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(54) Title: INHIBITORS OF COMPLEMENT ACTIVATION

(57) Abstract

The invention relates to factor D inhibitors, which bind to factor D and block the functional activity of factor D in complement activation. The inhibitors include antibody molecules, as well as homologues, analogues and modified or derived forms thereof, including immunoglobulin fragments like Fab, $(Fab')_2$ and Fv, small molecules, including peptides, oligonucleotides, peptidomimetics and organic compounds. A monoclonal antibody which bound to factor D and blocked its ability to activate complement was generated and designated 166–32. The hybridoma producing this antibody was deposited at the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110–2209, under Accession Number HB–12476.

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Inhibitors of Complement Activation

FIELD OF THE INVENTION

This invention relates to inhibitors specific to factor D and the use of such inhibitors in inhibiting complement system activation and inhibiting alternative pathway complement activation.

BACKGROUND OF THE INVENTION

The complement system plays a central role in the clearance of immune complexes and the immune response to infectious agents, foreign antigens, virus-infected cells and tumor cells. However, complement is also involved in pathological inflammation and in autoimmune diseases. Therefore, inhibition of excessive or uncontrolled activation of the complement cascade could provide clinical benefit to patients with such diseases and conditions.

The complement system encompasses two distinct activation pathways, designated the classical and the alternative pathways (V.M. Holers, In *Clinical Immunology: Principles and Practice*, ed. R.R. Rich, Mosby Press; 1996, 363-391). The classical pathway is a calcium/magnesium-dependent cascade which is normally activated by the formation of antigen-antibody complexes. The alternative pathway is magnesium-dependent cascade which is activated by deposition and activation of C3 on certain susceptible surfaces (*e.g.* cell wall polysaccharides of

yeast and bacteria, and certain biopolymer materials). Activation of the complement pathway generates biologically active fragments of complement proteins, *e.g.* C3a, C4a and C5a anaphylatoxins and C5b-9 membrane attack complexes (MAC), which mediate inflammatory activities involving leukocyte chemotaxis, activation of macrophages, neutrophils, platelets, mast cells and endothelial cells, vascular permeability, cytolysis, and tissue injury.

Factor D is a highly specific serine protease essential for activation of the alternative complement pathway. It cleaves factor B bound to C3b, generating the C3b/Bb enzyme which is the active component of the alternative pathway C3/C5 convertases. Factor D may be a suitable target for inhibition, since its plasma concentration in humans is very low (1.8 µg/ml), and it has been shown to be the limiting enzyme for activation of the alternative complement pathway (P.H. Lesavre and H.J. Müller-Eberhard. *J. Exp. Med.*, 1978; 148: 1498-1510; J.E. Volanakis et al., *New Eng. J. Med.*, 1985; 312: 395-401).

The down-regulation of complement activation has been demonstrated to be effective in treating several disease indications in animal models and in *ex vivo* studies, *e.g.* systemic lupus erythematosus and glomerulonephritis (Y. Wang et al., *Proc. Natl. Acad. Sci.*; 1996, 93: 8563-8568), rheumatoid arthritis (Y. Wang et al., *Proc. Natl. Acad. Sci.*, 1995; 92: 8955-8959), cardiopulmonary bypass and hemodialysis (C.S. Rinder, *J. Clin. Invest.*, 1995; 96: 1564-1572), hypercute rejection in organ transplantation (T.J. Kroshus et al., *Transplantation*, 1995; 60:

1194-1202), myocardial infarction (J. W. Homeister et al., *J. Immunol.*, 1993; 150: 1055-1064; H.F. Weisman et al., *Science*, 1990; 249: 146-151), reperfusion injury (E.A. Amsterdam et al., *Am. J. Physiol.*, 1995; 268: H448-H457), and adult respiratory distress syndrome (R. Rabinovici et al., *J. Immunol.*, 1992; 149: 1744-1750). In addition, other inflammatory conditions and autoimmune/immune complex diseases are also closely associated with complement activation (V.M. Holers, *ibid.*, B.P. Morgan. *Eur. J. Clin. Invest.*, 1994: 24: 219-228), including thermal injury, severe asthma, anaphylactic shock, bowel inflammation, urticaria, angioedema, vasculitis, multiple sclerosis, myasthenia gravis, membranoproliferative glomerulonephritis, and Sjögren's syndrome.

SUMMARY OF THE INVENTION

The invention includes factor D inhibitors, which bind to factor D and block the functional activity of factor D complement activation, including in alternative pathway complement activation. The inhibitors include antibody molecules, as well as homologues, analogues and modified or derived forms thereof, including immunoglobulin fragments like Fab, $(Fab')_2$ and Fv. Small molecules including peptides, oligonucleotides, peptidomimetics and organic compounds which bind to factor D and block its functional activity are also included.

A monoclonal antibody which bound to factor D and blocked its ability to activate complement was generated and designated 166-32. The hybridoma producing this antibody was deposited at the American Type Culture Collection,

10801 University Blvd., Manassas, VA 20110-2209, under Accession Number HB-12476.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the binding of anti-factor D monoclonal antibodies (MAbs) to purified human factor D in ELISA. The line marked with filled circles represents MAb 166-11. The line marked with filled triangles represents MAb 166-32. The line marked with filled diamonds represents MAb 166-188. The line marked with filled squares represents MAb 166-222. The Y-axis represents the reactivity of the MAbs with factor D expressed as optical density (OD) at 450 nm and the X-axis represents the concentration of the MAbs.

Fig. 2 shows the inhibition of alternative pathway (AP) hemolysis of unsensitized rabbit red blood cells (RBCs) by MAb 166-32 in the presence of 10% human serum. The line marked with filled squares represents MAb 166-32. The line marked with filled circles represents the irrelevant isotype-matched control MAb G3-519, which is specific to HIV envelope glycoprotein gp120. The Y-axis represents the % hemolysis inhibition, as further described in the text. The X-axis represents the concentration of the MAbs.

Fig. 3 shows the inhibition of alternative pathway (AP) hemolysis of unsensitized rabbit red blood cells (RBCs) by MAb 166-32 in the presence of 90% human serum. The line marked with filled squares represents MAb 166-32. The line marked with filled circles represents the irrelevant isotype-matched control MAb

G3-519, which is specific to HIV envelope glycoprotein gp120. The Y-axis represents the % hemolysis inhibition, as further described in the text. The X-axis represents the concentration of the MAbs.

Fig. 4 shows that MAb 166-32 does not inhibit classical pathway (CP) hemolysis of sensitized chicken RBCs, whereas the positive control anti-human C5 MAb 137-76 does. The line marked with filled circles represents MAb 137-76. The line marked with filled diamonds and with filled squares represents MAb 166-32 and the negative control MAb G3-519, respectively. The Y-axis represents the % hemolysis inhibition. The X-axis represents the concentration of the MAbs.

Fig. 5 shows the inhibition of alternative pathway (AP) hemolysis by MAb 166-32. Hemolysis was augmented by adding different concentrations of purified human factor D to a human serum depleted of its factor D by affinity chromatography using anti-factor D MAb 166-222. The assays were performed in the presence or absence of 0.3 µg/ml test MAbs. The line marked with filled squares represents no antibody added. The line marked with filled circles represents MAb 166-32. The line marked with filled triangles represents the irrelevant isotype-matched control MAb G3-519. The Y-axis represents the % hemolysis inhibition. The X-axis represents the concentration of factor D.

Fig. 6 shows the inhibition of factor-dependent EAC3b cell lysis by MAb 166-32. The alternative C3 convertase was assembled on EAC3b cells by incubation with factor B, factor P and factor D. Different concentrations of MAb 166-32 were

added to the incubation buffer to inhibit the activity of factor D. The line marked with filled squares represents MAb 166-32. The line marked with filled circles represents MAb G3-519. The Y-axis represents the % hemolysis inhibition. The X-axis represents the concentration of the MAbs.

Fig. 7 shows the inhibition of C3a production from zymosan by MAb 166-32. Zymosan activated the alternative complement pathway in the presence of human serum. The production of C3a was measured by using an ELISA assay kit. The line marked with filled squares represents MAb 166-32. The line marked with filled circles represents the irrelevant isotype-control MAb G3-519. The Y-axis represents the % inhibition of C3a production. The X-axis represents the concentration of the MAbs.

Fig. 8 shows the inhibition of sC5b-9 production from zymosan by MAb 166-32. Zymosan activated the alternative complement pathway in the presence of human serum. The production of sC5b-9 was measured by using an ELISA assay kit. The line marked with filled squares represents MAb 166-32. The line marked with filled circles represents the irrelevant isotype-control MAb G3-519. The Y-axis represents the % inhibition of sC5b-9 production. The X-axis represents the concentration of the MAbs.

Fig. 9 shows the inhibition of alternative pathway hemolysis of unsensitized rabbit RBCs by MAb 166-32 and its Fab. The line marked with filled circles represents MAb 166-32 (whole IgG). The line marked with filled squares represents

the Fab of the MAb 166-32. The Y-axis represents the % hemolysis inhibition. The X-axis represents the concentration of the MAbs.

Fig. 10 shows the inhibitory effect of MAb 166-32 on factor D in sera from different animal species in alternative pathway hemolysis of unsensitized rabbit RBCs. The line marked with filled squares represents human serum. The line marked with filled circles represents chimpanzee serum. The line marked with filled triangles represents rhesus monkey serum. The line marked with filled, inverted triangles represents baboon serum. The line marked with filled diamonds represents cynomolgus monkey serum. The line marked with open circles represents sheep serum. The line marked with open triangles represents canine serum. The Y-axis represents the % hemolysis inhibition. The X-axis represents the concentration of MAb 166-32.

Fig. 11 shows the reactivity of MAb 166-32 with different Baculovirus expressed factor D ("FD") mutants and hybrids in ELISA. The line marked with filled squares represents human factor D, FD/Hu. The line marked with filled circles represents pig factor D, FD/Pig. The line marked with filled triangles represents FD/Pighu. The line marked with filled, inverted triangles represents the hybrid protein, FD/Hupig. The line marked with filled diamonds represents the mutant protein, FD/VDA. The line marked with open circles represents the mutant protein, FD/L. The line marked with open squares represents the mutant protein, FD/RH.

The line marked with open diamonds represents the blank with no coating antigen. The recombinant proteins are further described in the text.

Fig. 12 shows the schematic representation of the expression vector plasmids for chimeric 166-32 Fab: (A) pSV2dhfrFd and (B) pSV2neo. Solid boxes represent the exons encoding the Fd or μ gene. Hatched segments represent the HCMV-derived enhancer and promoter elements (E-P), as indicated below. Open boxes are the dihydrofolate reductase (dhfr) and neo genes, as marked. The pSV2 plasmid consists of DNA segments from various sources: pBR322 DNA (thin line) contains the pBR322 origin of DNA replication (pBR ori) and the lactamase ampicillin resistance gene (Amp); SV40 DNA, represented by wider hatching and marked, contains the SV40 origin of DNA replication (SV40 ori), early promoter (5' to the dhfr and neo genes), and polyadenylation signal (3' to the dhfr and neo genes). The SV40-derived polyadenylation signal (pA) is also placed at the 3' end of the Fd gene.

Fig. 13 shows the inhibition of alternative pathway (AP) hemolysis of unsensitized rabbit RBCs. The line marked with filled squares represents the murine MAb 166-32. The line marked with filled circles represents chimeric MAb I66-32. The line marked with filled triangles represents isotype-matched negative control antibody G3-519. The Y-axis represents % inhibition of hemolysis. The X-axis represents the antibody concentration.

Fig. 14 shows the inhibition of alternative pathway (AP) hemolysis of unsensitized rabbit RBCs. The line marked with filled squares represents chimeric 166-32 IgG. The line marked with filled circles represents cFab/9aa. The line marked with filled triangles represents cFab. The Y-axis represents % inhibition of hemolysis. The X-axis represents the protein concentration of the IgG and Fab.

Fig. 15 shows the effects of anti-factor D MAb 166-32 treatment on the hemodynamic functions of isolated rabbit hearts perfused with human plasma. Left ventricular end-diastolic pressure (LVEDP) is represented by filled circles (for MAb 166-32) and filled squares (for MAb G3-519). Left ventricular developed pressure (LVDP) is represented by open circles (for MAb 166-32) and open squares (for MAb G3-519). MAb G3-519 is the isotype-matched irrelevant control.

Fig. 16 is a typical representation of left ventricular developed pressure (LVDP) by the two antibody groups in an isolated rabbit heart study. The upper panel represents a heart treated with the negative control antibody, MAb G3-519, and the lower panel a heart treated with MAb 166-32. The MAb G3-519 treated heart was not able to maintain LVDP after challenge with 4% human plasma, while the MAb 166-32 treated heart retained almost baseline LVDP after 60 minutes of perfusion with 4% human plasma.

Fig. 17 shows the concentration of Bb in lymphatic effluents at selected timepoints from isolated rabbit hearts perfused with 4% human plasma. Samples

from MAb 166-32 treated hearts (open circles) contained significantly less Bb than MAb G3-519 treated hearts (filled squares), $p < 0.05$.

Fig. 18 shows the alternative pathway hemolytic activity of plasma samples collected at different time points from the extracorporeal circuits treated with MAb 166-32 (filled squares) or MAb G3-519 (filled circles).

Fig. 19 shows the concentration of C3a in plasma samples collected at different time points from the extracorporeal circuits treated with MAb 166-32 (filled squares) or MAb G3-519 (filled circles).

Fig. 20 shows the concentration of sC5b-9 in plasma samples collected at different time points from the extracorporeal circuits treated with MAb 166-32 (filled squares) or MAb G3-519 (filled circles).

Fig. 21 shows the concentration of Bb in plasma samples collected at different time points from the extracorporeal circuits treated with MAb 166-32 (filled squares) or MAb G3-519 (filled circles).

Fig. 22 shows the concentration of C4d in plasma samples collected at different time points from the extracorporeal circuits treated with MAb 166-32 (filled squares) or MAb G3-519 (filled circles).

Fig. 23 shows the level of expression of CD11b on the surface of neutrophils obtained at different time points from the extracorporeal circuits treated with MAb 166-32 (filled squares) or MAb G3-519 (filled circles). The level of expression of

CD11b is represented by mean fluorescence intensity (MFI) obtained by immunocytofluorometic analyses.

Fig. 24 shows the level of expression of CD62P on the surface of platelets obtained at different time points from the extracorporeal circuits treated with MAb 166-32 (filled squares) or MAb G3-519 (filled circles). The level of expression of CD62P is represented by mean fluorescence intensity (MFI) obtained by immunocytofluorometric analyses.

Fig. 25 shows the concentration of neutrophil-specific myeloperoxidase (MPO) in plasma samples collected at different time points from the extracorporeal circuits treated with MAb 166-32 (filled squares) or MAb G3-519 (filled circles).

DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:1 shows the nucleotide sequence of human factor D.

SEQ ID NO:2 shows the amino acid sequence of human factor D.

SEQ ID NO:3 shows the nucleotide sequence of pig factor D.

SEQ ID NO:4 shows the amino acid sequence of pig factor D.

SEQ ID NO:5 is the primer used for cloning the V_K gene of MAb 166-32.

SEQ ID NO:6 was used as an anealed adaptor for cloning the V_K gene of MAb 166-32, as described below, and as a primer.

SEQ ID NO:7 was also used as an anealed adaptor for cloning the V_K gene of MAb 166-32, as described below.

SEQ ID NO:8 was used as a 3' primer for cloning the V_H gene of MAb 166-

32, as described below.

SEQ ID NO:9 was a primer used for cloning the V_H gene of MAb 166-32.

SEQ ID NO:10 was used as a primer for cloning the V_H gene of MAb 166-32.

SEQ ID NO:11 was the 5' primer for the PCR of the Fd gene of MAb 166-32.

SEQ ID NO:12 was the 3' primer for the PCR of the Fd gene of MAb 166-32.

SEQ ID NO: 13 was the 5' primer for the Fd gene PCR of MAb 166-32

SEQ ID NO: 14 was the 3' primer for the Fd gene PCR of MAb 166-32.

MAKING AND USING THE INVENTION

A. Generation of Monoclonal antibodies (MAbs) to human factor D

In one embodiment of the invention, anti-factor D MAbs can be raised by immunizing rodents (*e.g.* mice, rats, hamsters and guinea pigs) with either native factor D purified from human plasma or urine, or recombinant factor D or its fragments expressed by either eukaryotic or prokaryotic systems. Other animals can be used for immunization, *e.g.* non-human primates, transgenic mice expressing human immunoglobulins and severe combined immunodeficient (SCID) mice transplanted with human B lymphocytes. Hybridomas can be generated by conventional procedures by fusing B lymphocytes from the immunized animals with myeloma cells (e.g. Sp2/0 and NS0), as described by G. Köhler and C. Milstein (*Nature*, 1975: 256: 495-497). In addition, anti-factor D antibodies can be generated by screening of recombinant single-chain Fv or Fab libraries from human B lymphocytes in phage-display systems. The specificity of the MAbs to human

factor D can be tested by enzyme linked immunosorbent assay (ELISA), Western immunoblotting, or other immunochemical techniques. The inhibitory activity of the antibodies on complement activation can be assessed by hemolytic assays using unsensitized rabbit or guinea pig red blood cells (RBCs) for the alternative pathway, and using sensitized chicken or sheep RBCs for the classical pathway. The hybridomas in the positive wells are cloned by limiting dilution. The antibodies are purified for characterization for specificity to human factor D by the assays described above.

If used in treating inflammatory or autoimmune diseases in humans, the anti-factor D antibodies would preferably be used as chimeric, deimmunized, humanized or human antibodies. Such antibodies can reduce immunogenicity and thus avoid human anti-mouse antibody (HAMA) response. It is preferable that the antibody be IgG4, IgG2, or other genetically mutated IgG or IgM which does not augment antibody-dependent cellular cytotoxicity (S.M. Canfield and S.L. Morrison, *J. Exp. Med.*, 1991: 173: 1483-1491) and complement mediated cytolysis (Y.Xu et al., *J. Biol. Chem.*, 1994: 269: 3468-3474; V.L. Pulito et al., *J. Immunol.*, 1996; 156: 2840-2850).

Chimeric antibodies are produced by recombinant processes well known in the art, and have an animal variable region and a human constant region. Humanized antibodies have a greater degree of human peptide sequences than do chimeric antibodies. In a humanized antibody, only the complementarity

determining regions (CDRs) which are responsible for antigen binding and specificity are animal derived and have an amino acid sequence corresponding to the animal antibody, and substantially all of the remaining portions of the molecule (except, in some cases, small portions of the framework regions within the variable region) are human derived and correspond in amino acid sequence to a human antibody. See L. Riechmann et al., *Nature*, 1988; 332: 323-327; G. Winter, *United States Patent* No. 5,225,539; C.Queen et al., U.S. patent number 5,530,101.

