or release of additional proinflammatory cytokines, particularly IL-1 and IL-6 (see, for example, Rutgeerts *et al.*, 2004, Gastroenterology 126: 1593-1610). Inhibition of TNF-α inhibits the production of inflammatory cytokines including IL-1, IL-6, IL-8 and GM-CSF (Brennan *et al.*, 1989, Lancet 2: 244-247). Because of its role in inflammation, TNF-α has emerged as an important inhibition target in efforts to reduce the symptoms of inflammatory disorders. Various approaches to inhibition of TNF-α for the clinical treatment of disease have been pursued, including particularly the use of soluble TNF-α receptors and antibodies specific for TNF-α. Commercial products approved for clinical use include, for example, the antibody products Remicade™ (infliximab; Centocor, Malvern, PA; a chimeric monoclonal IgG antibody bearing human IgG1 constant and mouse variable regions), Humira™ (adalimumab or D2E7; Abbott Laboratories, described in U.S. patent No. 6,090,382) and the soluble receptor product Enbrel™ (etanercept, a soluble p75 TNFR2 Fc fusion protein; Immunex). The role of TNF-α in inflammatory arthritis is reviewed in, for example, Li & Schwartz, 2003, Springer Semin. Immunopathol. 25: 19-33. In RA, TNF-α is highly expressed in inflamed synovium, particularly at the cartilage-pannus junction (DiGiovine *et al.*, 1988, Ann. Rheum. Dis. 47: 768-772; Firestein *et al.*, 1990, J. Immunol. 144: 3347-3353; and Saxne *et al.*, 1988, Arthritis Rheum. 31 : 1041-1045). In addition to evidence that TNF-α increases the levels of inflammatory cytokines IL-1, IL-6, IL-8 and GM-CSF, TNF-α can alone trigger joint inflammation and proliferation of fibroblast-like synoviocytes (Gitter *et al.*, 1989, Immunology 66: 196-200), induce collagenase, thereby triggering cartilage destruction (Dayer *et al.*, 1985, J. Exp. Med. 162: 2163-2168; Dayer *et al.*, 1986, J. Clin. Invest. 77: 645-648), inhibit proteoglycan synthesis by articular chondrocytes (Saklatvala, 1986, Nature 322: 547-548; Saklatvala *et al.*, 1985, J. Exp. Med. 162: 1208-1222) and can stimulate osteoclastogenesis and bone resorption (Abu-Amer *et al.*, 2000, J. Biol. Chem. 275: 27307-27310; Bertolini *et al.*, 1986, Nature 319: 516-518). TNF-α induces increased release of CD14+ monocytes by the bone marrow. Such monocytes can infiltrate joints and amplify the inflammatory response via the RANK (Receptor Activator of NF-κB)-RANKL signaling pathway, giving rise to osteoclast formation during arthritic inflammation (reviewed in Anandarajah & Richlin, 2004, Curr. Opin. Rheumatol. 16: 338-343). TNF-α is an acute phase protein

which increases vascular permeability through its induction of IL-8, thereby recruiting macrophage and neutrophils to a site of infection. Once present, activated macrophages continue to produce TNF-α, thereby maintaining and amplifying the inflammatory response. Titration of TNF-α by the soluble receptor construct etanercept has proved effective for the treatment of RA, but not for treatment of Crohn's disease. In contrast, the antibody TNF-α antagonist infliximab is effective to treat both RA and Crohn's disease. Thus, the mere neutralization of soluble TNF-α is not the only mechanism involved in anti-TNF-based therapeutic efficacy. Rather, the blockade of other pro-inflammatory signals or molecules that are induced by TNF-α also plays a role (Rutgeerts *et al.*, supra). For example, the administration of infliximab apparently decreases the expression of adhesion molecules, resulting in a decreased infiltration of neutrophils to sites of inflammation. Also, infliximab therapy results in the disappearance of inflammatory cells from previously inflamed bowel mucosa in Crohn's disease. This disappearance of activated T cells in the lamina propria is mediated by apoptosis of cells carrying membrane-bound TNF-α following activation of caspases 8, 9 and then 3 in a Fas dependent manner (see Lugering *et al.*, 2001, Gastroenterology 121: 1145-1157). Thus, membrane- or receptor-bound TNF-α is an important target for anti-TNF-α therapeutic approaches. Others have shown that infliximab binds to activated peripheral blood cells and lamina propria cells and induces apoptosis through activation of caspase 3 (see Van den Brande *et al.*, 2003, Gastroenterology 124: 1774-1785). Intracellularly, the binding of trimeric TNF-α to its receptor triggers a cascade of signaling events, including displacement of inhibitory molecules such as SODD (silencer of death domains) and binding of the adaptor factors FADD, TRADD, TRAF2, c-IAP, RAIDD and TRIP plus the kinase RIPl and certain caspases (reviewed by Chen & Goeddel, 2002, Science 296: 1634-1635, and by Muzio & Saccani in :Methods in Molecular Medicine: Tumor Necrosis Factor, Methods and Protocols," Corti and Ghezzi, eds. (Humana Press, New Jersey; 2004), pp. 81-99). The assembled signalling complex can activate either a cell survival pathway, through NF-κB activation and subsequent downstream gene activation, or an apoptotic pathway through caspase activation. Similar extracellular downstream cytokine cascades and intracellular signal transduction pathways can be induced by TNF-α in other diseases. Thus, for other diseases or disorders in which the TNF-α molecule contributes to the pathology, inhibition of TNF-α presents an approach to treatment. Angiogenesis plays an important role in the active proliferation of inflammatory synovial tissue. RA synovial tissue, which is highly vascularized, invades the periarticular cartilage and bone tissue and leads to joint destruction. Vascular endothelial growth factor (VEGF) is the most potent angiogenic

cytokine known. VEGF is a secreted, heparin-binding, homodimeric glycoprotein existing in several alternate forms due to alternative splicing of its primary transcript (Leung *et al.*, 1989, Science 246: 1306-1309). VEGF is also known as vascular permeability factor (VPF) due to its ability to induce vascular leakage, a process important in inflammation. The identification of VEGF in synovial tissues of RA patients highlighted the potential role of VEGF in the pathology of RA (Fava *et al.*, 1994, J. Exp. Med. 180: 341-346; Nagashima *et al.*, 1995, J. Rheumatol. 22: 1624-1630). A role for VEGF in the pathology of RA was solidified following studies in which anti-VEGF antibodies were administered in the murine collagen-induced arthritis (CIA) model. In these studies, VEGF expression in the joints increased upon induction of the disease, and the administration of anti-VEGF antisera blocked the development of arthritic disease and ameliorated established disease (Sone *et al.*, 2001, Biochem. Biophys. Res. Comm. 281: 562-568; Lu *et al.*, 2000, J. Immunol. 164: 5922-5927).

Antibody Polypeptides

Antibodies are highly specific for their binding targets and although they are derived from nature's own defence mechanisms, antibodies face several challenges when applied to the treatment of disease in human patients. Conventional antibodies are large multi-subunit protein molecules comprising at least four polypeptide chains. For example, human IgG has two heavy chains and two light chains that are disulfide bonded to form the functional antibody. The size of a conventional IgG is about 150 kD. Because of their relatively large size, complete antibodies (e.g., IgG, IgA, IgM, etc.) are limited in their therapeutic usefulness due to problems in, for example, tissue penetration. Considerable efforts have focused on identifying and producing smaller antibody fragments that retain antigen binding function and solubility. The heavy and light polypeptide chains of antibodies comprise variable (V) regions that directly participate in antigen interactions, and constant (C) regions that provide structural support and function in non-antigen-specific interactions with immune effectors. The antigen binding domain of a conventional antibody is comprised of two separate domains: a heavy chain variable domain (VH) and a light chain variable domain (VL: which can be either Vκ or Vλ). The antigen binding site itself is formed by six polypeptide loops: three from the VH domain (H1, H2 and H3) and three from the VL domain (L1, L2 and L3). In vivo, a diverse primary repertoire of V genes that encode the VH and VL domains is produced by the combinatorial rearrangement of gene segments. C regions include the light chain C regions (referred to as CL regions) and the

heavy chain C regions (referred to as CH1, CH2 and CH3 regions). A number of smaller antigen binding fragments of naturally occurring antibodies have been identified following protease digestion. These include, for example, the "Fab fragment" (VL-CL-CH1-VH), "Fab' fragment" (a Fab with the heavy chain hinge region) and "F(ab')2 fragment" (a dimer of Fab' fragments joined by the heavy chain hinge region). Recombinant methods have been used to generate even smaller antigen-binding fragments, referred to as "single chain Fv" (variable fragment) or "scFv," consisting of VL and VH joined by a synthetic peptide linker.

Single Domain Antibodies

While the antigen binding unit of a naturally-occurring antibody (e.g., in humans and most other mammals) is generally known to be comprised of a pair of V regions (VL/VH), camelid species express a large proportion of fully functional, highly specific antibodies that are devoid of light chain sequences. The camelid heavy chain antibodies are found as homodimers of a single heavy chain, dimerized via their constant regions. The variable domains of these camelid heavy chain antibodies are referred to as VHH domains and retain the ability, when isolated as fragments of the VH chain, to bind antigen with high specificity (Hamers-Casterman *et al.*, 1993, Nature 363: 446-448; Gahroudi *et al.*, 1997, FEBS Lett. 414: 521-526). Antigen binding single VH domains have also been identified from, for example, a library of murine VH genes amplified from genomic DNA from the spleens of immunized mice and expressed in E. coli (Ward *et al.*, 1989, Nature 341: 544-546). Ward *et al.* named the isolated single VH domains "dAbs," for "domain antibodies". The term "dAb" will refer herein to a single immunoglobulin variable domain (VH, VHH or VL) polypeptide that specifically binds antigen. A "dAb" binds antigen independently of other V domains; however, as the term is used herein, a "dAb" can be present in a homo- or heteromultimer with other VH or VL domains where the other domains are not required for antigen binding by the dAb, i.e., where the dAb binds antigen independently of the additional VH, VHH or VL domains. Single immunoglobulin variable domains, for example, VHH, are the smallest antigen-binding antibody unit known. For use in therapy, human antibodies are preferred, primarily because they are not as likely to provoke an immune response when administered to a patient. Isolated non-camelid VH domains tend to be relatively insoluble and are often poorly expressed. Comparisons of camelid VHH with the VH domains of human antibodies reveals several key differences in the framework regions of the camelid VHH domain corresponding to the VR/VL interface of

the human VH domains. Mutation of these residues of human VH3 to more closely resemble the VHH sequence (specifically Gly 44 Glu, Leu 45 Arg and Trp 47 Gly) has been performed to produce "camelized" human VH domains (Davies & Riechmann, 1994, FEBS Lett. 339: 285-290) in an attempt to yield improved expression and solubility. Variable domain amino acid numbering used herein is consistent with the Kabat numbering convention (Kabat *et al.*, 1991, Sequences of Immunological Interest, 5th ed. U.S. Dept. Health & Human Services, Washington, D.C.).