Deimmunized antibodies are antibodies in which the T and B cell epitopes have been eliminated, as described in International Patent Application PCT/GB98/01473. They have no or reduced immunogenicity when applied *in vivo*.

Human antibodies can be made by several different ways, including by use of human immunoglobulin expression libraries (Stratagene Corp., La Jolla, California) to produce fragments of human antibodies (VH, VL, Fv, Fd, Fab, or $(Fab')_2$, and using these fragments to construct whole human antibodies using techniques similar to those for producing chimeric antibodies. Human antibodies can also be produced in transgenic mice with a human immunoglobulin genome. Such mice are available from Abgenix, Inc., Fremont, California, and Medarex, Inc., Annandale, New Jersey.

One can also create single peptide chain binding molecules in which the heavy and light chain Fv regions are connected. Single chain antibodies ("ScFv") and the method of their construction are described in U.S. Patent No. 4,946,778.

Alternatively, Fab can be constructed and expressed by similar means (M.J. Evans et al., *J. Immunol. Meth.*, 1995; 184: 123-138). All of the wholly and partially human antibodies are less immunogenic than wholly murine MAbs, and the fragments and single chain antibodies are also less immunogenic. All these types of antibodies are therefore less likely to evoke an immune or allergic response. Consequently, they are better suited for *in vivo* administration in humans than wholly animal antibodies, especially when repeated or long-term administration is necessary. In addition, the smaller size of the antibody fragment may help improve tissue bioavailability, which may be critical for better dose accumulation in acute disease indications.

Based on the molecular structures of the variable regions of the anti-factor D antibodies, one could use molecular modeling and rational molecular design to generate and screen small molecules which mimic the molecular structures of the binding region of the antibodies and inhibit the activities of factor D. These small molecules can be peptides, peptidomimetics, oligonucleotides, or organic compounds. The mimicking molecules can be used as inhibitors of complement activation in inflammatory indications and autoimmune diseases. Alternatively, one could use large-scale screening procedures commonly used in the field to isolate suitable small molecules form libraries of combinatorial compounds.

In one preferred embodiment of the invention, a chimeric Fab, having animal (mouse) variable regions and human constant regions is used therapeutically. The

Fab is preferred because:

1. it is smaller than a whole immunoglobulin and may provide better tissue permeation;
2. as monovalent molecule, there is less chance of immunocomplexes and aggregates forming; and
3. it can be produced in a microbial system, which can more easily be scaled-up than a mammalian system.

B. Applications of the Anti-Factor D Molecules

The anti-factor D binding molecules, antibodies, and fragments of this invention, can be administered to patients in an appropriate pharmaceutical formulation by a variety of routes, including, but not limited, intravenous infusion, intravenous bolus injection, and intraperitoneal, intradermal, intramuscular, subcutaneous, intranasal, intratracheal, intraspinal, intracranial, and oral routes. Such administration enables them to bind to endogenous factor D and thus inhibit the generation of C3b, C3a and C5a anaphylatoxins, and C5b-9.

The estimated preferred dosage of such antibodies and molecules is between 10 and 500 µg/ml of serum. The actual dosage can be determined in clinical trials following the conventional methodology for determining optimal dosages, *i.e.*, administering various dosages and determining which is most effective.

The anti-factor D molecules can function to inhibit *in vivo* complement

activation and/or the alternative complement pathway and inflammatory manifestations that accompany it, such as recruitment and activation of macrophages, neutrophils, platelets, and mast cells, edema, and tissue damage. These inhibitors can be used for treatment of diseases or conditions that are mediated by excessive or uncontrolled activation of the complement system. These include, but are not limited to: (1) tissue damage due to ischemia-reperfusion following acute myocardial infarction, aneurysm, stroke, hemorrhagic shock, crush injury, multiple organ failure, hypovolemic shock and intestinal ischemia; (2) inflammatory disorders, *e.g.*, burns, endotoxemia and septic shock, adult respiratory distress syndrome, cardiopulmonary bypass, hemodialysis; anaphylactic shock, severe asthma, angioedema, Crohn's disease, sickle cell anemia, poststreptococcal glomerulonephritis and pancreatitis; (3) transplant rejection, *e.g.*, hyperacute xenograft rejection; and (4) adverse drug reactions, *e.g.*, drug allergy, IL-2 induced vascular leakage syndrome and radiographic contrast media allergy. Autoimmune disorders including, but not limited to, systemic lupus erythematosus, myasthenia gravis, rheumatoid arthritis, Alzheimer's disease and multiple sclerosis, may also be treated with the inhibitors of the invention.

The anti-factor D molecules can also be used diagnostically to ascertain the presence of, or to quantify, factor D in a tissue specimen or a body fluid sample, such as serum, plasma, urine or spinal fluid. In this application, well known assay formats can be used, such as immunohistochemistry or ELISA, respectively. Such

diagnostic tests could be useful in determining whether certain individuals are either deficient in or overproduce factor D.

C. Animal Models of the Therapeutic Efficacy of Factor D Inhibitors

The therapeutic activity of factor D inhibitors in various disease indications described above can be confirmed by using available animal models for various inflammatory and autoimmune manifestations. The *in vitro* tests described below in the examples are adequate to establish their efficacy.

Animal models relevant to various complement-related clinical diseases in humans can also be used to confirm the *in vivo* efficacy of factor D inhibitors. These include, but not limited to: myocardial ischemia/reperfusion injury (H.F. Weisman et al., *Science*, 1990; 249: 146-151); myocardial infarction (J.W. Homeister et al., *J. Immunol.* 1993; 150: 1055-1064), systemic lupus erythematosus and glomerulonephritis (S.K. Datta. *Meth. Enzymol.*, 1988; 162: 385-442; D.J. Salvant and A.V. Cybulsky, *Meth. Enzymol.*, 1988; 162: 421-461), rheumatoid arthritis (Y. Wang et al., *Proc. Natl. Acad. Sci.*, 1995; 92: 8955-8959), adult respiratory distress syndrome (R. Rabinovici et al., *J. Immunol.*, 1992; 149: 1744-1750), hyperacute rejection in organ transplantation (T.J. Kroshus et al., *Transplantation*, 1995; 60: 1194-1202), burn injury (M.S. Mulligan et al., *J. Immunol.*, 1992; 148: 1479-1485), cardiopulmonary bypass (C.S. Rinder et al., *J. Clin. Invest.*, 1995; 96: 1564-1572).

Exemplification of how to make and use the invention, and verification of its

utility, appear below.

Example 1: Generation of anti-factor D MAbs

Male A/J mice (Harlan, Houston, TX), 8-12 weeks old, were injected subcutaneously with 25 g of factor D purified from human serum (Advanced Research Technologies, San Diego,CA) in complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) in 200 µl of phosphate-buffered saline (PBS) pH7.4. The factor D preparation were tested to be > 95% pure by sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). The factor D was tested and found to be biologically active in hemolysis as described below. At two-week intervals the mice were twice injected subcutaneously with 25 µg of human factor D in incomplete Freund's adjuvant. Then two weeks later and three days prior to sacrifice, the mice were again injected intraperitoneally with 25 µg of the same antigen in PBS. For each fusion, single cell suspensions were prepared from the spleen of an immunized mouse and used for fusion with Sp2/0 myeloma cells. 5 x 10^6 of the Sp2/0 and 5 x 10^6 spleen cells were fused in a medium containing 50% polyethylene glycol (M.W. 1450) (Kodak, Rochester, NY) and 5% dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO). The cells were then adjusted to a concentration of 1.5 x 10^5 spleen cells per 200 µl of the suspension in Iscove medium (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 0.1 mM hypoxanthine, 0.4 M aminopterin, and 16 M thymidine. Two hundred microliters of the cell suspension

were added to each well of about twenty 96-well microculture plates. After about ten days culture supernatants were withdrawn for screening for reactivity with purified factor D in ELISA.

Wells of Immulon 2 (Dynatech Laboratories, Chantilly, VA) microtest plates were coated by adding 50 µl of purified human factor at 50 ng/ml overnight at room temperature. The low concentration of factor D for coating enabled the selection of high-affinity antibodies. After the coating solution was removed by flicking of the plate, 200 µl of BLOTTO (non-fat dry milk) in PBS was added to each well for one hour to block the non-specific sites. An hour later, the wells were then washed with a buffer PBST (PBS containing 0.05% Tween 20). Fifty microliters of culture supernatants from each fusion well were collected and mixed with 50 µl of BLOTTO and then added to the individual wells of the microtest plates. After one hour of incubation, the wells were washed with PBST. The bound murine antibodies were then detected by reaction with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Fc specific) (Jackson ImmunoResearch Laboratories, West Grove, PA) and diluted at 1:2,000 in BLOTTO. Peroxidase substrate solution containing 0.1% 3,3,5,5 tetramethyl benzidine (Sigma, St. Louis, MO) and 0.0003% hydrogen peroxide (sigma) was added to the wells for color development for 30 minutes. The reaction was terminated by addition of 50 µl of 2M H_2SO_4 per well. The OD at 450 nM of the reaction mixture was read with a BioTek ELISA Reader (BioTek Instruments, Winooski, VM).

The culture supernatants from the positive wells were then tested by two assays: i) inhibition of alternative pathway hemolysis of unsensitized rabbit RBCs by pre-titered human serum by the method described below; and ii) inhibition of formation of C3a by zymosan treated with human serum as described below. The cells in those positive wells were cloned by limiting dilution. The MAbs were tested again for reactivity with factor D in the ELISA. The selected hybridomas were grown in spinner flasks and the spent culture supernatant collected for antibody purification by protein A affinity chromatography. Four MAbs were tested to be strongly reactive with human factor D in ELISA. These MAbs are designated 166-11, 166-32, 166-188, and 166-222 (Fig. 1). Among them, MAb 166-32 (IgG1) strongly inhibited the alternative pathway hemolysis of unsensitized rabbit RBCs as described below.

Example 2: Determination the kinetic constants of the anti-factor D MAbs by surface plasmon resonance method

The kinetic constants for the binding of MAbs 166-11, 166-32, 166-188, and 166-22 to human factor D were determined by surface plasmon resonance-based measurements using the BIAcore instrument (Pharmacia Biosensor AB, Uppsala, Sweden). All the binding measurements were performed in HEPES-buffered saline (HBS) (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant P20) at 25°C. To measure the binding rate constants of factor D to the MAbs, a rabbit anti-mouse IgG (H+L) was immobilized onto a CM5 sensorchip by amine coupling using N-hydroxysuccinimide and N-ethyl-N'-(3-diethylaminopropyl)

carbodimide. Each individual MAb was then captured onto the coated sensorchip before the injection of factor D at different concentrations. To measure the association rate constants (k_{assoc}), five dilutions of factor D (2.5 nM, 5 nM, 10 nM, 15 nM and 20 nM) were made based on the concentration indicated by the manufacturer, and were injected to the flowcell at the flow rate of 5 µl/min. To measure the dissociation rate constants (k_{dissoc}), 100 nM factor D was injected into the flowcell at the flow rate of 5 µl/min. The data, in the form of sensorgrams, was analyzed using the data-fitting programs implemented in the BIAcore system. Since MAb 166-32 has a very fast k_{assoc} which is beyond the reliability limit of the assay format due to the limitation of the mass transport effect, an additional binding format was also used to measure its kinetic rate constants. Factor D was immobilized onto the sensorchip by amine coupling as described above while MAb 166-32 of different dilutions (5 nM, 10 nM, 15 nM, 20 nM and 25 nM for the measurement of k_{assoc} and 200 nM for the measurement of k_{dissoc} flowed to the sensorchip at the flow rate of 5 µl/min. The data, in the form of sensorgrams, was analyzed as described above. The kinetic constants of factor D binding to the MAbs on BIAcore is shown in Table 1 below. MAbs 166-32 and 166-222 have very high affinity to factor D, with equilibrium dissociation constant (K_D) less than 0.1 nM.

Table 1 Kinetic Constants of Factor D Binding to MAbs on BIAcore

MAbs	k_{assoc} (x 10^5 $M^{-1}s^{-1}$)	k_{dissoc} (x $10^{-4}s^{-1}$)	K_D (x $10^{-10}M$)[c]
166-32[a]	>10	1.1	<1
166-32[b]	4.6	0.76	1.6
166-188[a]	8.75	2.1	2.4
166-11[a]	8.0	1.0	1.24
166-222[a]	>10	0.8	<1

[a] Factor D was used as the analyte which flowed onto the sensorchip coated with anti-factor D MAb captured by rabbit anti-mouse IgG during the determination.

[b] MAb 166-32 was used as the analyte and factor D was crosslinked to the sensorchip by the amine-coupling method.

[c] K_D, equilibrium dissociation constant, = k_{dissoc}/k_{assoc}

Example 3: Inhibition of complement-activated hemolysis

To study the functional activity of the anti-factor D MAbs in inhibiting complement activation *in vitro*, two hemolytic assays were used.

For the alternative pathway, unsensitized rabbit RBCs were washed three times with gelatin/veronal-buffered saline (GVB/Mg-EGTA) containing 2 mM $MgCl_2$ and 1.6 mM EGTA. EGTA at a concentration of 10mM was used to inhibit the classical pathway (K. Whaley et al., in A.W. Dodds (Ed.), *Complement: A Practical Approach*. Oxford University Press, Oxford, 1997, pp. 19-47). The washed cells were re-suspended in the same buffer at 1.7 x 10^8 cells/ml. In each well of a round-bottom 96-well microtest plate, 50 µl of normal human serum (20%) was mixed with 50 µl of GVB/Mg-EGTA or serially diluted test MAb then 30 µl of the washed rabbit RBCs suspension were added to the wells containing the mixtures. Fifty microliters of normal human serum (20%) was mixed with 80 µl of GVB/Mg-EGTA to give the serum color background. For negative control, an isotype-matched anti-HIV-1 gp120 MAb, G3-519, was used. The final mixture was incubated at 37°C for 30 minutes. The plate was then shaken on a micro-test plate shaker for 15 seconds. The plate was then centrifuged at 300 x g for 3 minutes. Supernatants (80 µl) were collected and transferred to wells on a flat-bottom 96-well microtest plates for measurement of OD at 405 nm. The percent inhibition of hemolysis is defined as 100 x [(OD without MAb – OD serum color background) – (OD with MAb – OD

serum color background)] / (OD without MAb – OD serum color background).

Fig. 2 shows the data that MAb 166-32 strongly inhibits in a dose-dependent manner the alternative pathway hemolysis of unsensitized rabbit RBCs in the presence of 10% human serum, whereas the irrelevant isotype-matched control MAb G3-519 does not. MAb G3-519 is specific to HIV envelope glycoprotein gp120.

In assays to test the inhibitory activity of MAb166-32 in 90% human serum, frozen human serum was thawed and pre-treated with EGTA at a final concentration of 10 mM. Ten microliters of serially diluted MAb 166-32 or G3-519 were added to 90 μl of EGTA-treated human serum in duplicate wells of a 96-well microtest plate for 15 minutes at room temperature. Thirty microliters of the washed rabbit RBC's were added to each well. The plate was incubated at 37°C for 30 minutes. The plate was shaken on a plate-shaker for 15 seconds and then centrifuged at 300 x g for 3 minutes. Supernatants (80 μl) were collected and transferred to wells on a flat-bottom 96-well microtest plate for measurement of OD at 405 nm. Each plate contained two wells containing 100 μl of 90% human serum and 30 μl of the buffer as serum color background and also two wells containing the RBCs lysed with 100 μl of 90% human serum, in the absence of monoclonal antibody, to represent total lysis. Fig.3 shows the data that MAb 166-32 strongly inhibits in a dose-dependent manner the alternative pathway hemolysis of unsensitized rabbit RBCs even in the presence of 90% human serum.

For the classical pathway, chicken RBCs (5 x 10^7 cells/ml) in gelatin/veronal-buffered saline (GVB^{++}) containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ were sensitized with purified rabbit anti-chicken RBC immunoglobulins at 8 µg/ml (Inter-Cell Technologies, Hopewell, NJ) for 15 minutes at 4°C. The cells were then washed with GVB^{++}. The final human serum concentration used was 2%.

Fig. 4 shows the data that MAb 166-32 and the irrelevant control G3-519 do not inhibit the classical pathway hemolysis of sensitized chicken RBCs, whereas the positive control anti-human C5 MAb 137-76 does. The data from Figs. 2, 3 and 4 indicate that MAb 166-32 is specific to the inhibition of the alternative pathway of complement activation.

Example 4: Specificity of MAb 166-32 to factor D

Two hemolytic assays, as described below, were used to demonstrate the specificity of MAb166-32 to human factor D.

(1) Inhibition of factor D dependent hemolytic assays using unsensitized rabbit RBCs

A human serum sample was first depleted of factor D by passing it through an affinity column packed with 3M Emphaze Biosupport Medium (Pierce, Rockford, IL) coupled with the anti-factor D MAb 166-222. The flow-through serum was tested to be inactive in triggering alternative pathway hemolysis due to the complete depletion of factor D. The procedure of this assay is similar to that described in Example 3 described above, except purified factor D of varying concentrations was added to the factor D depleted serum to reconstitute the hemolytic activity. Under

these conditions, the hemolysis of rabbit RBCs was factor D dependent. It was shown that the reconstituted hemolytic activity is linearly proportional to the concentration of the supplemented factor D (from 0.01 µg/ml to 2 µg/ml (Fig. 5)). The data from Fig. 5 also shows that 0.3 µg/ml of MAb 166-32 can completely inhibit hemolysis of unsensitized rabbit RBCs in the presence of 0.1 µg/ml supplemented factor D, whereas the negative control MAb G3-519 has no effect on the factor D dependent hemolysis. These data suggest that MAb 166-32 can effectively inhibit the biological activity of human factor D at a molar ratio of 1:2 (MAb 166-32 to factor D). Therefore MAb 166-32 is a potent, high-affinity antibody to factor D. The antibody has the potential to be used clinically to treat diseases or indications caused by activation of the alternative complement pathway.