WO 03/035694 (Muyldermans) reports that a Trp 103 Arg mutation improves the solubility of non- camelid VH domains. Davies & Riechmann, (1995, Biotechnology N.Y. 13: 475-479) also report production of a phage-displayed repertoire of camelized human VH domains and selection of clones that bind hapten with affinities in the range of 100-400 nM, but clones selected for binding to protein antigen had weaker affinities. The antigen binding domain of an antibody comprises two separate regions: a heavy chain variable domain (VH) and a light chain variable domain (VL: which can be either Vκ or Vλ). The antigen binding site itself is formed by six polypeptide loops: three from VH domain (H1, H2 and H3) and three from VL domain (L1, L2 and L3). A diverse primary repertoire of V genes that encode the VH and VL domains is produced by the combinatorial rearrangement of gene segments. The VH gene is produced by the recombination of three gene segments, VH, D and JH. In humans, there are approximately 51 functional VH segments (Cook and Tomlinson, 1995, Immunol. Today, 16: 237), 25 functional D segments (Corbett *et al.*, 1997 J. Mol. Biol., 268: 69) and 6 functional JH segments (Ravetch *et al.*, 1981, Cell, 27: 583-591), depending on the haplotype. The VH segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the VH domain (H1 and H2), whilst the VH, D and JH segments combine to form the third antigen binding loop of the VH domain (H3). The VL gene is produced by the recombination of only two gene segments, VL and JL. In humans, there are approximately 40 functional Vκ segments (Schable and Zachau (1993) Biol. Chem. Hoppe Seyler, 374: 1001-1022), 31 functional Vλ segments (Williams *et al.*, 1996, J. Mol. Biol., 264: 220-232; Kawasaki *et al.*, 1997, Genome Res., 7: 250-261), 5 functional Jκ segments (Hieter *et al.*, 1982, J. Biol. Chef,., 257: 1516-1522) and 4 functional Jλ segments (Vasicek and Leder, 1990, J. Exp. Med., 172: 609-620), depending on the haplotype. The VL segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the VL domain (L1 and L2), whilst the VL and JL segments combine to form the third antigen binding loop of the VL domain (L3).

Antibodies selected from this primary repertoire are believed to be sufficiently diverse to bind almost all antigens with at least moderate affinity. High affinity antibodies are produced by "affinity maturation" of the rearranged genes, in which point mutations are generated and selected by the immune system on the basis of improved binding. Analysis of the structures and sequences of antibodies has shown that five of the six antigen binding loops (H1, H2, L1, L2, L3) possess a limited number of main-chain conformations or canonical structures (Chothia and Lesk, 1987, Mol. Biol., 196: 901-917; Chothia *et al.*, 1989, Nature, 342: 877-883). The main-chain conformations are determined by (i) the length of the antigen binding loop, and (ii) particular residues, or types of residue, at certain key position in the antigen binding loop and the antibody framework. Analysis of the loop lengths and key residues has enabled us to predict the main-chain conformations of H1, H2, L1, L2 and L3 encoded by the majority of human antibody sequences (Chothia *et al.*, 1992, J. Mol. Biol., 227: 799-817; Tomlinson *et al.*, 1995, EMBO J., 14: 4628-4638; Williams *et al.*, 1996, J. Mol. Biol., 264: 220-232). Although the H3 region is much more diverse in terms of sequence, length and structure (due to the use of D segments), it also forms a limited number of main- chain conformations for short loop lengths which depend on the length and the presence of particular residues, or types of residue, at key positions in the loop and the antibody framework (Martin *et al.*, 1996, J. Mol. Biol, 263: 800-815; Shirai *et al.*, 1996, FEBS Letters, 399: 1-8).

Bispecific antibodies comprising complementary pairs of VH and VL regions are known in the art. These bispecific antibodies must comprise two pairs of VH and VLs, each VH/VL pair binding to a single antigen or epitope. Methods described involve hybrid hybridomas (Milstein & Cuello, Nature, 1983, 305:537-40), minibodies (Hu *et al.*, 1996, Cancer Res 30 56:3055-3061), diabodies (Holliger *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90, 6444-6448; WO 94/13804), chelating recombinant antibodies (CRAbs; Neri *et al.*, 1995, J. Mol. Biol. 246, 367-373), bisFv (e.g. Atwell *et al.*, 1996, Mol. Immunol. 33, 1301-1312), "knobs in holes" stabilized antibodies (Carter *et al.*, 1997, Protein Sci. 6, 781-788). In each case, each antibody species comprises two antigen-binding sites, each fashioned by a complementary pair of VH and VL domains. Each antibody is thereby able to bind to two different antigens or epitopes at the same time, with the binding to EACH antigen or epitope mediated by a VH and its complementary VL domain. Each of these techniques presents its particular disadvantages; for instance in the case of hybrid hybridomas, inactive VH/VL pairs can greatly reduce the fraction of bispecific IgG. Furthermore, most bispecific approaches rely on the association of the different VH/VL pairs or the

association of VH and VL chains to recreate the two different VH/VL binding sites. It is therefore impossible to control the ratio of binding sites to each antigen or epitope in the assembled molecule and thus many of the assembled molecules will bind to one antigen or epitope but not the other. In some cases it has been possible to engineer the heavy or light chains at the sub-unit interfaces (Carter *et al.*, 1997) in order to improve the number of molecules which have binding sites to both antigens or epitopes, but this never results in all molecules having binding to both antigens or epitopes. There is some evidence that two different antibody binding specificities might be incorporated into the same binding site, but these generally represent two or more specificities that correspond to structurally related antigens or epitopes or to antibodies that are broadly cross- reactive. For example, cross-reactive antibodies have been so described, usually where the two antigens are related in sequence and structure, such as hen egg white lysozyme and turkey lysozyme (McCafferty *et al.*, WO 92/01047) or to free hapten and to hapten conjugated to carrier (Griffiths *et al.*, 1994, EMBO J 13:14 3245- 60). In a further example, WO 02/02773 (Abbott Laboratories) describes antibody molecules with "dual specificity". The antibody molecules referred to are antibodies raised or selected against multiple antigens, such that their specificity spans more than a single antigen. Each complementary VH/VL pair in the antibodies of WO 02/02773 specifies a single binding specificity for two or more structurally related antigens; the VH and VL domains in such complementary pairs do not each possess a separate specificity. The antibodies thus have a broad single specificity which encompasses two antigens, which are structurally related. Furthermore natural autoantibodies have been described that are polyreactive (Casali & Notkins, 1989, Ann. Rev. Immunol. 7, 515-531), reacting with at least two (usually more) different antigens or epitopes that are not structurally related. It has also been shown that selections of random peptide repertoires using phage display technology on a monoclonal antibody will identify a range of peptide sequences that fit the antigen binding site. Some of the sequences are highly related, fitting a consensus sequence, whereas others are very different and have been termed mimotopes (Lane & Stephen, 1993, Current Opinion in Immunology, 5, 268-271). It is therefore clear that a natural four-chain antibody, comprising associated and complementary VH and VL domains, has the potential to bind to many different antigens from a large universe of known antigens. It is less clear how to create a binding site to two given antigens in the same antibody, particularly those which are not necessarily structurally related. Protein engineering methods have been suggested that may have a bearing on this. For example, it has also been proposed that a catalytic antibody could be created with a binding activity to a metal ion through one variable domain, and to a hapten

(substrate) through contacts with the metal ion and a complementary variable domain (Barbac *et al.*, 1993, Proc. Natl. Acad. Sci USA 90, 6385-6389). However in this case, the binding and catalysis of the substrate (first antigen) is proposed to require the binding of the metal ion (second antigen). Thus the binding to the VH/VL pairing relates to a single but multi component antigen. Methods have been described for the creation of bispecific antibodies from camel antibody heavy chain single domains in which binding contacts for one antigen are created in one variable domain, and for a second antigen in a second variable domain. However the variable domains were not complementary. Thus a first heavy chain variable domain is selected against a first antigen, and a second heavy chain variable domain against a second antigen, and then both domains are linked together on the same chain to give a bispecific antibody fragment (Conrath *et al.*, J. Biol. Chem. 270, 27589-27594). However the camel heavy chain single domains are unusual in that they are derived from natural camel antibodies which have no light chains, and indeed the heavy chain single domains are unable to associate with camel light chains to form complementary VH and VL pairs. Single heavy chain variable domains have also been described, derived from natural antibodies which are normally associated with light chains (from monoclonal antibodies or from repertoires of domains; see EP-A-0368684). These heavy chain variable domains have been shown to interact specifically with one or more related antigens but have not been combined with other heavy or light chain variable domains to create a ligand with specificity for two or more different antigens. Furthermore, these single domains have been shown to have a very short *in vivo* half-life. Therefore, such domains are of limited therapeutic value. It has been suggested to make bispecific antibody fragments by linking heavy chain variable domains of different specificity together (as described above). The disadvantage with this approach is that isolated antibody variable domains may have a hydrophobic interface that normally makes interactions with the light chain and is exposed to solvent and may be "sticky" allowing the single domain to bind to hydrophobic surfaces. Furthermore, in the absence of a partner light chain, the combination of two or more different heavy chain variable domains and their association, possibly via their hydrophobic interfaces, may prevent them from binding to one or both of the ligands they are able to bind in isolation. Moreover, in this case the heavy chain variable domains would not be associated with complementary light chain variable domains and thus may be less stable and readily unfold (Worn & Pluckthun, 1998, Biochemistry 37: 13120-7).

Human/mouse chimeric antibodies have been created in which antibody variable region sequences from the mouse genome are combined with antibody constant region sequences from the human genome. The chimeric antibodies exhibit the binding characteristics of the parental mouse antibody, and the effector functions associated with the human constant region. The antibodies are produced by expression in a host cell, including for example Chinese Hamster Ovary (CHO), NS0 myeloma cells, COS cells and SP2 cells.

Such chimeric antibodies have been used in human therapy, however antibodies to these chimeric antibodies have been produced by the human recipient. Such anti-chimeric antibodies are detrimental to continued therapy with chimeric antibodies.

It has been suggested that human monoclonal antibodies are expected to be an improvement over mouse monoclonal antibodies for *in vivo* human therapy. From work done with antibodies from Old World primates (rhesus monkeys and chimpanzees) it has been postulated that these non-human primate antibodies will be tolerated in humans because they are structurally similar to human antibodies (Ehrlich PH *et al.*, 1988, Human and primate monoclonal antibodies for *in vivo* therapy. Clin Chem. 34:9 pg 1681-1688). Furthermore, because human antibodies are non-immunogenic in Rhesus monkeys (Ehrlich *et al.*, 1987, Hybridoma; 6:151-60), it is likely that the converse is also applicable and primate antibodies will be non-immunogenic in humans. These monoclonal antibodies are secreted by hybridomas constructed by fusing lymphocytes to a human x mouse heteromyeloma.