(2) Inhibition of the formation of alternative C3 convertase on EAC3b cells

EAC3b cells are sheep RBCs coated with human C3b (purchased from the National Jewish Center of Immunology and Respiratory Medicine, Denver, CO). In this assay, the alternative C3 convertase was assembled on the surface of EAC3b cells by addition of factor B, factor P (properdin) and factor D. EAC3b cells (5×10^8), which were then washed three times in $DGVB^{++}$ medium (50% veronal buffered saline, pH7.2, containing 0.075 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.1% gelatin, 2.5% (w/v) dextrose, and 0.01% sodium azide). The washed cells were then resuspended in 1.5 ml of $DGVB^{++}$, factor P (30 µg) and factor B (20 µg). The concentrations of factor P and factor B were pre-determined to be in excess. Fifty microliters of the cell

suspension was added to each well of a round-bottom 96-well microtest plate. Then 50 µl of a mixture of factor D (1.2 ng/ml) and serially diluted MAb 166-32 or MAb G3-519 was added to the wells containing to the cells for incubation for 15 minutes at 30°C. The concentration of factor D (1.2 ng/ml) was pre-determined to give over 90% hemolysis under these conditions. After incubation, the cells were washed twice in GVB-EDTA medium (gelatin/veronal-buffered saline containing 10 mM EDTA). The cells were then resuspended in 30 µl of GVB-EDTA medium. To initiate hemolysis, 100 µl of guinea pig serum (Sigma) (diluted 1:10 in GVB-EDTA) were added to each well. The mixtures were then incubated at 37°C for 30 minutes. The microtest plate was then centrifuged at 300 x g for 3 minutes. The supernatant was collected for OD measurement at 405 nm.

Fig. 6 shows the results of the experiments that MAb 166-32 inhibits the lysis of EAC3b cells, whereas the irrelevant MAb G3-519 does not. MAb 166-32 inhibits factor D from cleavage of factor B, therefore preventing the formation of C3 convertase on the surface of EAC3b cells.

Example 5: Inhibition of the generation of C3a from complement-activated zymogen by MAb 166-32

To further ascertain the functional specificity of MAb 166-32 to factor D, the effect of the MAb on alternative complement activation on zymosan (activated yeast particles) was examined. Zymosan A (from *Saccharomyces cerevisiae*, Sigma) (1 mg/ml) was washed three times in GVB/Mg-EGTA and then resuspended in the same medium at 1 mg/ml. Twenty-five microliters of MAb 166-32 or G3-519 in

different concentrations were mixed with 25 µl of human serum (diluted 1:5 in GVB/Mg-EGTA) in a microtube and incubated for 15 minutes at room temperature. The blank contained no antibody but the plain medium and the serum. After incubation, 50 µl of washed zymosan suspension were added to each tube for incubation for 30 minutes at 37°C. The microtubes were then centrifuged at 2000 x g for 5 minutes, the supernatants were collected and mixed with equal volume of Specimen Stabilizing Solution (Quidel, San Diego, CA). The samples were frozen at -25°C until being assayed. The concentration of C3a and sC5b-9 in the samples were measured by quantitative ELISA kits (Quidel) according to the procedures provided by the manufacturer.

Fig. 7 shows that MAb 166-32 inhibits the generation of C3a from complement-activated zymosan, whereas the irrelevant MAb G3-519 has no effect. These data suggest that MAb 166-32 inhibits the formation of C3 convertase by factor D. The complete inhibition of factor D by MAb 166-32 can effectively block the formation of C3 convertase as indicated by the inability to generate C3a. This will lead to the inhibition of C5 convertase in the subsequent steps of the complement cascade, as evidenced by the inhibition of sC5b-9 (MAC) formation (Fig. 8).

Example 6: Inhibition of complement-activated hemolysis by the Fab of MAb 166-32

In order to examine whether monovalent form of MAb 166-32 is effective in inhibiting the alternative complement pathway as the parental bivalent MAb 166-32, the Fab of MAb 166-32 was prepared by papain digestion using a commercial

reagent kit (Pierce). The Fab was then tested for inhibitory activity on the alternative pathway hemolysis using unsensitized rabbit RBCs as described above.

Fig. 9 shows the data of the experiments that both the whole IgG and Fab show similar potency in blocking the alternative complement activation. These results suggest that monovalent form of MAb 166-32 is active and it retains the similar potency against factor D as its parental bivalent antibody. This property is important for the consideration of using Fab or single-chain Fv as the alternative products. One advantage of using the latter monovalent forms is that they will have better tissue penetration because of their smaller size. Inasmuch as the Fab of MAb 166-32 is active, it is likely that the binding epitope on factor D recognized by the MAb is functionally important.

Example 7: Effects of MAb 166-32 on alternative pathway hemolysis using sera from different animal species

In order to study the cross-reactivity of MAb 166-32 with factor D from different animal species, alternative pathway hemolytic assays were performed using sera from different animal species. Fresh sera from different animal species (human, rhesus monkey, chimpanzee, baboon, cynomolgus monkey, sheep, dog, mouse, hamster, rat, rabbit, guinea pig and pig) were first tested for the CH50 values, which are defined as the dilution of the serum to achieve 50% lysis of unsensitized rabbit RBCs. The inhibitory activity of MAb 166-32 on the same hemolytic activity (CH50) of each serum was then tested and compared.

Fig. 10 shows that MAb 166-32 has strong inhibitory activity against sera

from human, rhesus monkey, and chimpanzee and moderate inhibitory activity against sera from baboon, cynomolgus monkey, sheep and dog. The antibody does not inhibit sera from mouse, hamster, rat, rabbit, guinea pig and pig. These data suggests that MAb 166-32 binds to an epitope on factor D shared by humans, rhesus monkeys, chimpanzees, baboons, cynomolgus monkeys, sheep and dogs.

Example 8: Construction of human Factor D mutants for epitope mapping of MAb 166-32

To delineate the binding epitope on human factor D recognized by MAb 166-32, the reactivity of the antibody with human factor D on Western blots was first tested. MAb 166-32 did not react with SDS-denatured human factor D (either reduced or non-reduced) immobilized on nitrocellulose membrane. This result indicates the MAb 166-32 binds native but not denatured factor D.

Since MAb 166-32 does not inhibit the hemolytic activity of mouse and pig factor D as described in Example 7, it is likely that MAb 166-32 binds to a site on human factor D that has a high degree of difference in the amino acid sequence from those of mouse and pig factor D. Based on this concept, various factor D mutants and hybrids were made by replacing amino acid residues in human factor D with the corresponding amino acid residues in the pig counterpart, for mapping the binding epitope of Mab 166-32, as described below.

(1) Construction of factor D mutants and hybrids

Human factor D gene segments were obtained by polymerase chain reaction (PCR) using human adipocyte cDNA (Clontech, San Francisco, CA) as the template

and appropriate oligonucleotide primers. Amplified DNA fragments were digested with *Bam*HI and *Eco*RI restriction enzymes and the digested product was inserted at the *Bam*HI and *Eco*RI sites of the Baculovirus transfer vector pVL1393 (Pharmingen, San Diego, CA) to give the wild type pVL1393-factor D/Hu. The human factor D is designated as factor D/Hu. The nucleotide sequence and the deduced amino acid sequence of the mature human factor D protein are shown in SEQ ID NOS: 1and 2 (R.T. White et al., *J. Biol. Chem.*, 1992; 267:9210-9213; GenBank accession number: M84526).

Pig factor D cDNA clone pMon24909 was obtained as a gift from J.L. Miner of University of Nebraska (GenBank accession number: U29948). The *Bam*HI-*Eco*RI fragments of pMon24909 were cloned into pVL1393 to give pVL1393-factor D/Pig. The pig factor D is designated as factor D/Pig. The nucleotide sequence and the deduced amino acid sequence of the mature pig factor D protein are shown in SEQ ID NOS: 3 and 4.

Three human factor D mutants were constructed by using appropriate primers and overlapping PCR. The amino acid mutations were designed by replacing the amino acid residues in the human sequence with the corresponding amino acid residues of the pig sequence when the amino acid sequences of human and pig factor D are aligned for homology comparison. The first mutant, factor D/VDA, contained three amino acid mutations: V113E, D116E, and A118P. (This is a short-hand method of designating mutations in which, for example, V113E

means that the valine at amino acid residue number 113 in human factor D was changed to glutamic acid in pig factor D). The second mutant, factor D/RH, contained two amino acid mutations: R156L and H159Y. The third mutant, factor D/L, contained a single mutation: L168M. DNA sequences encoding these mutants were confirmed by DNA sequencing. After digesting with appropriate enzymes, DNA fragments were inserted at the *Bam*HI and *Eco*RI sites of the Baculovirus transfer vector pVL1393 to give pVL1393-factorD/VDA, pVL1393-factorD/RH and pVL1393-factorD/L, respectively.

Two chimeric human-pig factor D hybrids were also constructed by using appropriate primers and overlapping PCR. The first hybrid, factor D/Hupig, contained 52 human factor D-derived amino acids at the N-terminus, and the remaining amino acids were derived from the pig factor D. The other hybrid, factor D/Pighu, contained 52 pig factor D-derived amino acids at the N-terminus and the remaining amino acids were derived from the human factor D. The *Bam*HI and *Eco*RI-digested DNA fragments were inserted at the *Bam*HI and *Eco*RI sites of the Baculovirus transfer vector pVL1393 to give pVL1393-factorD/Hupig and pVL1393-factorD/Pighu.

(2) Expression of factor D mutants and hybrids

The procedures for transfection of the plasmids, generation of recombinant Baculoviruses, and production of the recombinant factor D proteins in insect cells Sf9 were done according to the manufacturer's manual (Baculovirus Expression

Vector System, Pharmingen).

(3) Purification of factor D mutants and hybrids

Factor D mutant and hybrid proteins from the culture supernatants of infected Sf9 cells were purified by affinity chromatography using purified sheep anti-human factor D polyclonal antibodies (The Binding Site Limited, San Diego, CA). Three milliliters of sheep anti-human factor D antibodies (13.2 mg/ml) were equilibrated in a coupling buffer (0.1 M borate and 0.75 M Na_2SO_4, pH 9.0) and coupled with 4 ml of Ultralink Biosupport Medium (Pierce) for 2 hours at room temperature. The beads were washed first with 50 mM diethylamine, pH 11.5 to saturate all the remaining reactive sites and then with a buffer containing 10 mM Tris, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, and 0.02% NaN_3, pH 8.0. The gel was stored in the buffer at 4°C.

Culture supernatant harvested from 100 ml spinner culture of Sf9 cells infected with the various Baculovirus mutants were passed through the sheep anti-factor D affinity column which was pre-equilibrated with PBS to remove the storage buffer. The bound factor D proteins were eluted with 50 mM diethylamine, pH 11.5. The collected fractions were immediately neutralized to pH 7.0 with 1 M Hepes buffer. Residual salts were removed by buffer exchange with PBS by Millipore membrane ultrafiltration (M.W. cut-off: 3,000) (Millipore Corp., Bedford, MA). Protein concentrations were determined by the BCA method (Pierce).

(4) Factor D ELISA

The reactivity of MAb 166-32 with the various factor D mutants and hybrids was tested by ELISA. Different wells of 96-well microtest plates were coated with the proteins (factor D/Hu, factor D/Pig, factor D/Hupig, factor D/Pighu, factor D/VDH, factor D/RH, and factor D/L) by addition of 100 µl of each protein at 0.5 µg/ml in PBS. After overnight incubation at room temperature, the wells were treated with PBSTB (PBST containing 2% BSA) to saturate the remaining binding sites. The wells were then washed with PBST. One hundred microliters of serially diluted Mab 166-32 (1 µg/ml to 0.5 ng/ml) were added to the wells for 1 hour at room temperature. The wells were then washed with PBST. The bound antibody was detected by incubation with diluted HRP-goat anti-mouse IgG (Fc) (Jackson ImmunoResearch) for 1 hour at room temperature. Peroxidase substrate solution was then added for color development as described above. The OD was measured using an ELISA reader at 450 nm.

Fig. 11 shows that MAb 166-32 reacts with factor D/Hu, factor D/Pighu, and factor D/VDA, but not factor D/Pig, factor D/Hupig, factor D/RH, and factor D/L. The ELISA results indicate that amino acid residues Arg156, His159 and Leu168 of human factor D are essential for the binding of MAb 166-32. This is consistent with the fact that MAb 166-32 did not bind factor D/Hupig when the C-terminus portion of human factor D was replaced with that of pig. Amino acid residues Arg156, His159 and Leu168 are located in a so-called "methionine loop" constituted by a disulfide linkage between Cys154 and Cys170 with a methionine residue at position

169 (J.E. Volanakis et al., In: *The Human Complement System in Health and Disease*, J.E. Volanakis and M.M. Franks, eds., Marcel Dekker, 1998, pp. 49-81). Structurally, the "methionine loop" is a member of a rigid type 1 β turn. It is found to be exposed on the surface of the factor D molecule based on the data from X-ray crystallography studies (S.V.L. Narayana et al., *J. Mol. Biol.*, 1994, 235: 695-708). However, the contribution of the "methionine loop" to substrate specificity and catalysis of factor D has never been studied (J.E. Volanakis et al., *Protein Sci.*, 1996; 5: 553-564). The data here have demonstrated for the first time that the "methionine loop" plays an important role in the functional activity of factor D. MAb 166-32 and its Fab, when bound to this region on factor D, can effectively inhibit the catalysis of factor B.

Example 9: Cloning of anti-factor D MAb 166-32 variable region genes and construction and expression of chimeric 166-32 IgG and its Fab

In order to reduce the immunogenicity of MAb 166-32 when used in humans, a chimeric form of MAb 166-32 was made by replacing the mouse constant regions with human constant regions of IgG1. Two forms of chimeric Fab of the antibody were also made by replacing the mouse constant regions with their human counterparts. The cloning of MAb 166-32 variable region genes and the construction and expression of the chimeric 166-32 antibody and its Fab are described below.

(1) Cloning of anti-factor D MAb variable region genes

Total RNA was isolated from the hybridoma cells secreting anti-Factor D MAb

166-32 using RNAzol following the manufacturer's protocol (Biotech, Houston, TX). First strand cDNA was synthesized from the total RNA using oligo dT as the primer. PCR was performed using the immunoglobulin constant (C) region-derived 3' primers and degenerate primer sets derived from the leader peptide or the first framework region of murine V_H or V_K genes as the 5' primers. Although amplified DNA was noted for V_H, no DNA fragment of expected lengths was amplified for V_K. Both V_H and V_K genes were cloned by anchored PCR .

Anchored PCR was carried out as described by Chen and Platsucas (*Scand. J. Immunol.*, 1992; 35: 539-549). For cloning the V_K gene, double-stranded cDNA was prepared using the *Not*I-MAK1 primer (5'-TGCGGCCGCTGTAGGTGCTGTCTTT-3' SEQ ID NO:5). Annealed adaptors AD1 (5'-GGAATTCACTCGTTATTCTCGGA-3' SEQ ID NO:6) and AD2 (5'-TCCGAGAATAACGAGTG-3' SEQ ID NO:7) were ligated to both 5' and 3' termini of the double-stranded cDNA. Adaptors at the 3' ends were removed by *Not*I digestion. The digested product was used as the template in PCR with the AD1 oligonucleotide as the 5' primer and MAK2 (5'-CATTGAAAGCTTTGGGGTAGAAGTTGTTC-3' SEQ ID NO:8) as the 3' primer. DNA fragments of approximately 500 bp were cloned into pUC19. Twelve clones were selected for further analysis. Seven clones were found to contain the CDR3 sequence specific for the Sp2/0 V message, and presumably were derived from the aberrant κ light chain messages of the fusion partner for the 166-32 hybridoma cell line. The *Not*I-MAK1 and MAK2 oligonucleotides were derived from the murine C_K

region, and were 182 and 84 bp, respectively, downstream from the first bp of the Cκ gene. Three clones were analyzed by DNA sequencing, yielding sequences encompassing part of murine Cκ, the complete Vκ, and the leader peptide.

For cloning the V_H gene, double-stranded cDNA was prepared using the *NotI*-MAG1 primer (5'-CGCGGCCGCAGCTGCTCAGAGTGTAGA-3' SEQ ID NO:9). Annealed adaptors AD1 and AD2 were ligated to both 5' and 3' termini of the double-stranded cDNA. Adaptors at the 3' ends were removed by *NotI* digestion. The digested product was used as the template in PCR with the AD1 oligonucleotide and MAG2 (5'-CGGTAAGCTTCACTGGCTCAGGGAAATA-3' SEQ ID NO:10) as primers. DNA fragments of 500 to 600 bp in length were cloned into pUC19. The *NotI*-MAG1 and MAG2 oligonucleotides were derived from the murine Cγ 1 region, and were 180 and 93 bp, respectively, downstream from the first bp of the murine Cγ 1 gene. Three clones were analyzed by DNA sequencing, yielding sequences encompassing part of murine Cγ 1, the complete V_H, and the leader peptide.

(2) Construction of expression vectors for chimeric166-32 IgG and Fab

The V_H and Vκ genes were used as templates in PCR for adding the Kozak sequence to the 5' end and the splice donor to the 3' end. After the sequences were analyzed to confirm the absence of PCR errors, the V_H and Vκ genes were inserted into expression vector cassettes containing human Cγ 1 and C, respectively, to give pSV2neoV_H-huCγ1 and pSV2neoV-huCκ. CsCl gradient-

purified plasmid DNAs of the heavy- and light-chain vectors were used to transfect COS cells by electroporation. After 48 hours, the culture supernatant was tested by ELISA to contain approximately 200 ng/ml of chimeric IgG. The cells were harvested and total RNA was prepared. First strand cDNA was synthesized from the total RNA using oligo dT as the primer. This cDNA was used as the template in PCR to generate the Fd and κ DNA fragments. For the Fd gene, PCR was carried out using (5'-AAGAAGCTTGCCGCCACCATGGATTGGCTGTGGAACT-3' SEQ ID NO:11) as the 5' primer and a CH1-derived 3' primer (5'-CGGGATCCTCAAACTTTCTTGTCCACCTTGG-3' SEQ ID NO:12). DNA sequence was confirmed to contain the complete V_H and the CH1 domain of human IgG1. After digestion with the proper enzymes, the Fd DNA fragments were inserted at the *Hind*III and *Bam*HI restriction sites of the expression vector cassette pSV2dhfr-TUS to give pSV2dhfrFd (Fig. 12A).