EP 0 605 442 discloses chimeric antibodies which bind human antigens. These antibodies comprise the whole variable region from an Old World monkey and the constant region of a human or chimpanzee antibody. One of the advantages suggested in this reference for these constructs is the ability to raise antibodies in Old World monkeys to human antigens which are less immunogenic in humans compared with antibodies raised in a mouse host.

New World primates (infraorder- Platyrrhini) comprise at least 53 species commonly divided into two families, the *Callithricidae* and *Cebidae*. The *Callithricidae* consist of marmosets and tamarins. The *Cebidae* includes the squirrel monkey, titi monkey, spider monkey, woolly monkey, capuchin, uakaris, sakis, night or owl monkey and the howler monkey.

Evolutionarily distant primates, such as New World primates, are not only sufficiently different from humans to allow antibodies against human antigens to be generated, but are sufficiently similar to humans to have antibodies similar to human antibodies so that the host does not generate an anti-antibody immune response when such primate-derived antibodies are introduced into a human.

Previous studies have characterised the expressed immunoglobulin heavy chain repertoire of the *Callithrix jacchus* marmoset (von Budingen *et al.*, 2001, Immunogenetics; 53:557-563). Six IGHV subgroups were identified which showed a high degree of sequence similarity to their human IGHV counterparts. The framework regions were more conserved when compared to the complementarity determining regions (CDRs). The degree of similarity between *C. jacchus* and human IGHV sequences was less than between non-human Old World primates and humans.

SUMMARY OF THE INVENTION

In a first aspect the present invention provides a chimeric antibody polypeptide comprising an antigen binding site, wherein the antigen binding site comprises a human variable domain having at least one New World Primate CDR.

In a second aspect the present invention provides a method of producing an antibody polypeptide according to the first aspect of the invention, the method comprising the steps of:

(i) providing an acceptor sequence encoding a human variable domain; and

(ii) replacing a CDR sequence of the variable domain with a donor CDR sequence, wherein the donor sequence is a New World Primate CDR sequence.

In a third aspect the present invention provides a chimeric domain antibody (dAb) which binds human TNF-α, the dAb comprising an immunoglobulin heavy or light chain variable domain, wherein said variable domain comprises at least one New World Primate CDR.

In a fourth aspect the present invention provides a pharmaceutical composition comprising an effective amount of an antibody polypeptide according to the first or third aspects of the invention, together with a pharmaceutically acceptable carrier or diluent.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the amino acid (SEQ ID No:6) and nucleotide sequence (SEQ ID No:5) of the acceptor dAb.

Figure 2 shows the nucleotide and amino acid sequences of eleven (11) marmoset and six (6) Owl monkey Vκ gene segments.

Figure 3 shows the acceptor dAb amino acid and nucleotide sequence (both strands). The restriction digest sites for Kpn I and San DI which excises a region including the CDR2 is indicated in the figure. CDR2 residues removed are indicated in underline.

Figure 4 shows sequence alignments showing oligonucleotides used during cloning and final sequence confirmation of the nucleotide (A) and amino acid (B) sequences shown in Figure 2.

Figure 5 demonstrates the ability of CDR2-grafted dAbs to inhibit the binding of TNF to recombinant TNF receptor. The dAbs tested were as follows: Owl Monkey 1 (CDR= YAATKLQS; SEQ ID No:1), Owl Monkey 2 (CDR=YEASSLQS; SEQ ID No:2), Marmoset 1 (CDR=YEASKLQS; SEQ ID No:3), Marmoset 2 (CDR=YSASNLET; SEQ ID No:4) and Acceptor dAb (CDR=YSASELQS; SEQ ID No:49).

Figure 6 demonstrates the improved ability of Compounds 100 and 123 to neutralise the cytotoxic activity of TNF on mouse L929 fibroblasts relative to Compound 145.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect the present invention provides a chimeric antibody polypeptide comprising an antigen binding site, wherein the antigen binding site comprises a human variable domain having at least one New World Primate CDR.

In a third aspect the present invention provides a chimeric domain antibody (dAb) which binds human TNF-α, the dAb comprising an immunoglobulin heavy or light chain variable domain, wherein said variable domain comprises at least one New World Primate CDR.

In a fourth aspect the present invention provides a pharmaceutical composition comprising an effective amount of an antibody polypeptide according to the first or third aspects of the invention, together with a pharmaceutically acceptable carrier or diluent.

In an embodiment of the present invention the human variable domain comprises at least one human framework region having an amino acid sequence encoded by a human germline antibody gene segment, or an amino acid sequence comprising up to 5 amino acid differences relative to the amino acid sequence encoded by the human germline antibody gene segment.

The human variable domain preferably comprises four human framework regions, FR1, FR2, FR3 and FR4 having amino acid sequences encoded by a human germline antibody gene segment, or the amino acid sequences which collectively contain up to 10 amino acid differences relative to the amino acid sequences encoded by said human germline antibody gene segment.

Preferably the human germline antibody gene segment selected from the group consisting of DP47, DP45, DP48 and DPK9.

The New World Primate CDR may be any CDR, however, it is preferred that the New World Primate CDR is CDR2.

Alternatively the New World Primate CDR is CDR1 or CDR3.

It is also preferred that the New World Primate CDR sequence is a germline New World Primate CDR sequence.

The antibody polypeptide of the present invention is preferably selected from a dAb, scFv, Fab, $(Fab')_2$, Fv, disulphide bonded Fv, IgG, and a diabody.

The antibody polypeptide of the present invention is preferably directed against TNF-α.

In another preferred embodiment the human variable domain amino acid sequence comprises a Kpn1 restriction site spaced from a SanD1 restriction site, said CDR of the human variable domain being between the restriction sites.

It is also preferred that the New World Primate CDR sequence is obtainable from New World Primate DNA by PCR using primer pair VK1BL (SEQ ID No:11) / VK1BL35a (SEQ ID No:12) or primer pair VK1BL (SEQ ID No:11 / VK1BL35b (SEQ ID No:13).

The present invention also provides a chimeric domain antibody (dAb) which binds to human TNF-α, wherein the dAb is a human dAb that binds human TNF-α in which at least one of the CDRs is replaced with the corresponding CDR from a New World Primate.

The present invention also provides a method of producing an antibody polypeptide according to the first aspect of the invention, the method comprising the steps of:

(i) providing an acceptor sequence encoding a human variable domain; and

(ii) replacing a CDR sequence of the variable domain with a donor CDR sequence, wherein the donor sequence is a New World Primate CDR sequence.

It is preferred that in step (ii) said CDR of said human variable domain is replaced by said donor New World Primate CDR using restriction digestion and annealing of an oligonucleotide encoding the donor CDR into the acceptor sequence.

It is preferred that the method further comprises affinity maturing the variable domain produced in step (ii).

As used herein the term "New World Primate CDR" refers to a CDR sequence obtained from a New World Primate. The term encompasses modification of 1, 2 or 3 amino acids within the sequence which may be used to achieve improved antigen binding characteristics or lower immunogenicity. The term does not, however, extend to cover modifications which result in the New World Primate CDR sequence being identical to a human CDR sequence.

As used herein the term "human framework region" refers to a framework region obtained from a human or a human framework region having an amino acid sequence encoded by a human germline antibody gene segment, or an amino acid sequence comprising up to 5 amino acid differences relative to the amino acid sequence encoded by the human germline gene segment. The term also encompasses modification of the amino acid sequence of the framework region in order to obtain improved antigen binding characteristics or lower immunogenicity such as disclosed in US 4,816,567, US 5,585,089 and US 20030039649 the disclosures of which are incorporated herein by reference in their entirety. Typically

where modifications are made the total number of residues changed will be 10 or less collectively over the framework regions.

In a preferred embodiment the variable domain comprises four framework regions, wherein at least one framework region comprises an amino acid sequence derived from a corresponding framework region encoded by a human germline immunoglobulin gene.

In a further preferred embodiment the four framework regions comprise amino acid sequences derived from corresponding framework regions encoded by human germline immunoglobulin genes.

In yet a further preferred embodiment the human germline immunoglobulin gene is selected from the group consisting of DP47, DP45, DP48 and DPK9.

The term "domain" as used herein is meant a folded protein structure which retains its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain.

The term immunoglobulin or antibody "variable domain" as used herein is a term of art, and includes a folded polypeptide domain comprising sequences characteristic of immunoglobulin or antibody heavy or light chain variable domains and which specifically binds an antigen.

The term "immunoglobulin" as used herein refers to a family of polypeptides which retain the immunoglobulin fold characteristic of antibody molecules, which contains two β sheets and, usually, a conserved disulphide bond. Members of the immunoglobulin superfamily are involved in many aspects of cellular and non-cellular interactions in vivo, including widespread roles in the immune system (for example, antibodies, T- cell receptor molecules and the like), involvement in cell adhesion (for example the ICAM molecules) and intracellular signalling (for example, receptor molecules, such as the PDGF receptor). The present invention is applicable to all immunoglobulin superfamily molecules which possess binding domains. Preferably, the present invention relates to antibody polypeptides.

New World primates (infraorder- Platyrrhini) comprise at least 53 species commonly divided into two families, the *Callithricidae* and *Cebidae*. The *Callithricidae* consist of marmosets and tamarins. The *Cebidae* includes the squirrel monkey, titi monkey, spider monkey, woolly monkey, capuchin, uakaris, sakis, night or owl monkey and the howler monkey.

Evolutionarily distant primates, such as New World primates, are not only sufficiently different from humans to allow antibodies against human antigens to be generated, but are sufficiently similar to humans to have antibodies similar to human antibodies so that the host does not generate an anti-antibody immune response when such primate-derived antibodies are introduced into a human.

Previous studies have characterised the expressed immunoglobulin heavy chain repertoire of the *Callithrix jacchus* marmoset (von Budingen H-C *et al.*, 2001, Immunogenetics; 53:557-563). Six IGHV subgroups were identified which showed a high degree of sequence similarity to their human IGHV counterparts. The framework regions were more conserved when compared to the complementarity determining regions (CDRs). The degree of similarity between *C. jacchus* and human IGHV sequences was less than between non-human Old World primates and humans.

In certain embodiments of the present invention the New World primate CDR is from the family *Callithricidae*.

In yet a further embodiment of the present invention the New World primate CDR is selected from the group consisting of marmosets, tamarins, squirrel monkey, titi monkey, spider monkey, woolly monkey, capuchin, uakaris, sakis, night or owl monkey and the howler monkey. More preferably, the New World primate is a marmoset.

In yet a further embodiment of the present invention the at least one New World primate CDR is substantially identical to a CDR encoded by a New World primate germline immunoglobulin gene.

The term "antibody" as used herein, is intended to refer to immunoglobulin molecules comprised of two heavy chains or immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (HCVR or V_H) and a heavy chain constant region. The heavy chain constant region comprises

three domains, C_H1, C_H2 and C_H3. Each light chain is comprised of a light chain variable region (LCVR or V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The term "antibody polypeptide" as used herein refers to a polypeptide comprising one or more components or derivatives of an immunoglobulin that exhibit the ability to bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full length antibody. Examples of binding fragments encompassed within the term "antibody polypeptide" include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_H1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_H1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody; (v) a dAb fragment (Ward *et al*, 1989, Nature 341:544-546) which consists of a single V_H domain, or a V_L domain (van den Beucken *et al*, 2001, J. Mol. Biol, 310, 591-601); and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); (see eg Bird *et al.*, 1988, Science 242:423-426 and Huston *et al.*, 1988 Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain Fvs are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain Fvs and related molecules such as diabodies or triabodies are also encompassed. Diabodies are bivalent antibodies in which V_H and V_L domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., Holliger, *et al.*, 1993, Proc. Natl. Acad. Sci. USA, 90:6444-6448; Poljak, *et al.*, 1994, Structure, 2:1121-1123).

Thus in certain embodiments of the present invention the antibody polypeptide is selected from the group consisting of a dAb, scFv, Fab, $F(ab')_2$, Fv, disulphide bonded Fv, a diabody and IgG.

Preferably, the antibody polypeptide further comprises a human or non-human primate constant region sequence. Examples of non-human primates include, but are not limited to, chimpanzees, oranguatangs and baboons.

The constant region sequence (Fc portion) is preferably obtained from a human or non-human primate immunoglobulin sequence. The primate sequence may be a New World primate or an Old World primate sequence. Suitable Old World primates include chimpanzee, or other hominid ape eg. gorilla or orang utan, which because of their close phylogenetic proximity to humans, share a high degree of homology with the human constant region sequence. Sequences which encode for human or primate constant regions are available from databases including e.g. The National Centre for Biotechnology Information protein and nucleotide databases, The Kabat Database of Sequences of Proteins of Immunological Interest.

In a preferred embodiment of the present invention the antibody polypeptide is a domain antibody (dAb).

Domain antibodies (dAb) are small functioning binding units of antibodies and correspond to the variable regions of either the heavy (V_H) or light (V_L) chains of antibodies. Domain antibodies have a molecular weight of approximately 13 kDa, or less than one tenth the size of a full antibody.

Antibody light chains are referred to as either kappa or lambda light chains and the heavy chains as gamma, mu, delta, alpha or epsilon. The variable region gives the antibody its specificity. Within each variable region are regions of hypervariability, otherwise known as complementarity determining regions (CDRs) which are flanked by more conserved regions referred to as framework regions. Within each variable region are three CDRs and four framework regions.

In contrast to conventional antibodies, domain antibodies are well expressed in bacterial, yeast and mammalian systems. Their small size allows for higher molar quantities per gram of product, thus providing a significant increase in potency per dose. In addition, domain antibodies can be used as a building block to create therapeutic products such as

multiple targeting dAbs in which a construct containing two or more variable domains bind to two or more therapeutic targets, or dAbs targeted for pulmonary or oral administration.

An increase in binding is demonstrated by a decrease in K_D (k_{off}/k_{on}) for the antibody or antigen binding portion thereof. An increase in potency is demonstrated in biological assays. For example, assays that can be used to measure the potency of the antibody or antigen-binding portion thereof include the TNFα-induced L929 cytotoxicity neutralisation assay, IL-12-induced human PHA-activated peripheral blood mononuclear cell (PBMC) proliferation assay, and RANKL mediated osteoclast differentiation of mouse splenocytes (Stern, 1990, Proc. Natl. Acad. Sci. USA 87:6808 – 6812; Kong, *et al.*, 1990, Nature 397:315 – 323; Matthews and Neale in *Lymphokines and Interferons, a Practical Approach*, 1987, M.J. Clemens, A.G. Morris and A.J.H. Gearing, eds., IRL Press, p. 221).

The CDR sequences may be obtained from several sources, for example, databases e.g. The National Centre for Biotechnology Information protein and nucleotide databases www.ncbi.nlm.nih.gov, The Kabat Database of Sequences of Proteins of Immunological Interest www.kabatdatabase.com, or the IMGT database www.imgt.cines.fr. Alternatively, the CDR regions can be predicted from the V_H and V_L domain repertoire (see for example Kabat and Wu, 1971, Ann. NY Acad. Sci. 190:382-393). The CDR sequence may be a genomic DNA or a cDNA.

There are a number of ways in which a replacement CDR may be grafted into a variable domain sequence and such methods will be familiar to those skilled in the art. The preferred method of the present invention involves replacement of the CDR2 in the variable region domain via primer directed mutagenesis. This method consists of annealing a synthetic oligonucleotide encoding a desired mutations to a target region where it serves as a primer for initiation of DNA synthesis *in vitro*, extending the oligonucleotide by a DNA polymerase to generate a double-stranded DNA that carries the desired mutations, and ligating and cloning the sequence into an appropriate expression vector.

In one embodiment of the invention, the variable domain sequence into which the CDR is grafted is the "dAb acceptor sequence" (designated Compound 128; SEQ ID No:6) provided in Figure 1.

As used herein the term "chimeric" is meant that the antibody polypeptide or domain antibody includes sequences from more than one species.

The anti-human TNF-α dAb according to the invention can be used to detect human TNF-α for example in a biological sample, such as serum or plasma using a conventional immunoassay, such as an enzyme linked immunosorbent assay (ELISA), a radioimmunoassay (RIA) or tissue immunohistochemistry. The anti-human TNF-α dAb according to the invention can be assayed in biological fluids by a competition immunoassay using recombinant human TNF-α standards labelled with a detectable substance and an unlabelled anti-human TNF-α antibody.

The anti-human TNF-α dAb according to the invention may also be used to detect TNF-α from species other than humans eg. chimpanzee, marmoset, rhesus, mouse, pig.

The anti-human TNF-α dAb according to the invention may also be used in cell culture applications where it is desired to inhibit TNF-α activity.

The invention also provides a method for treating a disorder characterised by human TNF-α activity in a human subject, comprising administering to the subject a pharmaceutical composition according to the second aspect of the invention.

A disorder characterised by human TNF-α activity is intended to include diseases and other disorders in which the presence of TNF-α in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor which contributes to a worsening of the disorder. Preferably, the disorder characterised by human TNF-α activity is selected from the group consisting of inflammation, inflammatory diseases, sepsis, including septic shock, endotoxic shock, gram negative sepsis and toxic shock syndrome; autoimmune disease, including rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis and gouty arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome; infectious disease, including fever and myalgias due to infection and cachexia secondary to infection; graft versus host disease; tumour growth or metastasis; pulmonary disorders including adult respiratory distress syndrome, shock lung, chronic pulmonary inflammatory disease, pulmonary sarcoidosis, pulmonary fibrosis and silicosis; inflammatory bowel disorders including Crohn's disease and ulcerative colitis; cardiac disorders; inflammatory bone disorders, hepatitis, coagulation disturbances, burns, reperfusion injury, keloid formation and scar tissue formation.

In a fourth aspect, the invention provides a pharmaceutical composition comprising an effective amount of the antibody polypeptide according to the first aspect of the invention or a chimeric domain antibody according to the third aspect of the invention, together with a pharmaceutically acceptable carrier or diluent.

A "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like which are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol, and the like as well as combinations thereof. In many cases it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable substances such as wetting or minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers.

The composition may be in a variety of forms, including liquid, semi-solid and solid dosage forms, such as liquid solutions (eg injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. Preferably, the composition is in the form of an injectable solution for immunization. The administration may be intravenous, subcutaneous, intraperitoneal, intramuscular, transdermal, intrathecal, and intra-arterial.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The compositions can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (i.e. antibody polypeptide) into the required amount in an appropriate solvent with one or a combination of ingredients listed above, followed by filtered sterilisation.

The composition may also be formulated as a sterile powder for the preparation of sterile injectable solutions. The proper fluidity of a solution can be maintained by for example, use of a coating such as lecithin and/or surfactants.

In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems.

Compatible polymers may be used such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters and polylactic acid.

The composition may also be formulated for oral administration. In this embodiment, the antibody polypeptide may be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet.

The composition may also be formulated for rectal administration.

Supplementary active compounds can also be incorporated into the composition. The antibody polypeptide may be co-formulated with and/or co-administered with one or more additional therapeutic agents eg. anti-inflammatory compounds, soluble TNF-α receptor or a chemical agent that inhibits human TNF-α production, or antibodies that bind other targets such as cytokines or cell surface molecules. Alternatively, it may be co-administered with a soluble immunochemical reagent such as protein A, C, G or L.

An effective amount may include a therapeutically effective amount or prophylactically effective amount of the antibody polypeptide of the invention. A therapeutically effective amount refers to an amount effective at dosages and for periods of time necessary, to achieve the desired therapeutic result. A prophylactically effective amount refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result.

In a preferred embodiment the composition is administered to mammals, preferably humans or primates.

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 non-limiting examples.

EXAMPLE 1

Materials and Methods

Isolation of New World primate VL genes

Marmoset (genus Callithrix, species unknown) and Owl monkey (Aotus trivirgatus) genomic DNA were obtained from the European Collection of Cell Cultures (ECACC),

catalogue numbers 85011419 and 90110510 respectively. Marmoset DNA was derived from cell line B95-8 while Owl monkey DNA came from cell line OMK 637-69.

Degenerate primers based on human Vκ leader sequences and recombination signal sequences (RSS) were derived from Walter and Tomlinson, Antibody Engineering: A Practical Approach (1996). The primers used for amplification of germline Vκ DNA were as follows:

Primer VK1BL
AATCKCAGGTKCCAGATG (SEQ ID No:11)

Primer VK1BL35a
GTTYRGGTKKGTAACACT (SEQ ID No:12)

Primer VK1BL35b
ATGMCTTGTWACACTGTG (SEQ ID No:13)

Genomic PCR (30 cycles) was performed using Taq polymerase with either primer pair VK1BLxVK1BL35a or VK1BLxVK1BL35b. There was overlap between the sequences cloned and the two primer sets used.