For the κ gene, PCR was carried out using (5'-AAGAAAGCTTGCCGCCACCATGTTCTCACTAGCTCT-3' SEQ ID NO:13) as the 5' primer and a Cκ-derived 3' primer (5'-CGGGATCCTTCTCCCTCTAACACTCT-3' SEQ ID NO:14). DNA sequence was confirmed to contain the complete Vκ and human Cκ regions. After digestion with proper restriction enzymes, the κ DNA fragments were inserted at the *Hind* III and *Bam*HI restriction sites of the expression vector cassette pSV2neo-TUS to give pSV2neoκ (Fig. 12B). The expression of both Fd and κ genes are driven by the HCMV-derived enhancer and promoter

elements. Since the Fd gene does not include the cysteine amino acid residue involved in the inter-chain disulfide bond, this recombinant chimeric Fab contains non-covalently linked heavy- and light-chains. This chimeric Fab is designated as cFab.

To obtain recombinant Fab with an inter-heavy and light chain disulfide bond, the above Fd gene was extended to include the coding sequence for additional 9 amino acids (EPKSCDKTH SEQ ID NO:12) from the hinge region of human IgG1. The *Bst*EII-*Bam*HI DNA segment encoding 30 amino acids at the 3' end of the Fd gene was replaced with DNA segments encoding the extended Fd. Sequence of the extended Fd with additional 9 amino acids from the hinge region of human IgG1 was confirmed by DNA sequencing. This Fd/9aa gene was inserted into the expression vector cassette pSV2dhfr-TUS to give pSV2dhfrFd/9aa. This chimeric Fab is designated as cFab/9aa.

(3) Expression of chimeric 166-32 IgG and Fab

To generate cell lines secreting chimeric 166-32 IgG, NS0 cells were transfected with purified plasmid DNAs of pSV2neoV_H-huCγ1 and pSV2neoV-huCκ by electroporation. Transfected cells were selected in the presence of 0.7mg/ml G418. Cells were grown in a 250-ml spinner flask using serum-containing medium.

To generate cell lines secreting chimeric 166-32 Fab, CHO cells were transfected with purified plasmid DNAs of pSV2dhfrFd (or pSV2dhfrFd/9aa) and pSV2neoκ by electroporation. Transfected cells were selected in the presence of

G418 and methotrexate. Selected cell lines were amplified in increasing concentrations of methotrexate. Cells were single-cell subcloned by limiting dilution. High-producing single-cell subcloned cell lines were then grown in 100-ml spinner culture using serum-free medium.

(4) Purification of chimeric 166-32 IgG

Culture supernatant of 100 ml spinner culture was loaded on a 10-ml PROSEP-A column (Bioprocessing, Inc., Princeton, NJ). The column was washed with 10 bed volumes of PBS. The bound antibody was eluted with 50 mM citrate buffer, pH 3.0. Equal volume of 1M Hepes, pH 8.0 was added to the fraction containing the purified antibody to adjust the pH to 7.0. Residual salts were removed by buffer exchange with PBS by Millipore membrane ultrafiltration (M.W. cut-off: 3,000). The protein concentration of the purified antibody was determined by the BCA method (Pierce).

(5) Purification of chimeric 166-32 Fab

Chimeric 166-32 Fab was purified by affinity chromatography using a mouse anti-idiotypic MAb to MAb 166-32. The anti-idiotypic MAb is designated as MAb 172-25-3. It was made by immunizing mice with MAb 166-32 conjugated with keyhole limpet hemocyanin (KLH) and screening for specific MAb 166-32 binding could be competed with human factor D.

The affinity chromatography matrix was prepared by mixing 25 mg MAb 172-25-3 with 5 ml of dry azlactone beads (UltraLink Biosupport Medium, Pierce) in a

coupling buffer (0.1 M borate and 0.75 M Na_2SO_4, pH 9.0) for 2 hours at room temperature. Then the residual reactive sites were blocked with 1 M ethanolamine, pH 9.0 for 2.5 hours at room temperature. The beads were then washed in a buffer containing 10 mM Tris, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100 and 0.02% NaN_3, pH 8.0) and stored then at 4°C.

For purification, 100 ml of supernatant from spinner cultures of CHO cells producing cFab or cFab/9aa were loaded onto the affinity column coupled with MAb 172-25-3. The column was then washed thoroughly with PBS before the bound Fab was eluted 50 mM diethylamine, pH 11.5. Residual salts were removed by buffer exchange as described above. The protein concentration of the purified Fab was determined by the BCA method (Pierce).

(6) SDS-PAGE of chimeric 166-32 IgG, cFab and cFab/9aa

The purified chimeric 166-32 IgG, cFab and cFab/9aa were analyzed for purity and molecular size by SDS-PAGE. The proteins were treated with sample buffers with or without mercaptoethanol. The samples were then run on pre-cast gels (12.5%) (Amersham Pharmacia Biotech, Uppsala, Sweden) together with pre-stained molecular weight standard (low molecular weight range) (BIO-RAD Laboratories, Hercules, CA) using the PhastSystem (Amersham Pharmacia Biotech). The gels were then stained in Coomassie Brilliant Blue solution (BIO-RAD) for 5 minutes and then de-stained in an aqueous solution containing 40% methanol and 10% acetic acid.

The results of an SDS-PAGE of cFab, cFab/9aa and chimeric IgG treated under both non-reducing and reducing condition, showed chimeric IgG has a protein band of 150 kD, and two protein bands of heavy (HC) and light (LC) chains of approximately 50 kD and 29 kD, respectively. As expected, cFab/9aa had only 1 protein band of about 40 kD under non-reducing condition, indicating that the heavy and light chains are linked by an inter-chain disulfide bond. On the other hand, cFab showed two protein bands under non-reducing condition, indicating that the heavy and light chains are not linked by an inter-chain disulfide bond.

(7) Determination of the activities of chimeric 166-32 IgG, cFab and cFab/9aa

The activities of chimeric 166-32 IgG, cFab and cFab/9aa were determined by using the alternative complement hemolytic assay described above. Fig. 13 shows that the murine and chimeric forms of MAb 166-32 have identical potency in inhibiting factor D. Fig. 14 shows that cFab and cFab/9aa have almost identical potency in inhibiting factor D. Most importantly, the potency of the two forms of chimeric Fab is identical to that of the chimeric IgG, although there are two binding sites per IgG molecule. Together, the results demonstrate that chimeric IgG, cFab and cFab/9aa retain the potency of the parental murine MAb 166-32.

Example 10: Protection of complement-mediated tissue damage by Mab 166-32 in an *ex vivo* model of rabbit hearts perfused with human plasma

Activation of the complement system contributes to hyperacute rejection of xenografts. It may occur as a result of binding of complement fixing antibodies, the

direct activation of complement *via* the alternative pathway on foreign cell surfaces, and/or the failure of complement regulation by the foreign organ (J.L. Platt et al., *Transplantation*, 1991; 52: 937-947). Depending on the particular species-species interaction, complement activation through either the classical or alternative pathway predominates, though in some cases both pathways may be operative (T. Takahashi et al., *Immunol. Res.*, 1997; 16: 273-297). Previous studies have shown that hyperacute rejection can occur in the absence of anti-donor antibodies *via* activation of the alternative pathway (P.S. Johnston et al., *Transplant. Proc.*, 1991; 23: 877-879).

To demonstrate the importance of the alternative complement pathway in tissue damage, the anti-factor D MAb 166-32 was tested using an *ex vivo* model in which isolated rabbit hearts were perfused with diluted human plasma. This model was previously shown to cause damage to the rabbit myocardium due to the activation of the alternative complement pathway (M.R. Gralinski et al., *Immunopharmacology*, 1996: 34: 79-88).

(1) Langendorff perfused rabbit hearts:

Male, New Zealand White rabbits (2.2-2.4 kg) were euthanized by cervical dislocation. The hearts were removed rapidly and attached to a cannula for perfusion through the aorta. The perfusion medium consisted of a recirculating volume (250 ml) modified Krebs-Henseleit (K-H) buffer (pH 7.4, 37°C) delivered at a constant rate of 20-25 ml/min. The composition of the buffer medium in millimoles

per liter was as follows: NaCl, 117; KCl, 4.0; $CaCl_2$. H_2O, 2.4; $MgCl_2.6H_2O$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.1; glucose, 5.0; monosodium L-glutamate, 5.0; sodium pyruvate, 2.0; and BSA, 0.25% (w/v). The K-H buffer passed through a gas porous "lung" consisting of Silastic™ Laboratory Grade Tubing (Dow Corning, Midland, MI), 55.49 meters in length, with an inner diameter of 1.47 mm and an outer diameter of 1.96 mm. The membranous "lung" was exposed continuously to a mixture of 95% O_2/5% CO_2 to obtain an oxygen partial pressure within the perfusion medium equal to 500 mm Hg. The hearts were paced throughout the protocol *via* electrodes attached to the right atrium. Pacing stimuli (3 Hz, 4 msec duration) were delivered from a laboratory square wave generator (Grass SD-5, Quincy, MA). The pulmonary artery was cannulated with polyethylene tubing to facilitate collection of the pulmonary artery effluent, representing the coronary venous return. The superior and inferior vena cava and the pulmonary veins were ligated to prevent exit of the perfusate from the severed vessels. A left ventricular drain, thermistor probe, and latex balloon were inserted *via* the left atrium and positioned in the left ventricle. The fluid-filled latex balloon was connected with rigid tubing to a pressure transducer to permit for measurement of left ventricular systolic and end-diastolic pressures. The left ventricular developed pressure is defined as the difference between the left ventricular systolic and end-diastolic pressures. The intraventricular balloon was expanded with distilled water to achieve an initial baseline left ventricular end-diastolic pressure of 5 mm Hg. Coronary perfusion pressure was measured with a

pressure transducer connected to a side-arm of the aortic cannula. All hemodynamic variables were monitored continuously using a multichannel recorder (Grass Polygraph 79D, Quincy, MA). The isolated hearts were maintained at 37°C throughout the experimental period by enclosing them in a temperature-regulated double-lumen glass chamber and passing the perfusion medium through a heated reservoir and delivery system.

(2) Antibody treatments:

Two treatment groups were used to determine the ability of anti-factor D MAb 166-32 to inhibit the effects of complement activation in isolated rabbit hearts perfused with human plasma. Group 1: Isotype-matched negative group, consisted of hearts perfused with 4% human plasma in the presence of 0.3 µg/ml of MAb G3-519 (n=6) specific to HIV-1 gp120. Group 2: Treatment group, consisted of hearts perfused with 4% human plasma in the presence of 0.3 µg/ml of MAb 166-32 (n=6). The human plasma was separated from freshly collected whole blood and stored at –80°C until use. This percentage of human plasma was chosen because it severely impairs myocardial function over a reasonable length of time and allows one to assess the efficacy of a treatment regimen. Higher concentrations of human plasma in this system cause the heart to rapidly develop contracture, making it difficult to analyze the effects of a drug at a low concentration. Preliminary studies had determined that 0.3 µg/ml was the minimal effective concentration that could protect the isolated heart from the effects of complement activation. All hearts

underwent 10-15 minutes of equilibration in the Langendorff apparatus before addition of either antibody to the perfusion medium. Ten minutes after the addition of antibody, 4 % human plasma was added to the perfusion medium (250 ml, recirculating). Hemodynamic variables, including coronary perfusion pressure (CPP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and left ventricular developed pressure (LVDP) were recorded before the addition of antibody (baseline), before the addition of 4% human plasma, and every 10 minutes thereafter, for 60 minutes.

MAb 166-32 (0.3 µg/ml) attenuated the increase in coronary perfusion pressure (CPP) when compared to hearts treated with MAb G3-519 (0.3 µg/ml) when exposed to 4% human plasma. A rise in CPP indicates coronary vascular resistance which is often associated with myocardial tissue damage. Isolated rabbit hearts perfused with MAb 166-32 maintained left ventricular end-diastolic pressure (LVEDP), in marked contrast to the results obtained with MAb G3-519 (Fig. 15). The latter group of hearts developed a progressive increase in LVEDP after exposure to 4% human plasma, indicating contracture or a failure of the ventricle to relax during diastole (Fig. 15). MAb 166-32 also attenuated the decrease in left ventricular developed pressure (LVDP) compared to the hearts treated with MAb G3-519 after exposure to diluted human plasma. (Fig. 15).

Fig. 16 depicts representative recordings of cardiac function obtained before and after 10, 30 and 60 minutes of perfusion in the presence of 4% human plasma.

The progressive increase in the LVEDP and the decrease in the LVDP of a MAb G3-519 treated rabbit heart is obvious after 10 minutes with progressive deterioration of ventricular function over the subsequent 50 minutes. On the other hand, preservation of ventricular function of a heart treated with MAb 166-32 is evident over the period of 60 minutes.

Taken together, the hemodynamic data indicate that anti-factor D MAb 166-32 protects isolated rabbit hearts from human complement-mediated injury as manifested by an overall maintenance of myocardial function after challenge with human plasma.

(3) Complement Bb ELISA:

Factor D catalyzes the cleavage of bound factor B, yielding the Ba and Bb fragments. The concentration of Bb present serves as an index of factor D activity. The concentrations of activated component Bb in the lymphatic fluid collected from the isolated rabbit hearts were measured using a commercially available ELISA kit (Quidel). The assays made use of a MAb directed against human complement Bb to measure the activation of human complement system during perfusion of rabbit hearts in the presence of human plasma. Lymphatic effluent from the severed lymphatic vessels was collected from the apex of the heart, snap-frozen in liquid nitrogen, and stored at –70°C until assayed. The flow rate of the lymphatic effluent was recorded and accounted for in order to normalize the Bb concentration.

Ten minutes after perfusion with 4% human plasma and at every time point thereafter, a significantly ($p < 0.05$) lower concentration of Bb was present in lymphatic effluents from hearts treated with MAb 166-32 as compared to hearts treated with MAb G3-519 (Fig. 17). The decrease in the production of the complement activation product Bb in the MAb 166-32 treated rabbit hearts confirms the inhibitory activity of the antibody on factor D.

(4) Immunohistochemical localization of C5b-9 deposition:

At the completion of the protocol, hearts were removed from the Langendorff apparatus, cut into transverse sections, and frozen in liquid nitrogen. The apex and atrial tissues were discarded. Sections were embedded in O.C.T. compound embedding medium (Miles, Inc., Ekhart, IN), cut to 3m, and placed on poly-L-lysine coated slides. After rinsing with phosphate-buffered saline (PBS), sections were incubated with 4% paraformaldehyde in PBS at room temperature. Heart sections were rinsed with PBS and incubated with 1% BSA for 15 minutes to minimize non-specific staining. After rinsing with PBS, sections were incubated with a murine anti-human C5b-9 MAb (Quidel) at a 1:1,000 dilution at room temperature for 1 hour. Sections were rinsed with PBS again and then incubated at room temperature for 1 hour with a goat anti-murine FITC conjugated antibody (Sigma) at a 1:320 dilution. After a final rinse with PBS, sections were mounted with Fluoromount-G (Electron Microscopy Sciences, Fort Washington, PA) and protected with a coverslip. Controls included sections in which the primary antibody was omitted and sections

in which an isotype-matched murine antibody IgG1 (Sigma) was substituted for the anti-C5b-9 MAb.

Heart sections from MAb 166-32 and MAb G3-519 treated hearts were examined for human MAC (or C5b-9) deposition by immunofluorescence staining. MAb 166-32 treated hearts exhibited a reduction in MAC deposition as compared to MAb G3-519 treated hearts.

In all, the data from the *ex vivo* studies of rabbit hearts demonstrate the efficacy of MAb 166-32 in preventing cardiac tissue injury as a result of the inhibition of the alternative complement pathway. Inhibition of complement activation has been shown to prolong the survival of xenografts (S.C. Makrides, *Pharmacological Rev.*, 1998, 50: 59-87). Therefore, MAb 166-32 could potentially be used as a therapeutic agent to protect xenografts from destruction by human plasma.

Example 11: Inhibitory effects of MAb 166-32 on complement activation and inflammatory reactions in an extracorporeal circulation model of cardiopulmonary bypass

Patients undergoing cardiopulmonary bypass (CPB) frequently manifest a generalized systemic inflammatory response syndrome. Clinically, these reactions are reflected in postoperative leukocytosis, fever, and extravascular fluid accumulation which may lead to prolonged recovery and occasionally with serious organ dysfunction (J.K. Kirklin et al., *J. Thorac. Cardiovasc. Surg.*, 1983; 86: 845-857; L. Nilsson et al., *Scand. J. Thorac. Cardiovasc. Surg.*, 1988; 22: 51-53; P.W. Weerwind et al., *J. Thorac. Cardiovasc. Surg.*, 1995; 110: 1633-1641). The

inflammatory responses consist of humoral and cellular changes that contribute to both tissue injury and impaired hemostasis. Complement activation has been implicated as the important cause of the systemic inflammatory reaction (P. Haslam et al., *Anaesthesia*, 1980; 25: 22-26; A. Salama et al., *N. Eng. J. Med.*, 1980; 318: 408-414; J. Steinberg et al., *J. Thorac. Cardiovasc. Surg.*, 1993; 106: 1008-1016). Complement activation is attributed to the interaction between the blood and the surface of the extracorporeal circuit constituting CPB machines (D. Royston, *J. Cardiothorac. Vasc. Anesth.*, 1997; 11: 341-354). Primary inflammatory substances are generated after activation of the complement system, including the anaphylatoxins C3a and C5a, the opsonin C3b, and the membrane attack complex C5b-9. C5a has been shown to upregulate CD11b (integrin) and CD18 (integrin) of MAC-1 complex in polymorphonuclear cells PMN (comprising mainly neutrophils) *in vitro* (M.P. Fletcher et al., *Am. J. Physiol.*, 1993; 265: H1750-H1761) and to induce lysosomal enzyme release by PMN. C5b-9 can induce the expression of P-selectin (CD62P) on platelets (T. Wiedmer et al., Blood, 1991; 78: 2880-2886), and both C5a and C5b-9 induce surface expression of P-selectin on endothelial cells (K.E. Foreman et al., *J. Clin. Invest.*,1994; 94: 1147-1155). C3a and C5a stimulate chemotaxis of human mast cells (K. Hartmann et al., *Blood*, 1997; 89: 2868-2870) and trigger the release of histamine (Y. Kubota, *J. Dermatol.*, 1992; 19: 19-26) which induces vascular permeability (T.J. Williams, *Agents Actions*, 1983; 13: 451-455).