PCR products were cloned into Invitrogen's TOPO TA cloning kit (Cat No K4500-01) and sequenced with M13 forward and pUC reverse primers. Sequence was confirmed in forward and reverse directions. In order to further confirm key sequences were not subject to PCR errors, the PCR and cloning process was repeated twice for marmoset sequences. Nucleotide (SEQ ID Nos:14-24 and SEQ ID Nos:36-41) and amino acid (SEQ ID Nos:25-35 and SEQ ID Nos:42-47) sequences are given in Figure 2. Marmoset sequences 1, 2 and 3 were confirmed. Sequences 4, 5, 6, 7 and 8 were seen only in the initial PCR. Sequences 9, 10 and 11 were seen only in the repeat (i.e. second) PCR and cloning.

Oligo Synthesis and Cloning into Acceptor Sequence

Four CDR sequences, namely YAATKLQS (SEQ ID No:1) from Owl monkey sequence 1 (SEQ ID No:42), YEASSLQS (SEQ ID No:2) from Owl monkey sequence 2 (SEQ ID No:43), YEASKLQS (SEQ ID No:3) from Marmoset sequence 1 (SEQ ID No:25), and YSASNLET (SEQ ID No:4) from Marmoset sequence 2 (SEQ ID No:26), were chosen from the amino acid sequences shown in Figure 2 as indicated. Owl Monkey sequence 5,

YYASSLQS (SEQ ID No:48) was found to be identical to GI6176295 an Aotus nancymaae (Ma's night monkey) cDNA sequence, all other sequences were unique.

An acceptor variable region (anti-TNF domain antibody) sequence in the expression vector (Domantis proprietary vector) was digested (25µg) sequentially with KpnI and SanDI which excises the majority of FR2 as well as CDR2 as indicated on the restriction digest map. The vector was then gel purified to remove the excised wild-type FR2 and CDR2 sequence.

Oligo annealing was performed by incubating oligo pairs (500 pmol of each as shown in Figure 4A and 4B) at 95°C for 5 minutes followed by 65°C for 5 minutes and then allowed to reach room temperature slowly on a hot block. Overlaps were then filled in during a Klenow reaction in the presence of dNTPs.

Affinity Maturation

The marmoset CDR-grafted dAb Compound 145 (SEQ ID No:7) was affinity matured by constructing 14 separate libraries, each a diversification of the sequence of SEQ ID No:7 at a single amino acid residue. The selected residues are shown shaded below.

DIQMTQSPSSLSASVGDRVTITCRASQSIDSYLHWYQQKPGKAPKLLIYSASNLETG
VPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQVVWRPFTFGQGTKVEIKR

The selection was based upon residues in CDR1 and CDR3 that are known to be diversified in the mature human Ig repertoire, and framework residues that have been observed to produce functional proteins after mutagenesis in related dAbs. For each of the selected residues, complimentary forward and reverse PCR primer pairs were designed with NKK degeneracy, and two initial PCR reactions were performed each with a single mutagenic primer and flanking primer. After clean-up, the two PCR products were annealed and then amplified using flanking primers alone (splicing by overlap extension of PCR; Lowman H.L. & Clackson T. (eds), Phage Display: A practical approach, Oxford University Press , Oxford, UK). Clones were initially screened by ELISA using solid-phase TNF, and positive clones were sequenced. dAb protein was purified from the best clones and evaluated for potency in receptor binding assays and L929 cytotoxicity assays. Compounds 100 (SEQ ID No:9) and 123 (SEQ ID No:8) were found to have improved TNF-neutralization relative to the parent dAb, Compound 145 (SEQ ID No:7).

Combination of the affinity-enhancing substitutions of Compounds 100 (SEQ ID No:9) and 123 (SEQ ID No:8), yielded an anti-TNF dAb with further improved potency in the L929 cytotoxicity assay (Compound 196; SEQ ID No:10).

Results

Potency of anti-TNF dAb clones in receptor binding assay (RBA) and cytotoxocity assay

The ability of the anti-TNF dAbs to inhibit TNF binding to its receptor and to neutralize TNF-mediated cytotoxicity of L929 cells was conducted as follows:

Receptor binding assay

dAbs diversified in the 14 selected positions were tested for the ability to inhibit the binding of TNF to recombinant TNF receptor 1 (p55). Briefly, Maxisorp plates were incubated overnight with 30 mg/ml anti-human Fc mouse monoclonal antibody (Zymed, San Francisco, USA). The wells were washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 and then blocked with 1% BSA in PBS before being incubated with 100 ng/ml TNF receptor 1 Fc fusion protein (R&D Systems, Minneapolis, USA). Each dAb was mixed with TNF which was added to the washed wells at a final concentration of 10 ng/ml. TNF binding was detected with 0.2 mg/ml biotinylated anti-TNF antibody (HyCult biotechnology, Uben, Netherlands) followed by 1 in 500 dilution of horse radish peroxidase labelled streptavidin (Amersham Biosciences, UK) and then incubation with TMB substrate (KPL, Gaithersburg, USA). The reaction was stopped by the addition of HCl and the absorbance was read at 450nm. Anti-TNF dAb activity lead to a decrease in TNF binding and therefore a decrease in absorbance compared with the TNF only control (Figure 5).

L929 Cytotoxicity Assay

Anti-TNF dAbs identified by the minilibrary diversification approach, including Compounds 100 (SEQ ID No:9) and 123 (SEQ ID No:8), were also tested for the ability to neutralise the cytotoxic activity of TNF on mouse L929 fibroblasts (Evans, T., 2000, Molecular Biotechnology 15, 243-248). Briefly, L929 cells plated in microtitre plates were incubated overnight with anti-TNF dAb, 100 pg/ml TNF and 1 mg/ml actinomycin D (Sigma, Poole, UK). Cell viability was measured by reading absorbance at 490nm following an incubation with [3-(4,5-dimethylthiazol-2-yl)-5-(3 -

carbboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (Promega, Madison, USA). Anti-TNF dAb activity lead to a decrease in TNF cytotoxicity and therefore an increase in absorbance compared with the TNF only control. The results, in comparison with the parent dAb Compound 145 (SEQ ID No:7) are presented in Figure 6.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

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

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2___

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS:

1. A chimeric antibody polypeptide comprising an antigen binding site, wherein the antigen binding site comprises a human variable domain having at least one New World Primate CDR.

2. The antibody polypeptide of claim 1, wherein the human variable domain comprises at least one human framework region having an amino acid sequence encoded by a human germline antibody gene segment, or an amino acid sequence comprising up to 5 amino acid differences relative to the amino acid sequence encoded by a human germline antibody gene segment.

3. The antibody polypeptide of claim 2, wherein the human variable domain comprises four human framework regions, FR1, FR2, FR3 and FR4 having amino acid sequences encoded by a human germline antibody gene segment, or the amino acid sequences of FR1, FR2, FR3 and FR4 collectively containing up to 10 amino acid differences relative to the amino acid sequences by said human germline antibody gene segment.

4. The antibody polypeptide according to claim 2 or 3, wherein the framework regions are encoded by a human germline antibody gene segment selected from the group consisting of DP47, DP45, DP48 and DPK9.

5. The antibody polypeptide of any preceding claim, wherein said New World Primate CDR is CDR2.

6. The antibody polypeptide of any one of claims 1 to 4, wherein said New World Primate CDR is CDR1 or CDR3.

7. The antibody polypeptide of any preceding claim, wherein said New World Primate CDR sequence is a germline New World Primate CDR sequence.

8. The antibody polypeptide of any preceding claim, wherein the antibody polypeptide is selected from a dAb, scFv, Fab, $(Fab')_2$, Fv, disulphide bonded Fv, IgG, and a diabody.

9. The antibody polypeptide of any preceding claim, wherein the antigen is TNF-α.

10. The antibody polypeptide of any preceding claim, wherein the New World Primate is a *Callithricidae*.

11. The antibody polypeptide of claim 10, wherein the New World Primate is a marmoset.

12. The antibody polypeptide of any preceding claim, wherein the human variable domain amino acid sequence comprises a Kpn1 restriction site spaced from a SanD1 restriction site, said CDR of the human variable domain being between the restriction sites.

13. The antibody polypeptide of any preceding claim, wherein said New World Primate CDR sequence is obtainable from New World Primate DNA by PCR using primer pair VK1BL (SEQ ID No:11) / VK1BL35a (SEQ ID No:12) or primer pair VK1BL (SEQ ID No: 11) / VK1BL35b (SEQ ID No:13).

14. A chimeric domain antibody (dAb) which binds to human TNF-α, wherein the dAb is a human dAb that binds human TNF-α in which at least one of the CDRs is replaced with the corresponding CDR from a New World Primate.

15. A chimeric dAb according to claim 1 wherein the replaced CDR is CDR2.

16. A chimeric dAb according to claim 1 or claim 2 wherein the New World Primate is a marmoset.

17. A method of producing a antibody polypeptide as defined in any one of claims 1 to 14, the method comprising

(i) Providing an acceptor sequence encoding a human variable domain; and

(ii) Replacing a CDR sequence of the variable domain with a donor CDR sequence, wherein the donor CDR sequence is a New World Primate CDR.

18. The method of claim 17, wherein in step (ii) said CDR of said human variable domain is replaced by said donor New World Primate CDR using restriction digestion and annealing of an oligonucleotide encoding the donor CDR into the acceptor sequence.

19. The method of claim 17 or 18, comprising (iii) affinity maturing the variable domain produced in step (ii).

FIGURE 1

```
1    GAC ATC CAG ATG ACC CAG TCT CCA TCC TCT CTG TCT GCA TCT GTA   45

1    Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val   15

46   GGA GAC CGT GTC ACC ATC ACT TGC CGG GCA AGT CAG AGC ATT GAT   90

16   Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Asp   30

91   AGT TAT TTA CAT TGG TAC CAG CAG AAA CCA GGG AAA GCC CCT AAG   135

31   Ser Tyr Leu His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys   45

136  CTC CTG ATC TAT AGT GCA TCC GAG TTG CAA AGT GGG GTC CCA TCA   180

46   Leu Leu Ile Tyr Ser Ala Ser Glu Leu Gln Ser Gly Val Pro Ser   60

181  CGT TTC AGT GGC ACT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC   225

61   Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile   75

226  AGC AGT CTG CAA CCT GAA GAT TTT GCT ACC TAC TAC TGT CAA CAG   270

76   Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln   90

271  GTT GTG TGG CGT CCT TTT ACG TTC GGC CAA GGG ACC AAG GTG GAA   315

91   Val Val Trp Arg Pro Phe Thr Phe Gly Gln Gly Thr Lys Val Glu   105

316  ATC AAA CGG   324

106  Ile Lys Arg
```

FIGURE 2

Marmoset Sequences

Marmoset nucleotide sequence 1 (SEQ ID No:14)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGGCAAAGTCACCAT
CACTTGCCGGGCGAGTCAGGACATTAACAAGTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
CAGTCCCTAAGCCCCTGATCTATGAGGCATCCAAATTGCAAAGTGGGGTCCCATCAAGGTTC
AGCGGCAGTGGATCTGGGACATATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATGC
TGCAACTTATTACTGTCAG