In vitro recirculation of whole blood in an extracorporeal bypass circuit has been used extensively as a model to simulate leukocytes (J. Kappelamyer et al., *Circ. Res.*, 1993; 72: 1075-1081; N. Moat et al., *Ann. Thorac. Surg.*, 1993; 56: 1509-1514; C.S. Rinder et al., *J. Clin. Invest.*, 1995: 96: 1564-1572) and platelets (V.L. Jr. Hennesy et al., *Am. J. Physiol.*, 1977; 2132: H622-H628; Y. Wachtfogel et al., *J. Lab. Clin. Med.*, 1985; 105: 601-607; C.S. Rinder et al., *ibid*) changes and complement activation (P.G. Loubser, *Perfusion*, 1987; 2: 219-222; C.S. Rinder et al., *ibid*; S.T. Baksaas et al., *Perfusion*, 1998; 13: 429-436) in CPB. The effectiveness of the anti-factor D MAb 166-32 to inhibit the cellular and complement activation in human whole blood was studied using this extracorporeal circulation model for CPB.

(1) Extracorporeal circuit preparation:

Extracorporeal circuits were assembled using a hollow-fiber pediatric membrane oxygenator with an integrated heat exchanger module (D 901 LILLPUT 1; DIDECO, Mirandola (MO), Italy), a pediatric venous reservoir with an integrated cardiotomy filter (D 752 Venomidicard; DIDECO), a perfusion tubing set (Sorin Biomedical, Inc., Irvine, CA) and a multiflow roller pump (Stockert Instruments GmbH, Munich, Germany). Oxygenator and circuitry were primed with Plasma-Lyte A solution (Baxter Healthcare Corp., Deerfield, IL). The prime was warmed to 32°C with a cooler-heater (Sarns; 3M Health Care, Ann Arbor, MI) and circulated at 500 ml/min, while the sweep gas flow was maintained at 0.25 liters per min using 100% oxygen.

The sweep gas was changed to a mixture of oxygen (95%) and carbon dioxide (5%) after the blood was added to the circuit. The pH, PCO_2, PO_2, and perfusate temperature were continuously monitored throughout the recirculation period. Sodium bicarbonate was added as required to maintain pH in the range of 7.25-7.40.

(2) Extracorporeal circuit operation and sampling:

450 ml of blood were drawn over 5-10 minutes from healthy volunteers on no medications into a transfer pack (Haemo-Pak; Chartermed, Inc., Lakewood, NJ) containing porcine heparin (5 units/ml, final concentration; Elkins-Sinn, Cherry Hill, NJ) and the anti-factor D MAb 166-32 or the isotype-matched negative control MAb G3-519 (18 µg/ml final concentration). This concentration of antibody is equivalent to about 1.5 times the molar concentration of factor D in the blood. Prior to the addition of the blood to the extracorporeal circuit, a blood sample was taken from the transfer pack as the "pre-circuit" sample, designated the "–10 minute sample". The blood was then added to the reservoir via the prime port. Prime fluid was simultaneously withdrawn distal to the oxygenator outlet to yield a final circuit volume of 600 ml and a final hematocrit of 25-28%. Blood was circulated with prime, and complete mixing was accomplished within 3 minutes; a baseline sample was drawn and designated as time 0. To mimic usual procedures of surgical operation under hypothermia, the circuit was then cooled to 27°C for 70 minutes after which

it was rewarmed to 37°C for another 50 minutes (for a total of 120 minutes of recirculation).

Blood samples were also drawn at 10, 25, 40, 55, 70, 80 and 120 minutes during the recirculation. Plasma samples were prepared by immediate centrifugation at 2,000 x g at 4°C. Aliquots for the alternative pathway hemolytic assays and neutrophil-specific myeloperoxidase assays were snap-frozen on dry ice and then stored at –80°C. Aliquots for measurement of complements C3a, C4d, sC5b-9, and Bb by ELISA were immediately mixed with equal volume of a Specimen Stabilizing Medium (Quidel), snap-frozen on dry ice, and then stored at –80°C. Samples of whole blood were also collected for immunostaining of the activation cell surface markers CD11b and CD62P on neutrophils and platelets, respectively. To prevent subsequent complement activation of the whole blood samples during the staining procedure, 10 µl of 1 M EDTA were added to every ml of whole blood to give a final concentration of 10 mM.

(3) Alternative pathway hemolytic assays:

The alternative complement activity in the plasma samples at different time points from the MAb 166-32 treated and the MAb G3-519 treated circuits were tested using rabbit red blood cells as described above. Fifty microliters of each sample (20%) were mixed with 50 µl of GVB/Mg-EGTA buffer before addition of 30 µl of rabbit red blood cells (1.7 x 10^8 cells/ml). After incubation at 37°C for 30

minutes, the supernatants were collected and OD read at 405 nm using an ELISA plate reader.

Fig. 18 shows that the alternative complement activity in the MAb 166-32 treated circuit was completely inhibited by the antibody, whereas MAb G3-519 had no effect on the complement activity when used in the corresponding circuit. The results indicate MAb 166-32 is a potent inhibitor of the alternative complement pathway. Even at a molar ratio of only 1.5: 1 (MAb : factor D), MAb 166-32 can completely inhibit the alternative complement activity.

(4) Assays of complement activation products:

In addition to the hemolytic assays described above, the plasma samples from the two extracorporeal circuits were are tested for the levels of C3a, sC5b-9, Bb, and C4d. These substances were quantitated using commercially available ELISA kits (Quidel) according to the manufacturerer's manuals. Like C5a, sC5b-9 is an alternative marker for C5 convertase activity in the complement cascade. Both C5a and sC5b-9 are produced as a result of cleavage of C5 by C5 convertase. Complement Bb is a specific marker for the activation of the alternative complement pathway, whereas C4d a specific marker for the activation of the classical pathway.

Figs. 19 and 20 show that MAb 166-32 inhibited effectively the production of C3a and sC5b-9 respectively, whereas the isotype-matched negative control MAb G3-519 did not. The specificity and potency of MAb 166-32 are further elucidated in Figs. 21 and 22. The production of Bb by the alternative complement pathway

was completely inhibited by MAb 166-32, whereas the levels of Bb in the G3-519 circuit increase over time during the recirculation. Interestingly, the levels of C4d in both MAb 166-32 and G3-519 circuit did not vary significantly over time. The latter results on Bb and C4d levels strongly indicate that the complement activation in extracorporeal circulation is mediated mainly *via* the alternative pathway.

In sum, the results indicate that MAb 166-32 is a potent inhibitor of the alternative complement pathway. Inhibition of factor D can abolish the complement activation in the subsequent steps of the cascade as manifested by the reduction in C3a and sC5b-9 formation.

(5) Assays for the activation of neutrophils and platelets:

The activation of neutrophils and platelets were quantitated by measuring the levels of the cell-surface expression of CD11b and CD62P on neutrophils and platelets, respectively. For CD11b labeling of neutrophils, 100 µl of whole blood collected from the circuits were immediately incubated with 20 µl of phycoerythrin (PE)-anti-CD11b antibody (clone D12, Becton Dickinson, San Jose, CA) for 10 minutes at room temperature in a microcentrifuge tube. Then 1.4 ml of FACS Lysing Solution (Becton Dickinson) was added for 10 minutes at room temperature to lyse red blood cells and to fix leukocytes. The microcentrifuge tubes were centrifuged at 300 x g for 5 minutes. The supernatant was aspirated and the cells resuspended in PBS for washing. The microcentrifuge tubes were spun again, the supernatant aspirated, and the cells finally resuspended in 0.5 ml of 1% paraformaldehyde

overnight prior to analysis using an EPIC-XL flow cytometer (Coulter Corp., Miami, FL). For double labeling to concomitantly identify the neutrophil population, 5 µl of fluorescein isothiocyanate (FITC)-anti-CD15 antibody (clone MMA, Becton Dickinson) were added for incubation together with PE-anti-CD11b antibody.

For CD62P labeling of platelets, 40 µl of whole blood collected from the circuits were immediately incubated with 20 µl of PE-anti-CD62P antibody (clone AC1.2, Becton Dickinson) for 10 minutes at room temperature in a microcentrifuge tube. Then the mixture was treated with FACS Lysing Solution as described above. The microcentrifuge tubes were centrifuged at 2,000 x g for 5 minutes. The platelets were washed in PBS, fixed in 1 % paraformaldehyde and then analyzed as described above. For double labeling to concomitantly identify the platelet population, 5 µl of FITC-anti-CD42a antibody were added for incubation together with PE-anti-CD62P antibody.

For flow cytometric measurement, the PMN (containing mainly neutrophils) and platelet populations were identified by live-gating based on forward- versus side-scatter parameters and specific staining with FITC-anti-CD15 antibody and FITC-anti-CD42a antibody, respectively. The background staining was gated using isotype-matched labeled antibodies. The intensity of expression of CD11b and CD62P was represented by mean fluorescence intensity (MFI).

Fig. 23 shows that neutrophils from the MAb 166-32 treated extracorporeal circuit showed substantially lower expression of CD11b as compared to those from

the MAb G3-519 treated circuit. These data together with the others above indicate that inhibition of the alternative complement activation by MAb 166-32 can prevent activation of neutrophils.

Similarly, Fig. 24 shows that platelets from the MAb 166-32 treated extracorporeal circuit showed substantially lower expression of CD62P as compared to those from the MAb G3-519 treated circuit. Again these data together with those above indicate that inhibition of the alternative complement activation by MAb 166-32 can prevent activation of platelets.

(6) Assay of neutrophil-specific myeloperoxidase (MPO)

The degree of activation of neutrophils was also measured using a commercial ELISA kit (R & D Systems, Inc., Minneapolis, MN) to quantitate the amount of neutrophil-specific myeloperoxidase (MPO) in the plasma samples from the extracorporeal circuits. MPO is stored in primary granules (azurophilic) of neutrophils. It is released when neutrophils undergo de-granulation during activation. Therefore MPO is a soluble marker for neutrophil activation. The assays were performed according to the manufacturer's manual. Briefly, samples were incubated in the cells of a microplate, which have been coated with a first MAb to MPO. The MPO-MAb complex is labeled with a biotin-linked polyclonal antibody prepared from goat MPO-antisera. The final step of the assay is based on a biotin-avidin coupling in which avidin has been covalently linked to alkaline phosphatase.

The amount of MPO in each sample is enzymatically measured upon addition of the substrate 4-nitrophenyl-phosphate (pNPP), by reading OD at 405 nm.

Fig. 25 shows that the levels of MPO in the MAb 166-32 treated circuit were substantially lower than those in the MAb G3-519 circuit. The results corroborate with those on the expression of CD11b in the immunofluorometric studies described above.

Taken together, the data on complement, neutrophils and platelets support the notion that effective inhibition of the alternative complement activation in extracorporeal circulation by the anti-factor D MAb 166-32 can abolish the formation of inflammatory substances C3a, C5a and sC5b-9 and thus reduce the activation of neutrophils and platelets. It is anticipated that MAb 166-32, as well as its fragments, homologues, analogues and small molecule counterparts thereof, will be effective in preventing or reducing clinical inflammatory reactions caused by CPB.

Example 12: Study of the effects of MAb 166-32 in a dog model of ischemia and reperfusion injury

A study was designed to examine whether MAb 166-32 would protect myocardial tissues from injury due to ischemia and reperfusion in dogs, although it was recognized at the outset that dog might not be a desirable animal model to study this indication for MAb 166-32. The ability of MAb 166-32 to neutralize dog factor D in hemolytic assays was at least 10 times less effective as compared to human factor D (see Example 7). Because of the limited amount of MAb 166-32

available at the time, MAb 166-32 was administered into the heart *via* the intracoronary blood vessel. It was hoped that the antibody would build up a concentration of at least 60 µg/ml in the coronary blood in order to inhibit completely dog factor D in the heart. The dosage was calculated to be 3.15 mg/kg/infusion for 6 infusions. MAb G3-519 was used as the isotype-matched control in the study.

Briefly, purpose-bred hound dogs were anaesthetized. A left thoracotomy was performed at the fourth intercostal space to expose the heart. The proximal left circumflex coronary artery was isolated and ligated for 90 minutes for induction of ischemia that was followed by 6 hours of reperfusion. The antibody was given 6 times at 30 minutes before ischemia, 10 minutes before reperfusion, and then 75, 150, 225, 300 minutes during reperfusion. Radioactive microspheres were injected at different time points to measure regional blood flow. At the end of the experiments, hearts were perfused with Evans blue dye and triphenyltetrazolium for measurement of area at risk and infarct size, respectively. Coronary lymph and whole blood from the jugular vein were collected before ischemia and at the end of the experiment. These samples were used to measure the concentration of the injected antibodies and alternative pathway hemolytic activity.

The results show that the highest achievable concentration of MAb 166-32 in the coronary lymph was about 30 µg/ml, which is well below the concentration required for complete inhibition of dog factor D in the coronary circulation. The antibody was also detected in the systemic circulation, suggesting that the injected

antibody dissipated outside of the heart. The data from the hemolytic assays show that alternative complement activity was not reduced; as is consistent with the fact that the concentration of the antibody was low. Therefore, it is not possible to draw a conclusion on the effect of MAb 166-32 in reperfusion from these experiments in dogs.

The foregoing description, terms, expressions and examples are exemplary only and not limiting. The invention includes all equivalents of the foregoing embodiments, both known and unknown. The invention is limited only by the claims which follow and not by any statement in any other portion of this document or in any other source.

WHAT IS CLAIMED IS:

1. An inhibitor of complement activation which binds factor D and at a molar ratio of about 1.5:1 (inhibitor: factor D) can inhibit complement activation.
2. An inhibitor of complement activation which binds to factor D and at a molar ratio of less than 80:1 (inhibitor: factor D) can inhibit complement activation.
3. An inhibitor of complement activation which binds to factor D and inhibits complement activation at a molar ratio of inhibitor to factor D such that it is suitable for therapeutic use.
4. The inhibitor of claims 1, 2 or 3 wherein the inhibition of complement activation is determined *in vitro*.
5. The inhibitor of claim 4 wherein the inhibition of complement activation is determined *in vitro* by an extracorporeal assay.
6. An Inhibitor of complement activation which binds to a region of human factor D between (and including) amino acid residue numbers Cys154 and Cys170.
7. The inhibitor of claim 6 which does not bind to human factor D if amino acid residues Arg156, His159 and Leu168 are absent.
8. The inhibitor of any of claims 1 to 3, 6 or 7 which is an antibody or a homologue, analogue or fragment thereof, a peptide, an oligonucleotide, a peptidomimetic or an organic compound.

9. The inhibitor of claim 8 wherein the antibody fragments are Fab, $(Fab')_2$, Fv or single chain Fv.

10. The inhibitor of claim 9 wherein the antibody is a chimeric, deimmunized, humanized, deimmunised or human antibody.

11. The monoclonal antibody 166-32.

12. The hybridoma producing the monoclonal antibody 166-32, deposited at the American Type Culture Collection under Accession number HB-12476.

13. A monoclonal antibody or a fragment, analogue or homologue thereof, or a peptide, oligonucleotide, peptidomimetic or an organic compound which binds to the same epitope on factor D as the antibody 166-32.

14. The fragment of claim 13 which is an are Fab, $(Fab')_2$, Fv or single chain Fv.

15. The chimeric form, having a mouse variable region and a human constant region, of the Fab fragment of claim 14.

16. A cell line producing the monoclonal antibody or fragment thereof which binds to the same epitope on factor D as the antibody 166-32.

17. A cell line producing the fragment of claim 14.

18. A cell line producing the chimeric Fab fragment of claim 15.

19. The chimeric form, having a mouse variable region and a human constant region, of the monoclonal antibody 166-32.

20. The chimeric form, having a mouse variable region and a human constant region, of the Fab fragment of the monoclonal antibody 166-32.

21. A method of treating diseases or conditions that are mediated by excessive or uncontrolled activation of the complement system comprising administering, *in vivo* or *ex vivo*, an inhibitor according to any claims 1 to 3, 6 and 7.

22. A method of treating diseases or conditions that are mediated by excessive or uncontrolled activation of the complement system comprising administering, *in vivo* or *ex vivo*, an inhibitor according to claim 8.

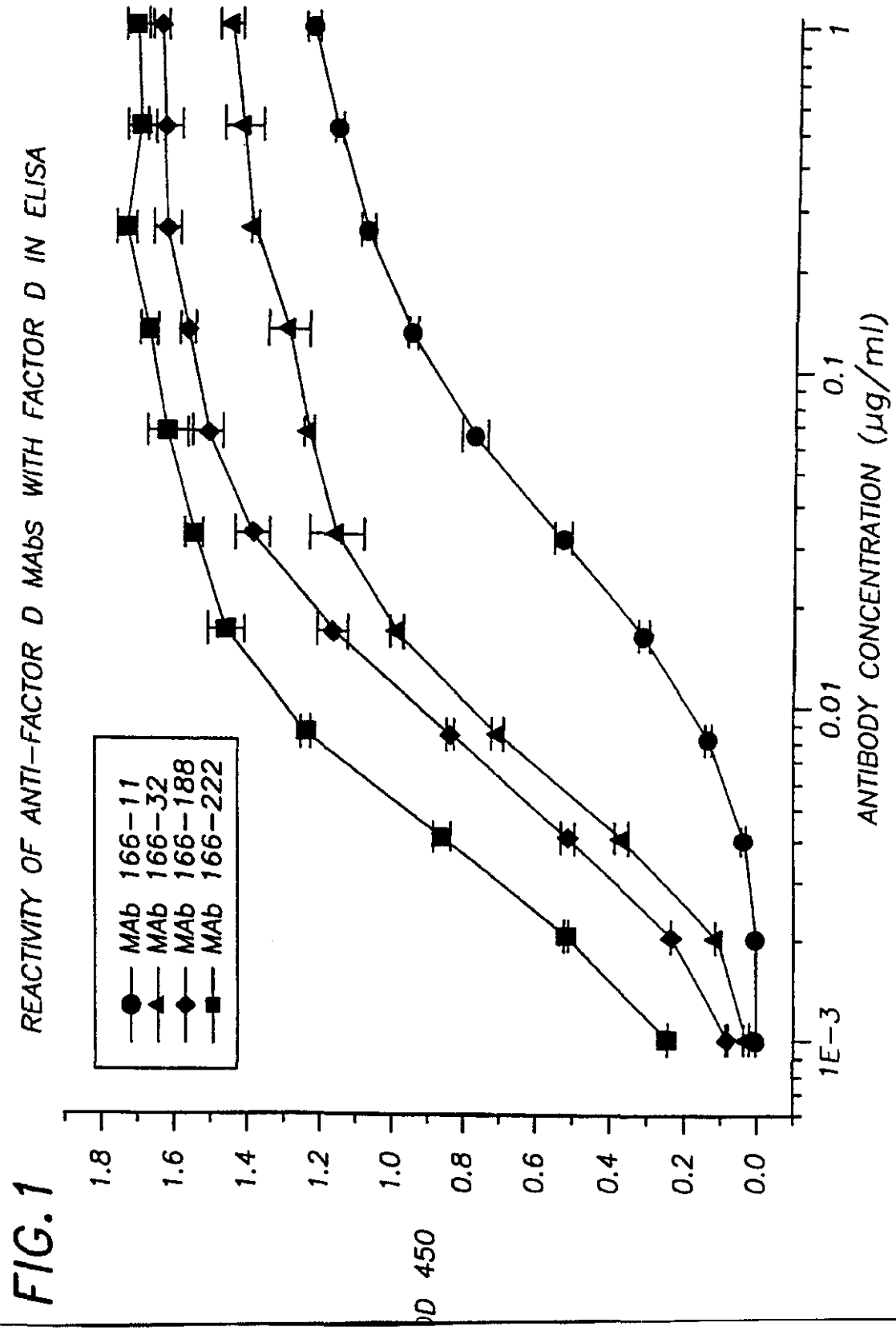
23. A method of treating diseases or conditions that are mediated by excessive or uncontrolled activation of the complement system comprising administering, *in vivo* or *ex vivo*, an inhibitor according to any claims 9 to 11, 13 to 15 or 19 and 20.