Marmoset nucleotide sequence 2 (SEQ ID No:15)

GACATCCAGATGATCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCAT
CACTTGCTGGGCAAGTCAGGGTATTAGCCACTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
AAGCCCCTAAGCTCCTGATCTATAGTGCATCAAATTTAGAAACAGGGGTCCCATCAAGGTTC
AGTGGAAGTGGATCCAGGACAGATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATAT
TGCAACATATTACTGTCAA

Marmoset nucleotide sequence 3 (SEQ ID No:16)

GACATCCAGATGACCCAGACTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCAT
CACTTGCCGGGCAAGTCAGGGTATTAGCAGCTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
AAGCCCCTAAGCTCCTGATCTATGGGGCATCAAATTTGGAAACAGGGGTCCCATCAAGATTC
AGCGGAAGTGGATCTGGGACAGATTTTACTCTCACCATCAGCAGTCTGCAGCCTGAAGATAT
TGCAACATATTACTGTCAA

Marmoset nucleotide sequence 4 (SEQ ID No:17)

GACATCCAGATGATCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCAT
CACTTGCTGGGCAAGTCAGGGTATTAGCCACTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
AAGCCCCTAAGCTCCTGATCTATAGTGCATCAAATTTAGGAACAGGGGTCCCATCAAGGTTC
AGTGGAAGTGGATCCAGGACAGATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATAT
TGCAACATATTACTGTCAA

Marmoset nucleotide sequence 5 (SEQ ID No:18)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGGCAAAGTCACCAT
CACTTGCCGGGCGTGTCAGGACATTAACAAGTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
CAGTCCCTAAGCCCCTGATCTATGAGGCATCCAAATTGCAAAGTGGGGTCCCATCAAGGTTC
AGCGGCAGTGGATCTGGGACATATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATGC
TGCAACTTATTACTGTCAG

Marmoset nucleotide sequence 6 (SEQ ID No:19)

GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTTACCAT
CACTTGCCGGGCGAGTCAGGGCATTAGTAATTATTTAGCCTGGTATCAGCAGAAACCAGGGA
AAACTCCTAGGCTCCTGATCTATGCTGCATCCAGTTTACAAACTGGGATTCCCTCTCGGTTC
AGCGGCAGTGGATCTGGGACAGACTACACTCTCACCATCAGCAGCCTGCAGTCTGAAGATGT
TGCAATTTATTACTGTCAA

Marmoset nucleotide sequence 7 (SEQ ID No:20)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGGCAAAGTCACCAT
CACTTGCCGGGCGAGTCAGGACATTAACAAGTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
CAGTCCCTAAGCCCCTGATCTATGAGGCATCCAAATTGCAAAGTGGGGTCCCATCAAGGCTC
AGCGGCAGTGGATCTGGGACATATTTCACTCTCACCATCAGCAGCCTGCAGCCTGAAGATGC
TGCAACTTATTACTGTCAG

Marmoset nucleotide sequence 8 (SEQ ID No:21)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGGCAAAGTCACCAT
CACTTGCCGGGCGAGTCAGGACATTAACAAGTGGTCAGCCTGGTATCAGCAGAAACCAGGGA
CAGTCCCTAAGCCCCTGATCTATGAGGCATCCAAATTGCAAAGTGGGGTCCCATCAAGGTTC
AGCGGCAGTGGATCTGGGACATATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATGC
TGCAACTTATTACTGTCAG

Marmoset nucleotide sequence 9 (SEQ ID No:22)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGGCAAAGTCACCGT
CACTTGCCGGGCGAGTCAGGACATTAACAAGTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
CAGTCCCTAAGCCCCTGATCTATGAGGCATCCAAATTGCAAAGTGGGGTCCCATCAAGGTTC
AGCGGCAGTGGATCTGGGACATATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATGC
TGCAACTTATTACTGTCAG

Marmoset nucleotide sequence 10 (SEQ ID No:23)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGGCAAAGTCACCAT
CACTTGCCGGGCGAGTCAGGACATTAACAAGTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
CAGTCCTTAAGCCCCTGATCTATGAGGCATCCAAATTGCAAAGTGGGGTCCCATCAAGGTTC
AGCGGCAGTGGATCTGGGACATATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATGC
TGCAACTTATTACTGTCAG

Marmoset nucleotide sequence 11 (SEQ ID No:24)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGGCAAAGTCACCAT
CACTTGCCGGGCGAGTCAGGACATTAACAAGTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
CAGTCCCTAAGCCCCTGATCTATGAGGCATCCAAATTGCAAAGTGGGGTCCCATTAAGGTTC
AGCGGCAGTGGATCTGGGACATATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATGC
TGCAACTTATTACTGTCAG

Marmoset amino acid sequence 1 (SEQ ID No:25)

DIQMTQSPSSLTASVGGKVTITCRASQDINKWLAWYQQKPGTVPKPLIYEASKLQSGVPSRF
SGSGSGTYFTLTISSLQPEDAATYYCQ

Marmoset amino acid sequence 2 (SEQ ID No:26)

DIQMIQSPSSLSASVGDRVTITCWASQGISHWLAWYQQKPGKAPKLLIYSASNLETGVPSRF
SGSGSRTDFTLTISSLQPEDIATYYCQ

Marmoset amino acid sequence 3 (SEQ ID No:27)

DIQMTQTPSSLSASVGDRVTITCRASQGISSWLAWYQQKPGKAPKLLIYGASNLETGVPSRF
SGSGSGTDFTLTISSLQPEDIATYYCQ

Marmoset amino acid sequence 4 (SEQ ID No:28)

DIQMIQSPSSLSASVGDRVTITCWASQGISHWLAWYQQKPGKAPKLLIYSASNLGTGVPSRF
SGSGSRTDFTLTISSLQPEDIATYYCQ

Marmoset amino acid sequence 5 (SEQ ID No:29)

DIQMTQSPSSLTASVGGKVTITCRACQDINKWLAWYQQKPGTVPKPLIYEASKLQSGVPSRF
SGSGSGTYFTLTISSLQPEDAATYYCQ

Marmoset amino acid sequence 6 (SEQ ID No:30)

DIQMTQSPSSLSASVGDRVTITCRASQGISNYLAWYQQKPGKTPRLLIYAASSLQTGIPSRF
SGSGSGTDYTLTISSLQSEDVAIYYCQ

Marmoset amino acid sequence 7 (SEQ ID No:31)

DIQMTQSPSSLTASVGGKVTITCRASQDINKWLAWYQQKPGTVPKPLIYEASKLQSGVPSRL
SGSGSGTYFTLTISSLQPEDAATYYCQ

Marmoset amino acid sequence 8 (SEQ ID No:32)

DIQMTQSPSSLTASVGGKVTITCRASQDINKWSAWYQQKPGTVPKPLIYEASKLQSGVPSRF
SGSGSGTYFTLTISSLQPEDAATYYCQ

Marmoset amino acid sequence 9 (SEQ ID No:33)

DIQMTQSPSSLTASVGGKVTVTCRASQDINKWLAWYQQKPGTVPKPLIYEASKLQSGVPSRF
SGSGSGTYFTLTISSLQPEDAATYYCQ

Marmoset amino acid sequence 10 (SEQ ID No:34)

DIQMTQSPSSLTASVGGKVTITCRASQDINKWLAWYQQKPGTVLKPLIYEASKLQSGVPSRF
SGSGSGTYFTLTISSLQPEDAATYYCQ

Marmoset amino acid sequence 11 (SEQ ID No:35)

DIQMTQSPSSLTASVGGKVTITCRASQDINKWLAWYQQKPGTVPKPLIYEASKLQSGVPLRF
SGSGSGTYFTLTISSLQPEDAATYYCQ

Owl Monkey sequences

Owl Monkey nucleotide sequence 1 (SEQ ID No:36)

GACATCCAGATGACCCAGTCTCCATCCTTCCTGTCTGCATCTGCAGGAGACAGAGTCACCAT
CACCTGCCAGGTGAGTCAGGGAATTAGCAGTGAATTACTCTGGTATCAGCAGAAACCAGGGA
AAGCCCCTATGCTCTTGATCTATGCTGCAACCAAATTGCAGTCGGGAATCCCATCTCGGTTC
AGTGGCCATGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCTGATGATTT
TGCTACTTATTACTGTCAA

Owl Monkey nucleotide sequence 2 (SEQ ID No:37)

GACATCCAGATGACCCAGTCTGCATTCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCAT
TACTTGCCAGGCGAGTCAGGGCATTACCAGTGATTTAGCCTGGTATCAGCAAAAGCCAGGGA
ACGCCTCTAAGCTCCTGATCTATGAGGCATCCAGTTTACAAAGCGAGGTCCCATCAAGGTTC
AGCGGCAGTGGATCTGGGAGAGATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATTT
TGTAACTTATTACTGTCAA

Owl Monkey nucleotide sequence 3 (SEQ ID No:38)

GACATCCAGATGACCCAGACTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCAT
CACTTGCCGGGCGAGTCAAGACATTTACAATTATTTAGCCTGGTATCAGCAGAAACCAGGGA
AAACTCCTAGGCTCTTGATCTATGCTGCATCCAGTTTGCAAACTGGGATTCCCTCTCGGTTC
AGTGGCAGTGGATCTGGGACAGACTACACTCTCACCATCAGCAGCCTGCAGCCTGATGATTT
TGCCACTTATTACTGTCAA

Owl Monkey nucleotide sequence 4 (SEQ ID No:39)

GACATCCAGATGACCCAGACTCCATCCTCCCTGCCTGCATCTGTAGGAGACAAAGTCACCAT
CACTTGCCGGGCAAGTCAGGGTATTAGCAGCTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
AAGCCCCTAAGCTCCTGATCCATAAGGCATCAAATTTGGAAACAGGGGTCCCATCAAGGTTC
AGTGGAAGTGGATCTGGGACAGATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATAT
CGCAACATATTACTGTCAA

Owl Monkey nucleotide sequence 5 (SEQ ID No:40)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGACAAAGTCACCAT
CACTTGCCGGGCAAGTCAGGGCATTAGCAATAATTTAGCCTGGTATCAGCAGAAACCAGGGA
AAGCCCCTAAGCCCCTGATCTATTATGCATCCAGTTTGCAAAGCGGGGTCCCATCAAGGTTC
AGCGGCAGTGGATCTGGGGCAGATTACACTCTCACCACCAGCAGCCTGCAGCCTGAAGATTT
TGCAACTTATTACTGTCAA

Owl Monkey nucleotide Sequence 6 (SEQ ID No:41)

GACAACCAGATGATCCAGTCTCCATCTTCCCTGACTGCATCTGTAGGAGACAGAGTCACCAT
CACTTGCCGAGCCAGTCAGAGTATTAGCAGCTGGTTAGCCTGGTATCAGCAGAAACCAGGGA
CAGTCCCTAAGCCTCTGATCTATGACGCATCCAAATTGCTAAGTGGGGTCCCATCAAGGTTC
AGTGGCTGTGGATCTGGGACAGATTTTACTCTCACCATCAGCAGCCTGCAGCCTGAAGATTT
TGCAACTTATTACTGTCAA

Owl Monkey amino acid sequence 1 (SEQ ID No:42)

DIQMTQSPSFLSASAGDRVTITCQVSQGISSELLWYQQKPGKAPMLLIYAATKLQSGIPSRF
SGHGSGTDFTLTISSLQPDDFATYYCQ

Owl Monkey amino acid sequence 2 (SEQ ID No:43)

DIQMTQSAFSLSASVGDRVTITCQASQGITSDLAWYQQKPGNASKLLIYEASSLQSEVPSRF
SGSGSGRDFTLTISSLQPEDFVTYYCQ

Owl Monkey amino acid sequence 3 (SEQ ID No:44)

DIQMTQTPSSLSASVGDRVTITCRASQDIYNYLAWYQQKPGKTPRLLIYAASSLQTGIPSRF
SGSGSGTDYTLTISSLQPDDFATYYCQ

Owl Monkey amino acid sequence 4 (SEQ ID No:45)

DIQMTQTPSSLPASVGDKVTITCRASQGISSWLAWYQQKPGKAPKLLIHKASNLETGVPSRF
SGSGSGTDFTLTISSLQPEDIATYYCQ

Owl Monkey amino acid sequence 5 (SEQ ID No:46)

DIQMTQSPSSLTASVGDKVTITCRASQGISNNLAWYQQKPGKAPKPLIYYASSLQSGVPSRF
SGSGSGADYTLTTSSLQPEDFATYYCQ

Owl Monkey amino acid sequence 6 (SEQ ID No:47)

DNQMIQSPSSLTASVGDRVTITCRASQSISSWLAWYQQKPGTVPKPLIYDASKLLSGVPSRF
SGCGSGTDFTLTISSLQPEDFATYYCQ

FIGURE 3

```
          D  I  Q  M  T  Q  S  P  S  S  L  S  A  S  V  G  D  R  V  T  I  T  C  R  A  S  Q

1         GACATCCAGATGACCCAGTCTCCATCCTCTCTGTCTGCATCTGTAGGAGACCGTGTCACCATCACTTGCCGGGCAAGTCA  80

          S  I  D  S  Y  L  H  W  Y  Q  Q  K  P  G  K  A  P  K  L  L  I  Y  S  A  S  E

81        GAGCATTGATAGTTATTTACATTGGTACCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATAGTGCATCCGAGT  160

81        CTCGTAACTATCAATAAATGTAACCATGGTCGTCTTTGGTCCCTTTCGGGGATTCGACGACTAGATATCACGTAGGCTCA  160

                                  KpnI

          L  Q  S  G  V  P  S  R  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L  Q  P

161       TGCAAAGTGGGGTCCCATCACGTTTCAGTGGCAGTGGATCTGGCACAGATTTCACTCTCACCATCAGCAGTCTGCAACCT  240

161       ACGTTTCACCCCAGGGTAGTGCAAAGTCACCGTCACCTACACCCTCTCTAAAGTGAGAGTGGTAGTCGTCAGACGTTGGA  240

                        BanDI

          E  D  F  A  T  Y  Y  C  Q  Q  V  V  W  R  P  F  T  F  G  Q  G  T  K  V  E  I  K

241       GAAGATTTTGCTACGTACTACTGTCAACACGTTGTGTGGCGTCCTTTTACGTTCGGCCAAGGGACCAAGGTGGAAATCAA  320

241       CTTCTAAAACGATGCATGATCACACTTGTCCAACACACCGCAGGAAAATGCAAGCCGGTTCCCTGGTTCCACCTTTAGTT  320

          R

321       ACGG    324

321       TGCC    324
```

FIGURE 4A

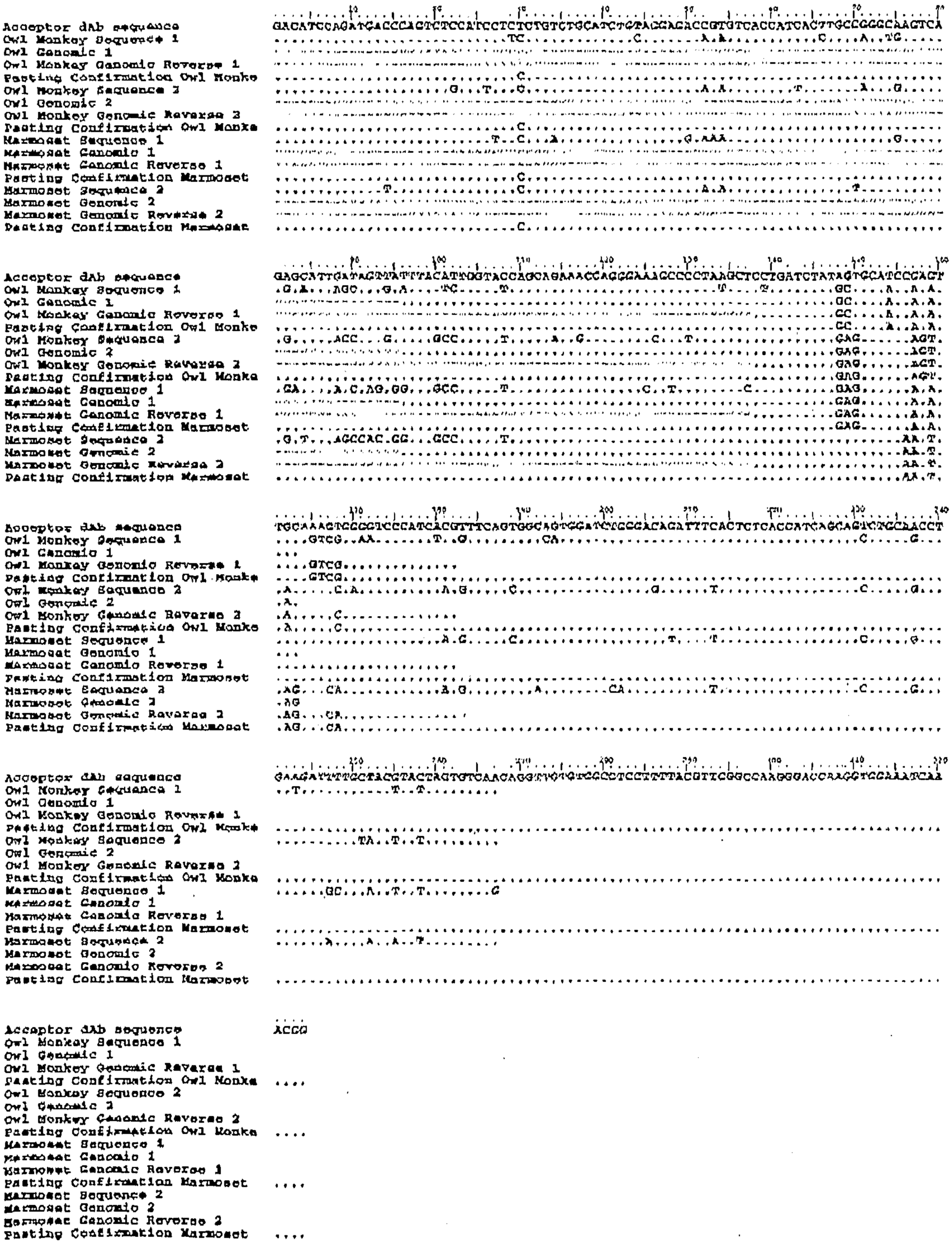

Acceptor dAb sequence
Owl Monkey Sequence 1
Owl Genomic 1
Owl Monkey Genomic Reverse 1
Pasting Confirmation Owl Monke
Owl Monkey Sequence 2
Owl Genomic 2
Owl Monkey Genomic Reverse 2
Pasting Confirmation Owl Monke
Marmoset Sequence 1
Marmoset Genomic 1
Marmoset Genomic Reverse 1
Pasting Confirmation Marmoset
Marmoset Sequence 2
Marmoset Genomic 2
Marmoset Genomic Reverse 2
Pasting Confirmation Marmoset