24. A method of treating complement-mediated conditions associated with cardiopulmonary bypass comprising administering, *in vivo* or *ex vivo*, an inhibitor according to any claims 1 to 3, 6 and 7.

25. A method of treating complement-mediated conditions associated with cardiopulmonary bypass comprising administering, *in vivo* or *ex vivo*, an inhibitor according to claim 8.

26. A method of treating complement-mediated conditions associated with cardiopulmonary bypass comprising administering, *in vivo* or *ex vivo*, an inhibitor according to any claims 9 to 11, 13 to 15 or 19 and 20.

FIG. 1


REACTIVITY OF ANTI-FACTOR D MAbs WITH FACTOR D IN ELISA
MAb 166-11
MAb 166-32
MAb 166-188
MAb 166-222
OD 450
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
1E-3
0.01
0.1
1
ANTIBODY CONCENTRATION (µg/ml)

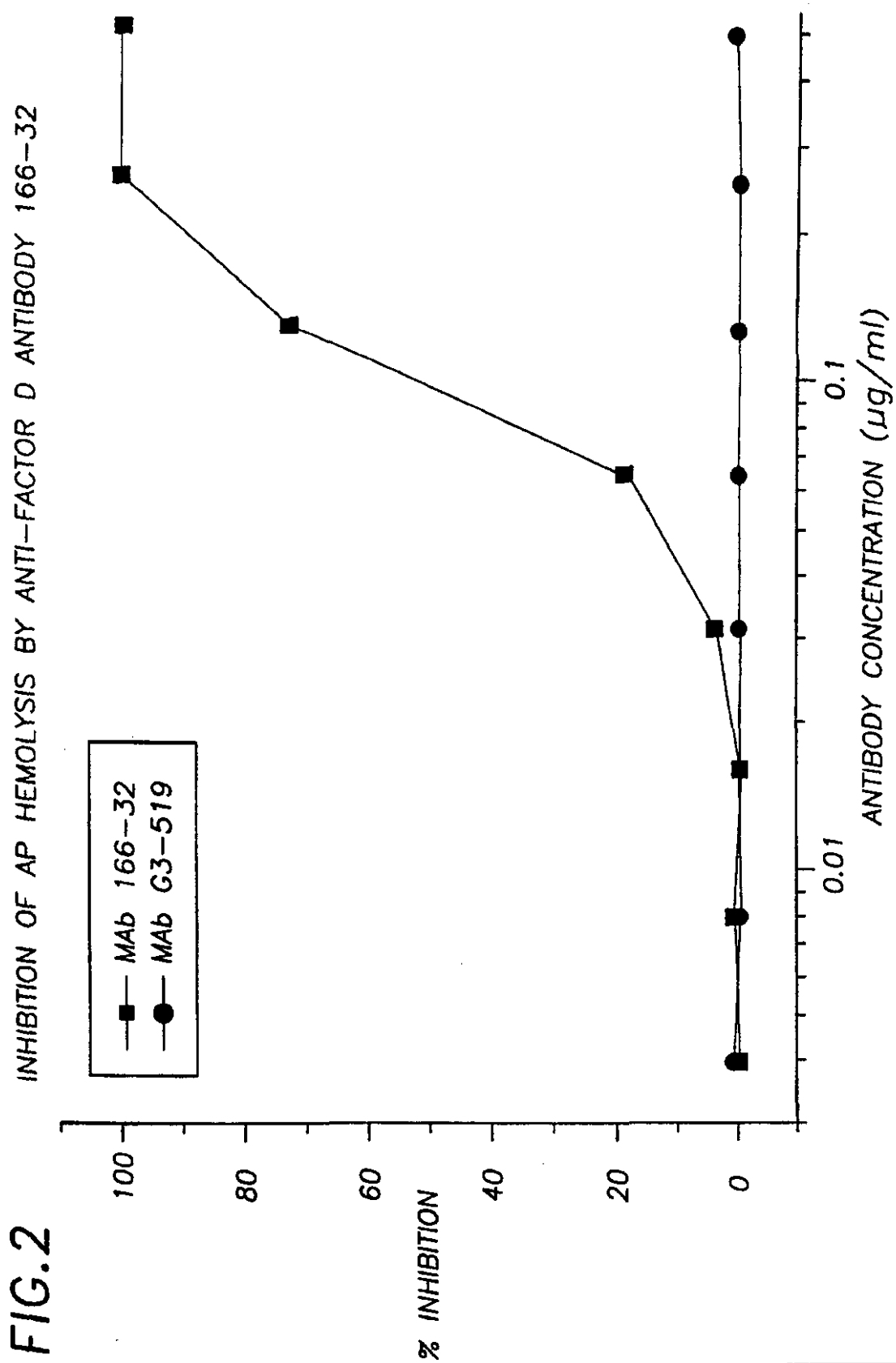

INHIBITION OF AP HEMOLYSIS BY ANTI-FACTOR D ANTIBODY 166-32
MAb 166-32
MAb G3-519
% INHIBITION
100
80
60
40
20
0
0.01
0.1
ANTIBODY CONCENTRATION (µg/ml)

FIG. 2

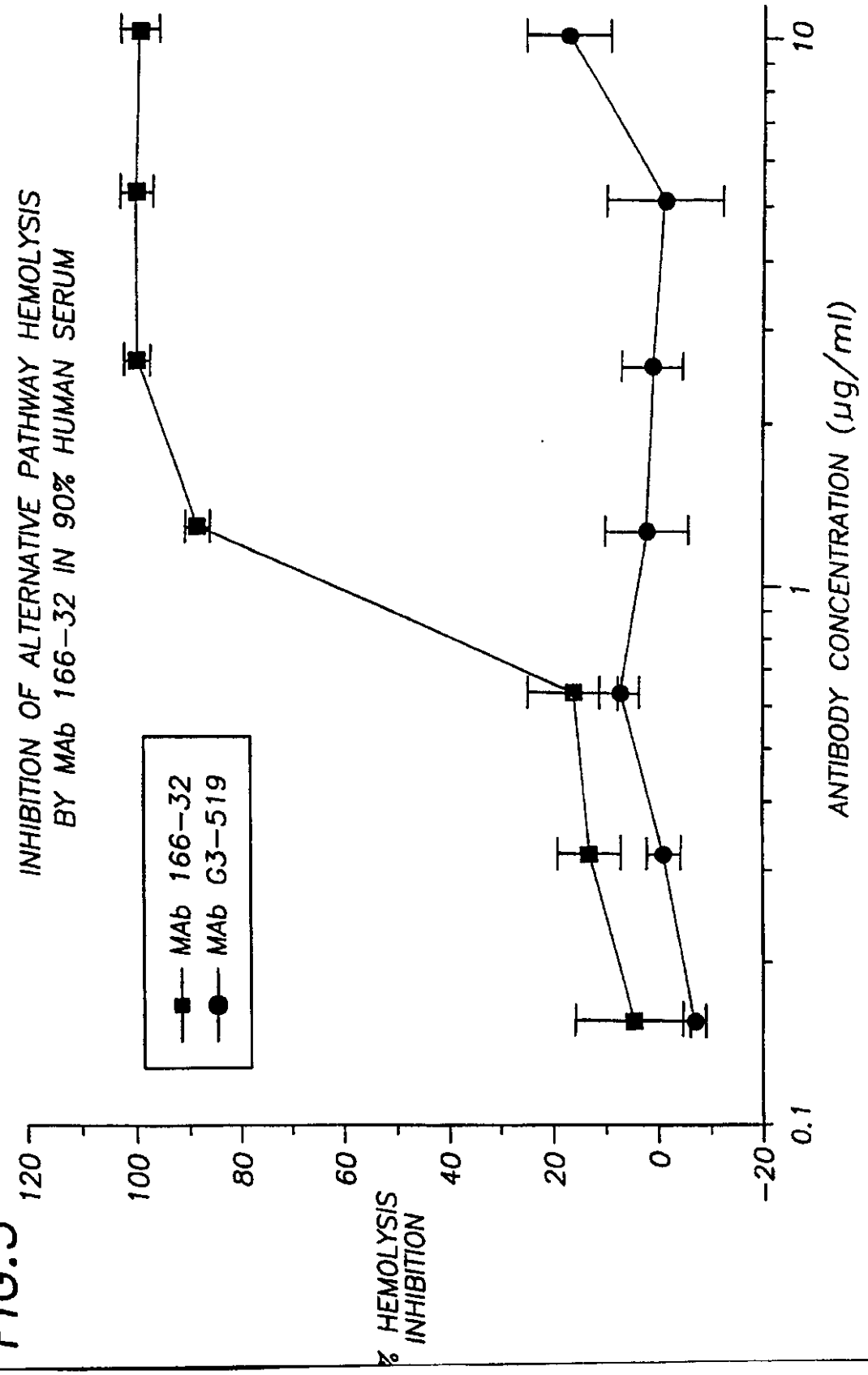

INHIBITION OF ALTERNATIVE PATHWAY HEMOLYSIS
BY MAb 166-32 IN 90% HUMAN SERUM
MAb 166-32
MAb G3-519
% HEMOLYSIS INHIBITION
120
100
80
60
40
20
0
-20
0.1
1
10
ANTIBODY CONCENTRATION (µg/ml)

FIG. 3

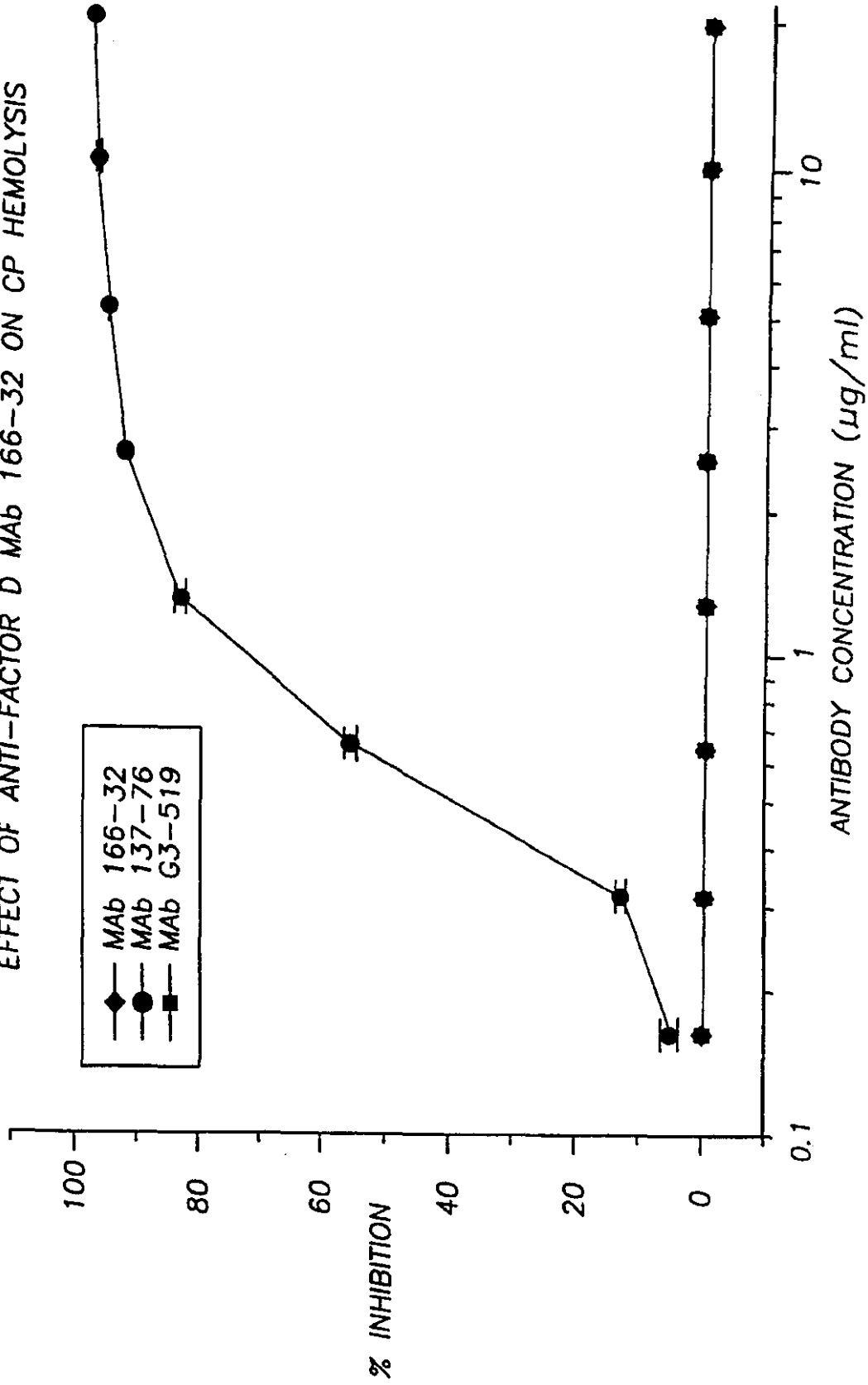

EFFECT OF ANTI-FACTOR D MAb 166-32 ON CP HEMOLYSIS
MAb 166-32
MAb 137-76
MAb G3-519
% INHIBITION
100
80
60
40
20
0
0.1
1
10
ANTIBODY CONCENTRATION (µg/ml)

FIG. 4

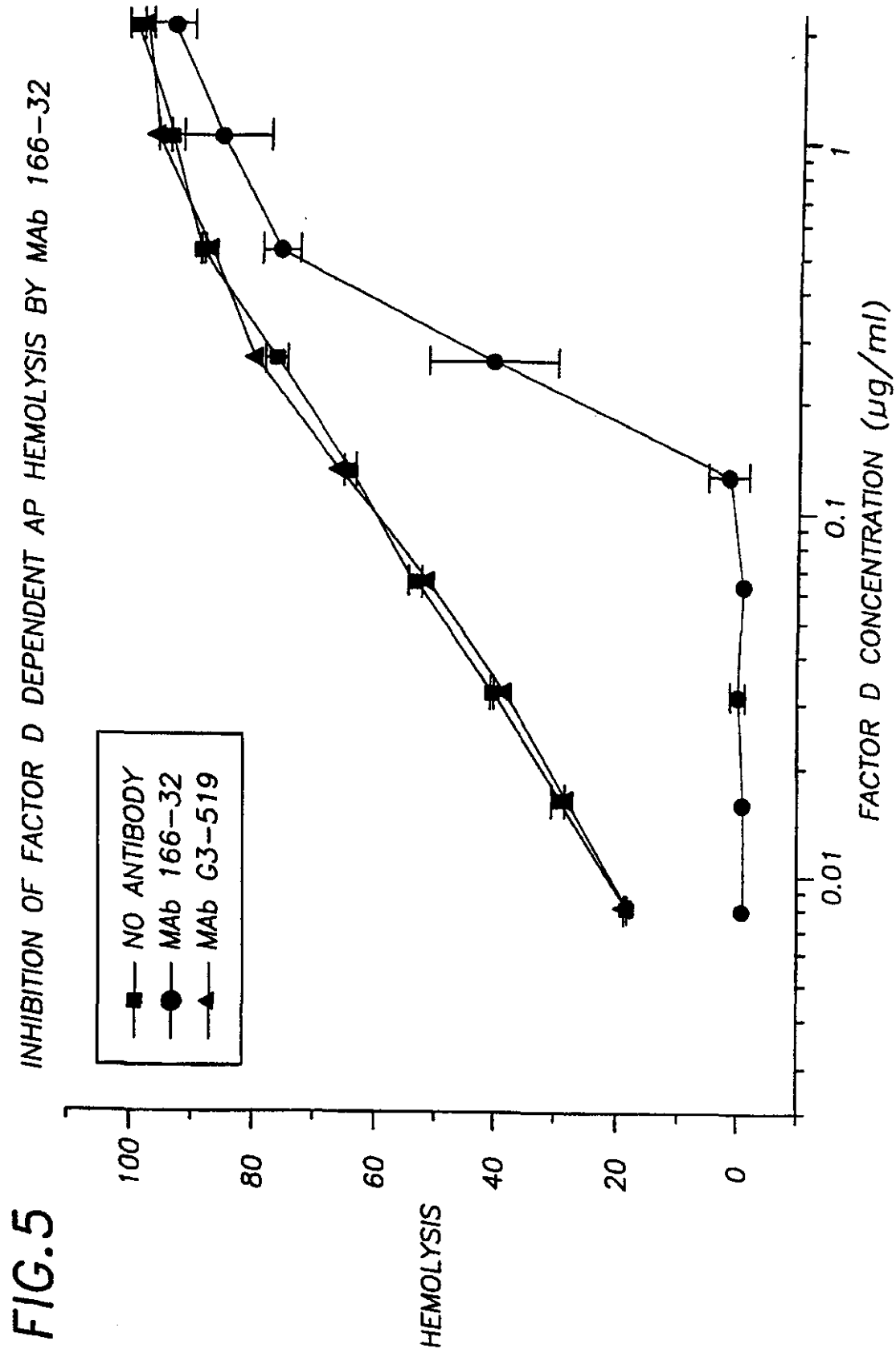

INHIBITION OF FACTOR D DEPENDENT AP HEMOLYSIS BY MAb 166-32
NO ANTIBODY
MAb 166-32
MAb G3-519
HEMOLYSIS
100
80
60
40
20
0
0.01
0.1
1
FACTOR D CONCENTRATION (µg/ml)

FIG.5

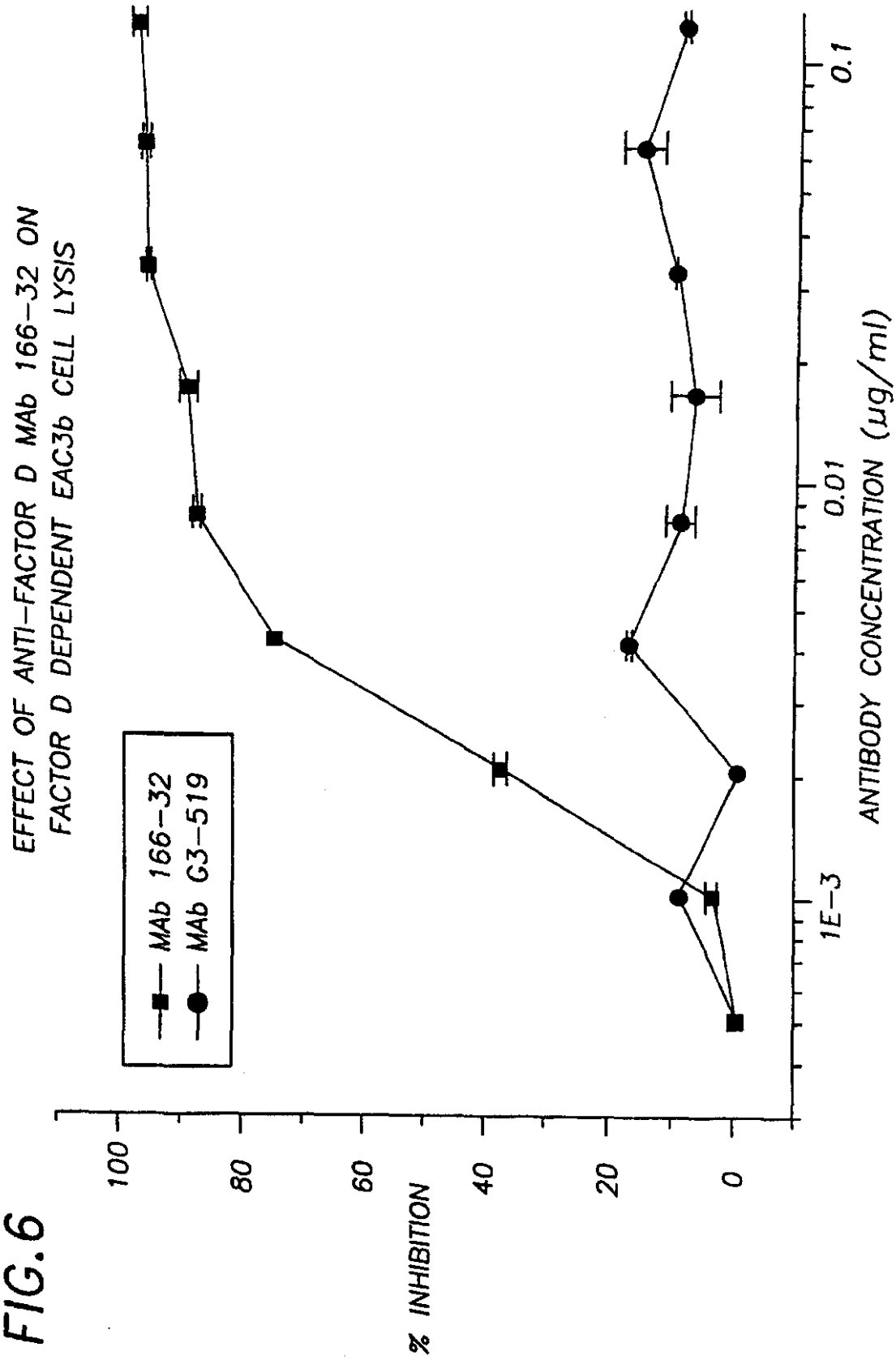

EFFECT OF ANTI-FACTOR D MAb 166-32 ON
FACTOR D DEPENDENT EAC3b CELL LYSIS
MAb 166-32
MAb G3-519
% INHIBITION
100
80
60
40
20
0
1E-3
0.01
0.1
ANTIBODY CONCENTRATION (µg/ml)

FIG. 6

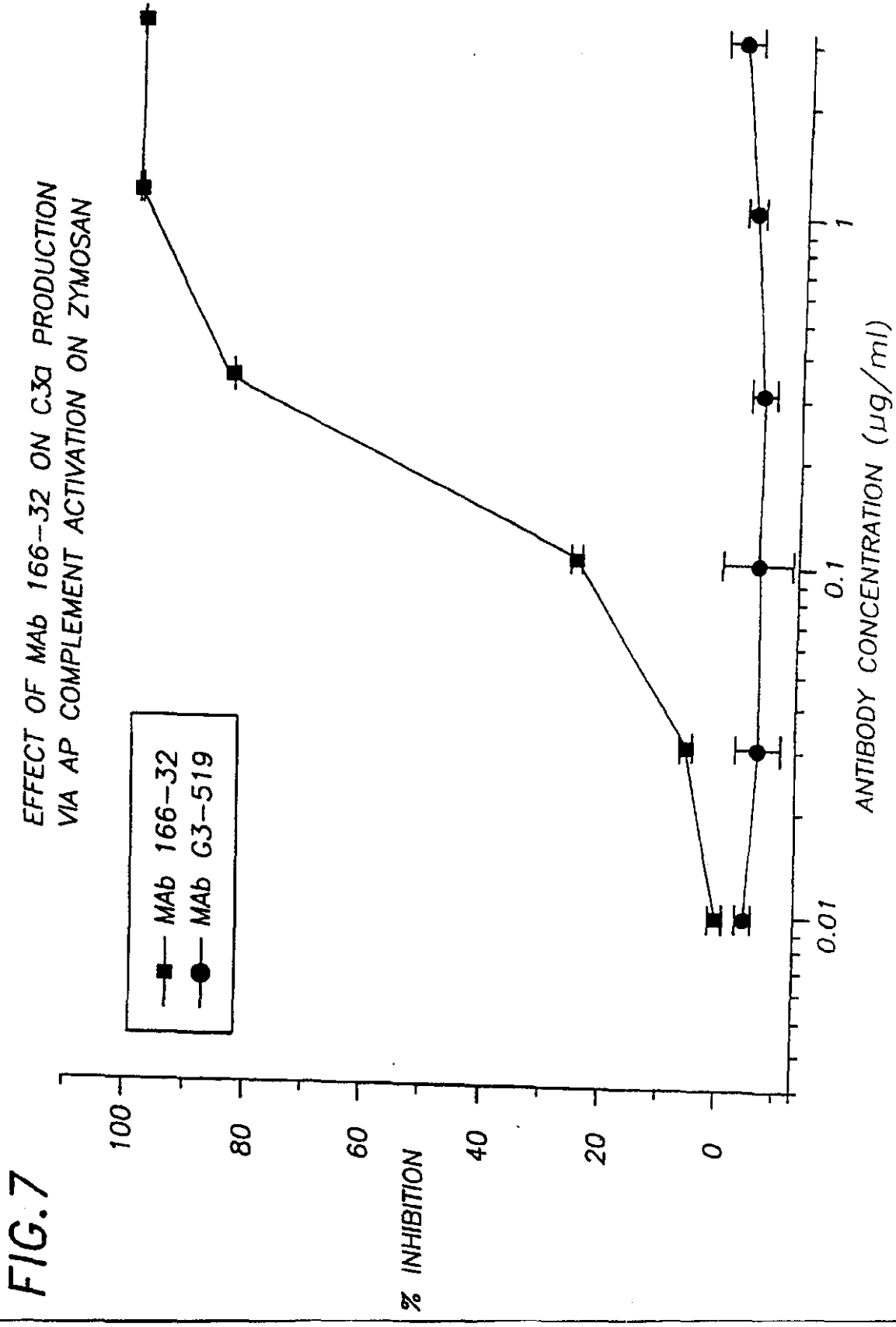

EFFECT OF MAb 166-32 ON C3a PRODUCTION
VIA AP COMPLEMENT ACTIVATION ON ZYMOSAN
MAb 166-32
MAb G3-519
% INHIBITION
100
80
60
40
20
0
0.01
0.1
1
ANTIBODY CONCENTRATION (µg/ml)

FIG. 7

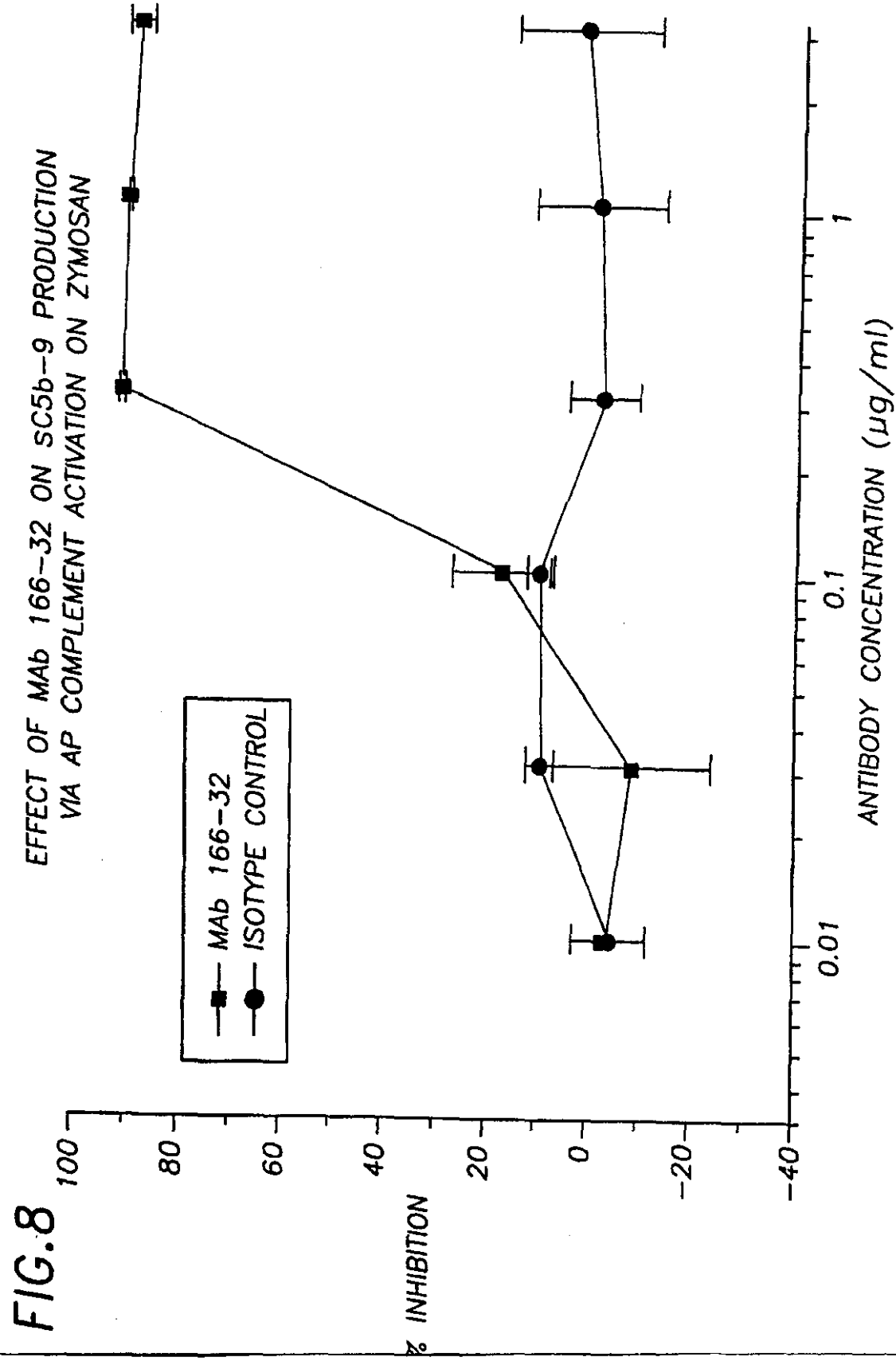

EFFECT OF MAb 166-32 ON SC5b-9 PRODUCTION
VIA AP COMPLEMENT ACTIVATION ON ZYMOSAN
MAb 166-32
ISOTYPE CONTROL
% INHIBITION
100
80
60
40
20
0
-20
-40
0.01
0.1
1
ANTIBODY CONCENTRATION (µg/ml)

FIG.8

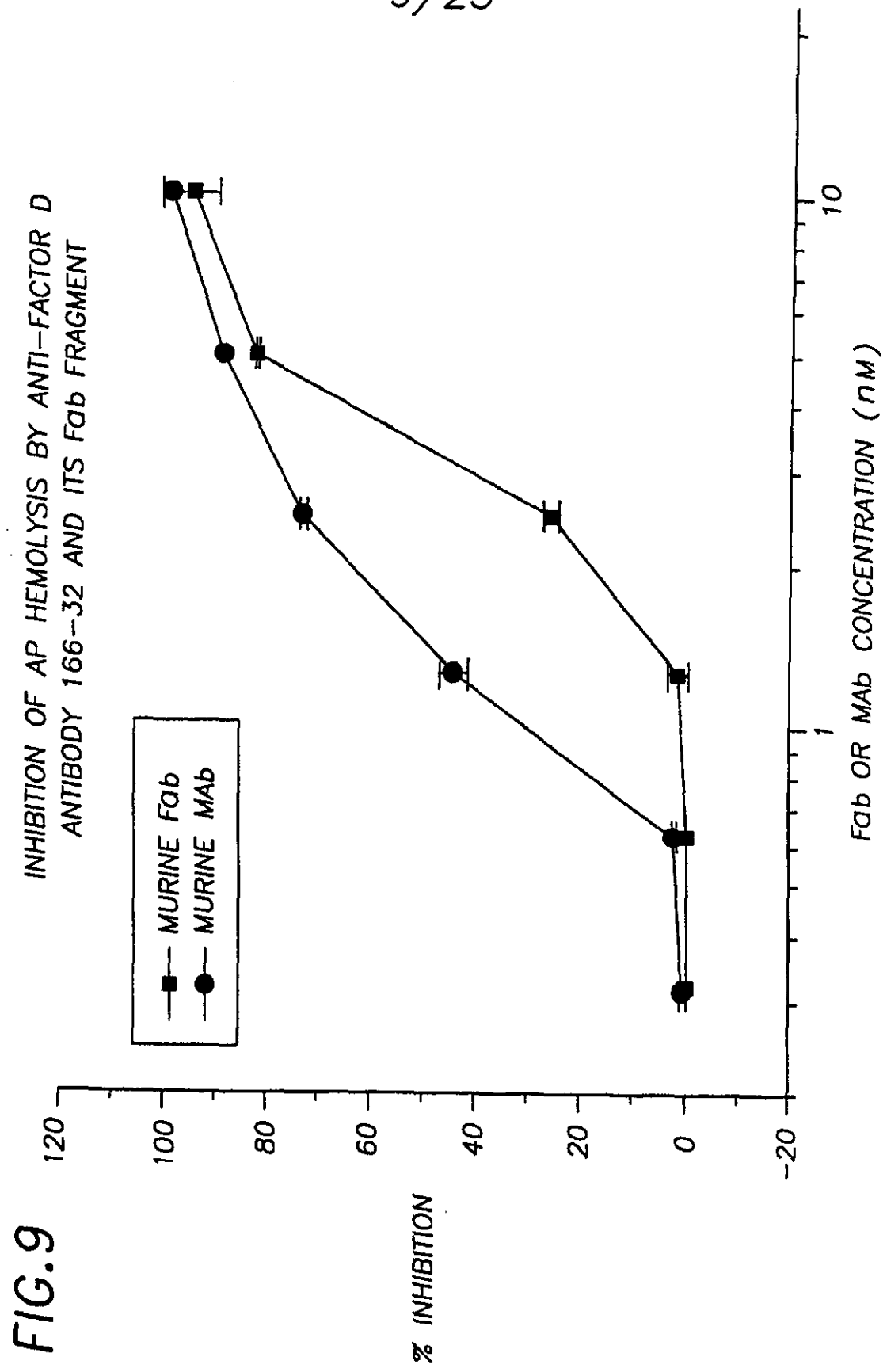
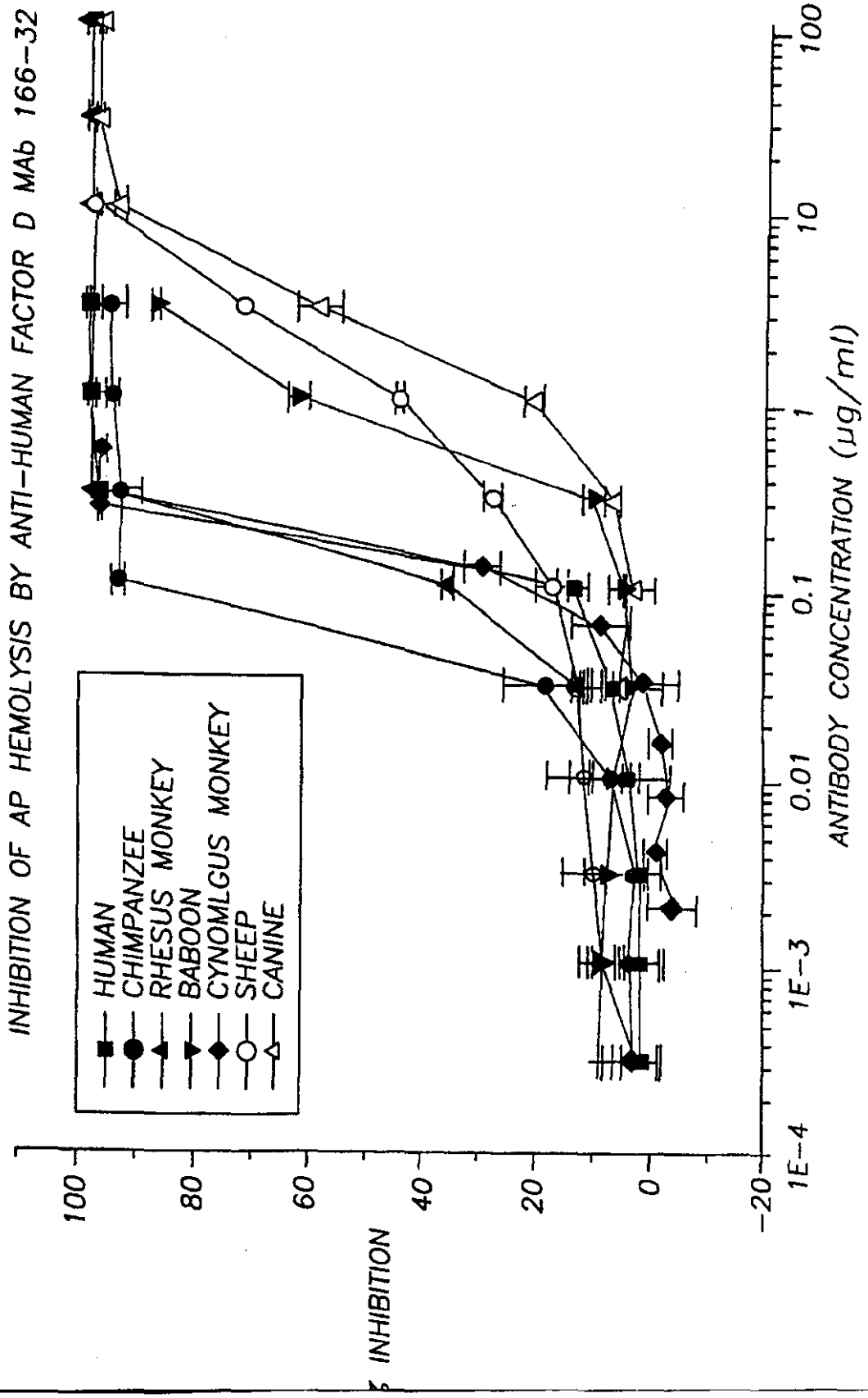