FIGURE 4B

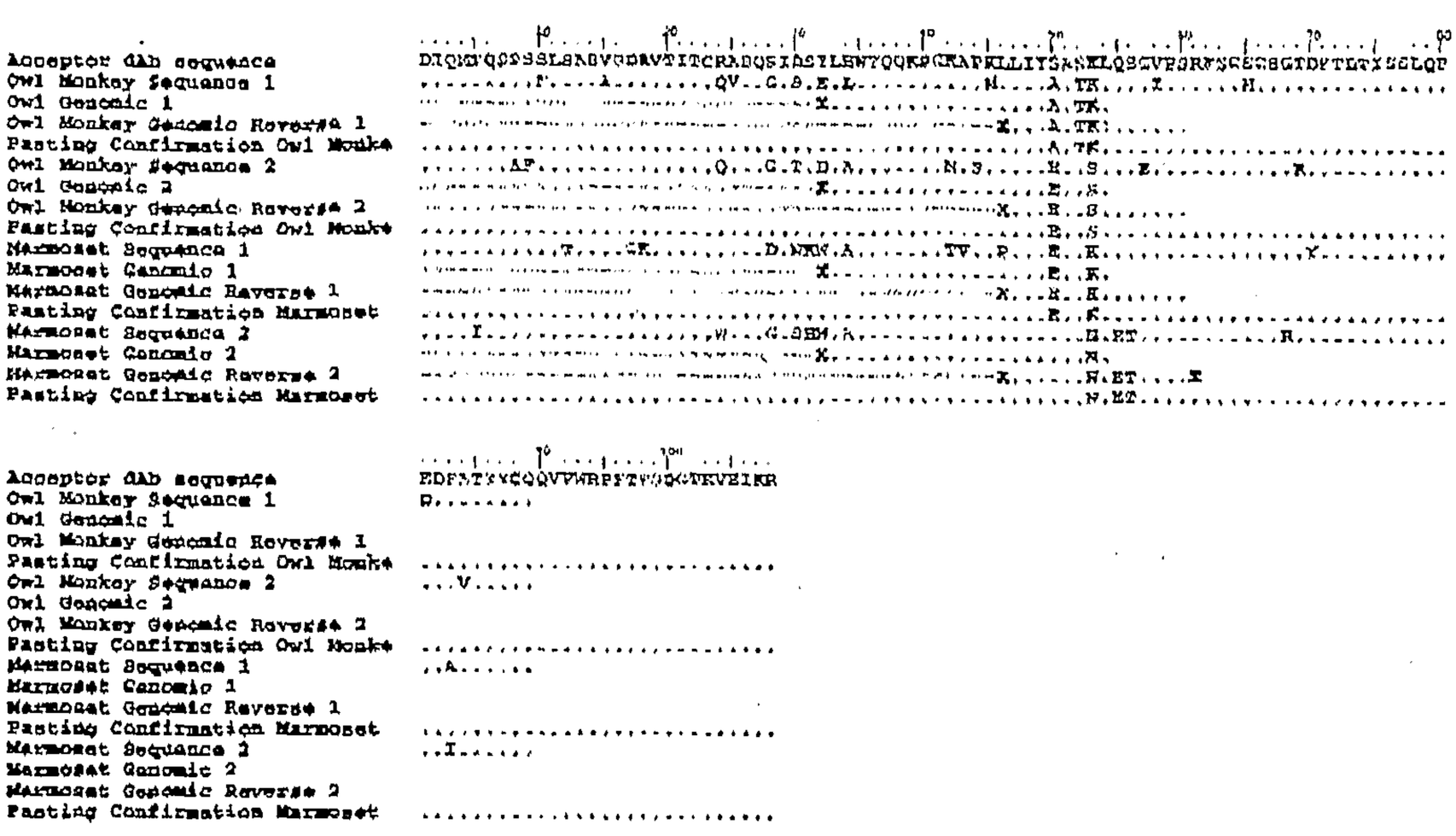

Acceptor dAb sequence
Owl Monkey Sequence 1
Owl Genomic 1
Owl Monkey Genomic Reverse 1
Pasting Confirmation Owl Monkey
Owl Monkey Sequence 2
Owl Genomic 2
Owl Monkey Genomic Reverse 2
Pasting Confirmation Owl Monkey
Marmoset Sequence 1
Marmoset Genomic 1
Marmoset Genomic Reverse 1
Pasting Confirmation Marmoset
Marmoset Sequence 2
Marmoset Genomic 2
Marmoset Genomic Reverse 2
Pasting Confirmation Marmoset
Acceptor dAb sequence
Owl Monkey Sequence 1
Owl Genomic 1
Owl Monkey Genomic Reverse 1
Pasting Confirmation Owl Monkey
Owl Monkey Sequence 2
Owl Genomic 2
Owl Monkey Genomic Reverse 2
Pasting Confirmation Owl Monkey
Marmoset Sequence 1
Marmoset Genomic 1
Marmoset Genomic Reverse 1
Pasting Confirmation Marmoset
Marmoset Sequence 2
Marmoset Genomic 2
Marmoset Genomic Reverse 2
Pasting Confirmation Marmoset

FIGURE 5

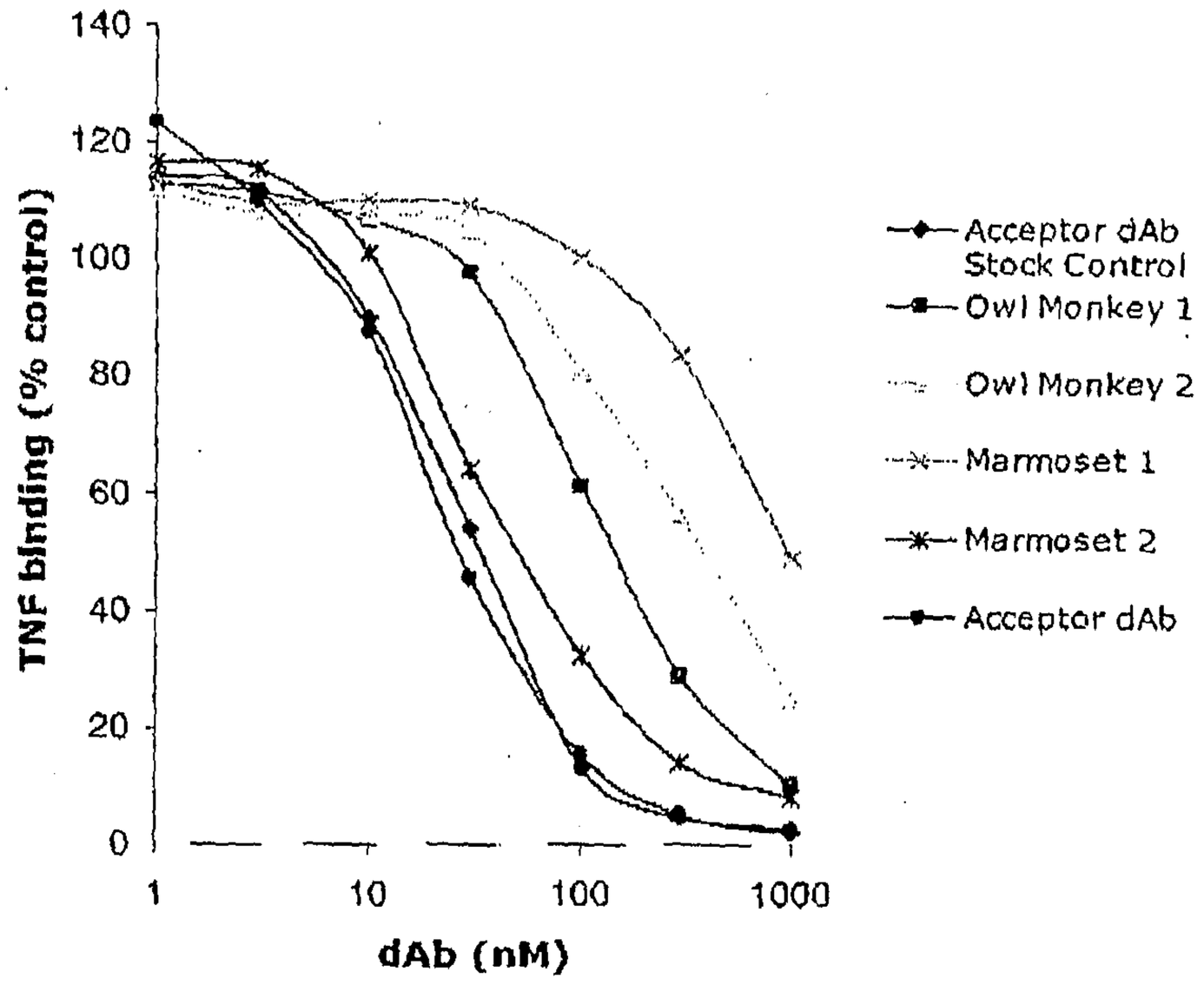
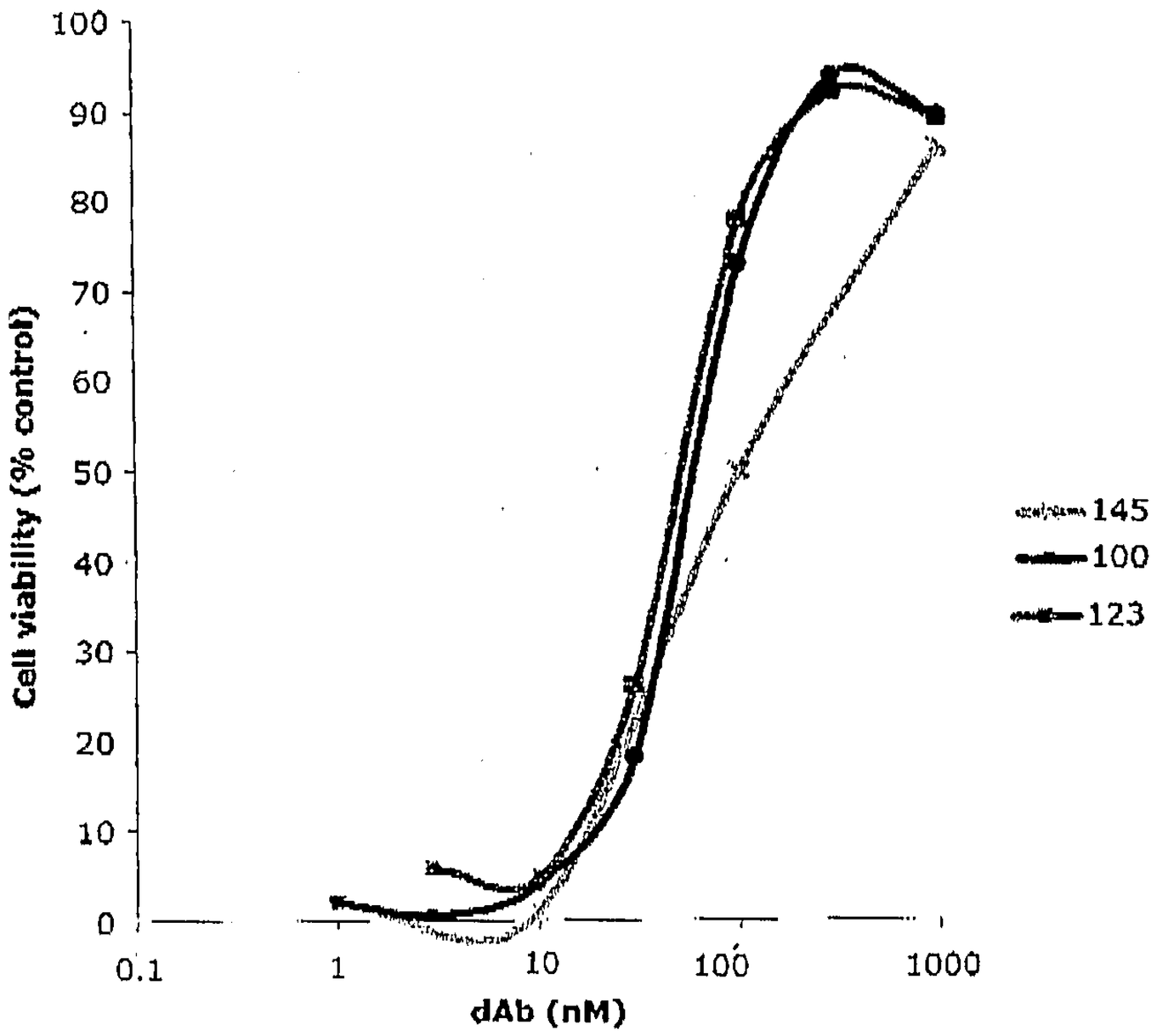

140
120
100
80
60
40
20
0
TNF binding (% control)
1
10
100
1000
dAb (nM)
Acceptor dAb Stock Control
Owl Monkey 1
Owl Monkey 2
Marmoset 1
Marmoset 2
Acceptor dAb

FIGURE 6


100
90
80
70
60
50
40
30
20
10
0
Cell viability (% control)
0.1
1
10
100
1000
dAb (nM)
145
100
123