INHIBITION OF AP HEMOLYSIS BY ANTI-FACTOR D
ANTIBODY 166-32 AND ITS Fab FRAGMENT
MURINE Fab
MURINE MAb
% INHIBITION
120
100
80
60
40
20
0
-20
1
10
Fab OR MAb CONCENTRATION (nM)

FIG. 9

FIG. 10


INHIBITION OF AP HEMOLYSIS BY ANTI-HUMAN FACTOR D MAb 166-32
HUMAN
CHIMPANZEE
RHESUS MONKEY
BABOON
CYNOMLGUS MONKEY
SHEEP
CANINE
% INHIBITION
100
80
60
40
20
0
-20
1E-4
1E-3
0.01
0.1
1
10
100
ANTIBODY CONCENTRATION (µg/ml)

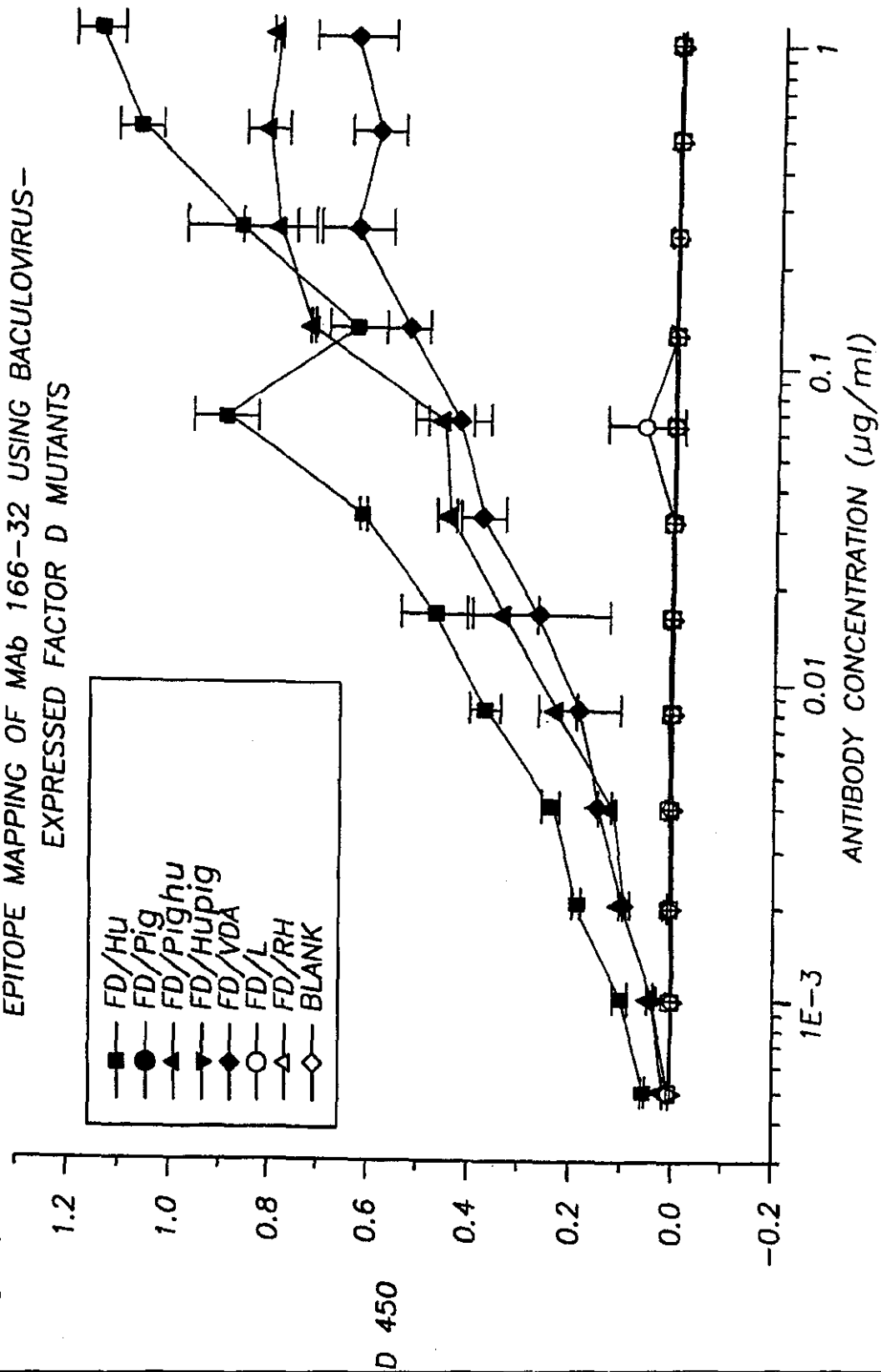

EPITOPE MAPPING OF MAb 166-32 USING BACULOVIRUS-EXPRESSED FACTOR D MUTANTS
FD/Hu
FD/Pig
FD/Pighu
FD/Hupig
FD/VDA
FD/L
FD/RH
BLANK
D 450
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
1E-3
0.01
0.1
1
ANTIBODY CONCENTRATION (µg/ml)

FIG. 11

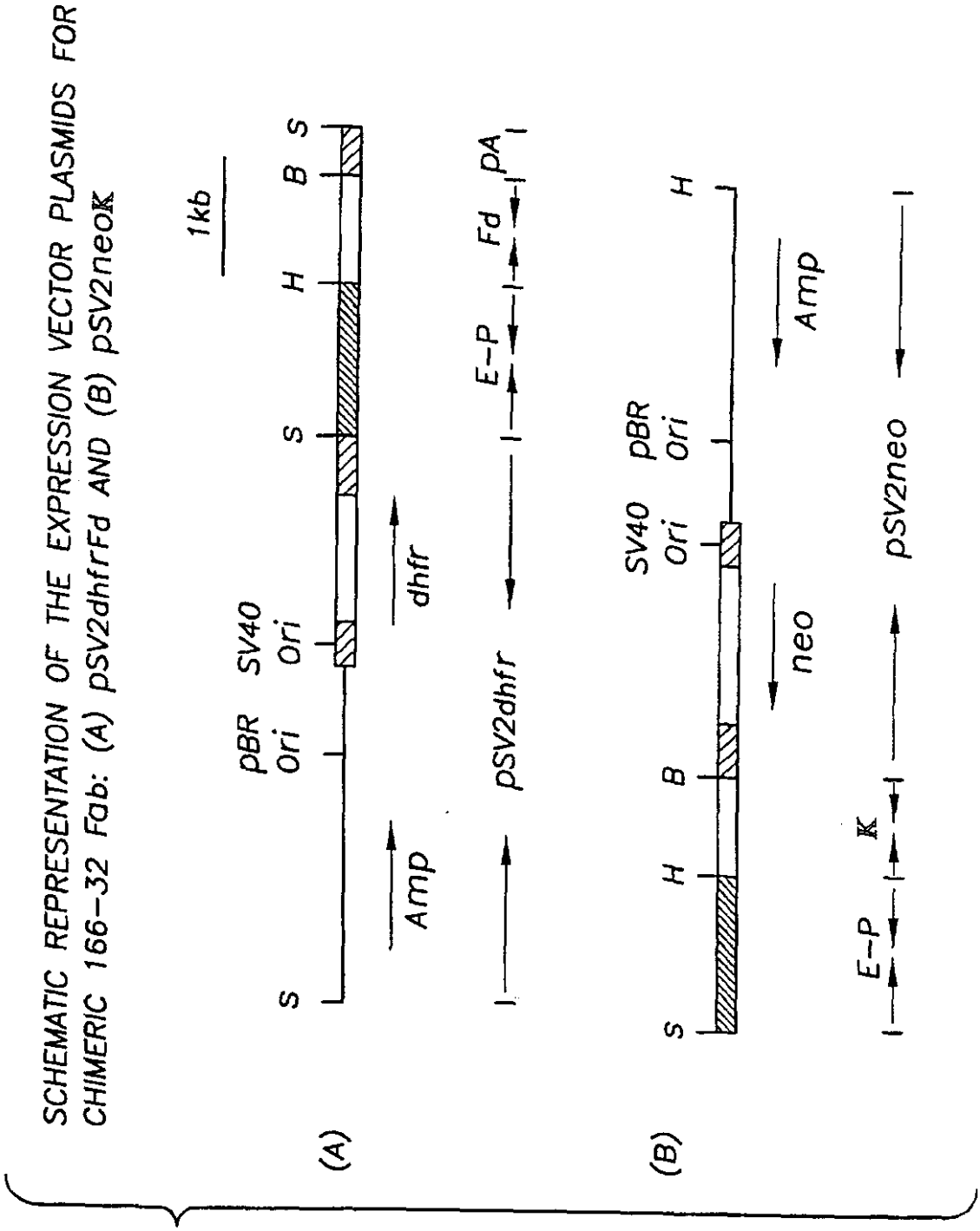


FIG. 12

SCHEMATIC REPRESENTATION OF THE EXPRESSION VECTOR PLASMIDS FOR CHIMERIC 166-32 Fab: (A) pSV2dhfrFd AND (B) pSV2neoK

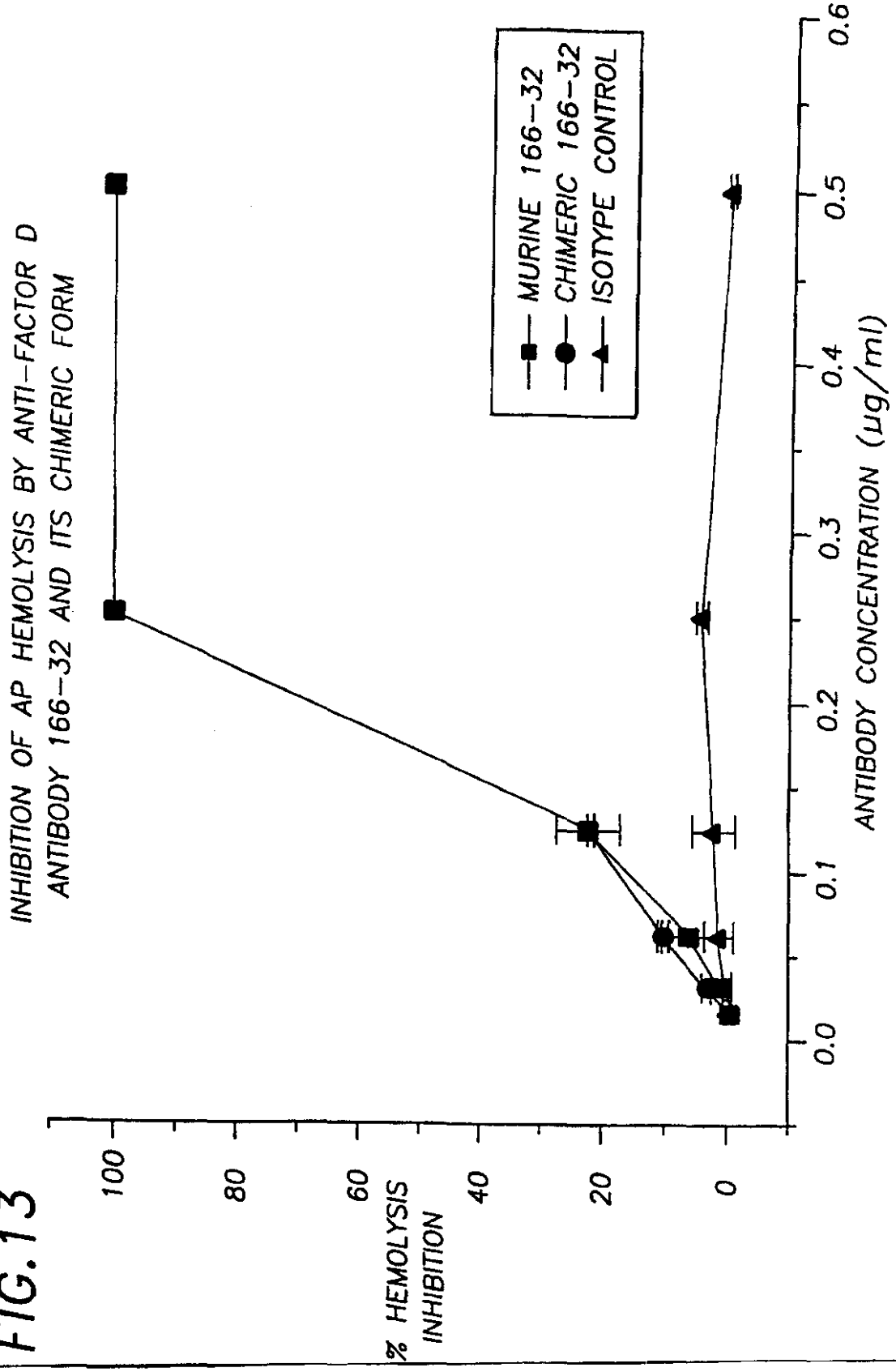

INHIBITION OF AP HEMOLYSIS BY ANTI-FACTOR D ANTIBODY 166-32 AND ITS CHIMERIC FORM
MURINE 166-32
CHIMERIC 166-32
ISOTYPE CONTROL
% HEMOLYSIS INHIBITION
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ANTIBODY CONCENTRATION (µg/ml)

FIG. 13

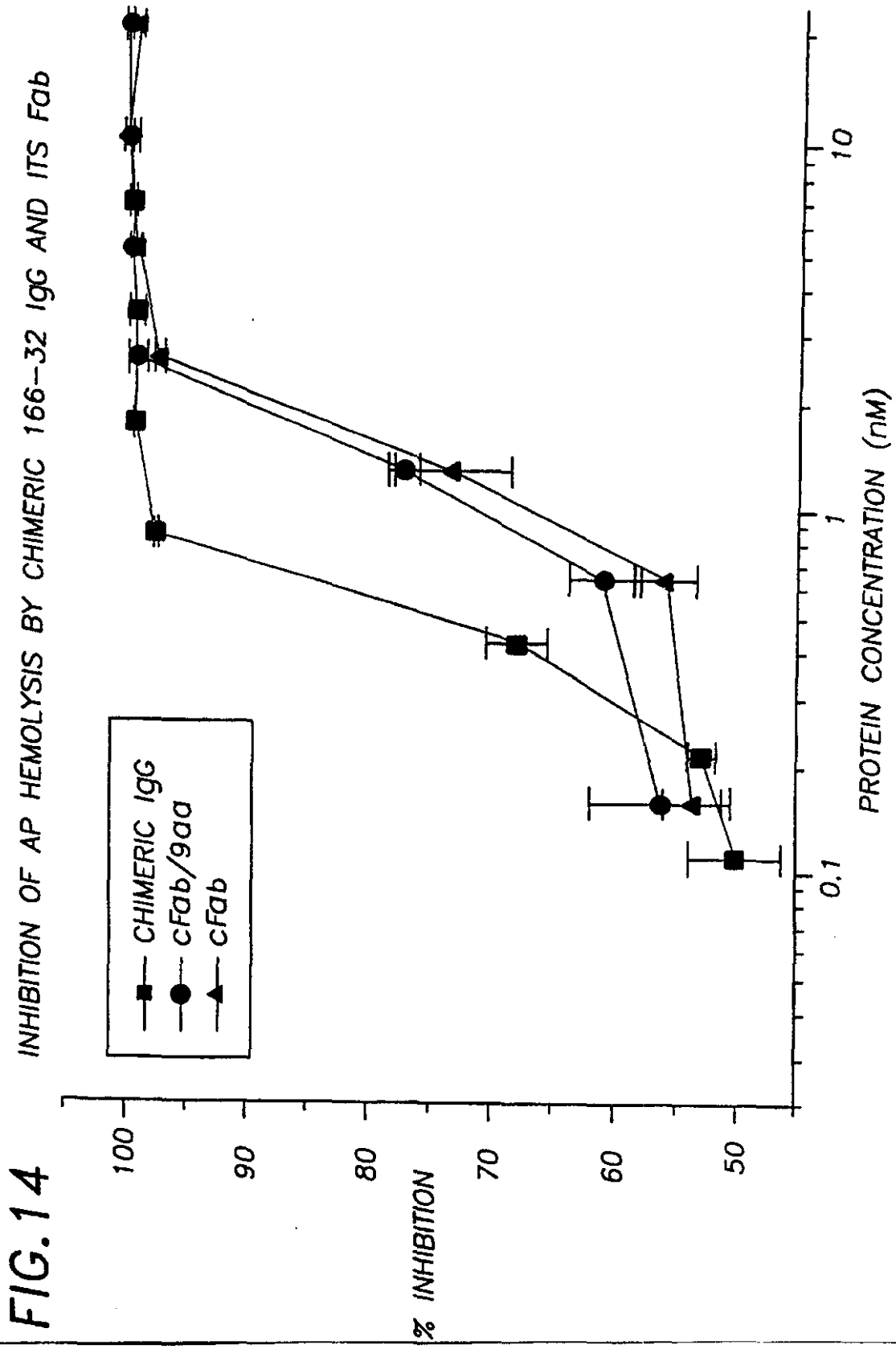
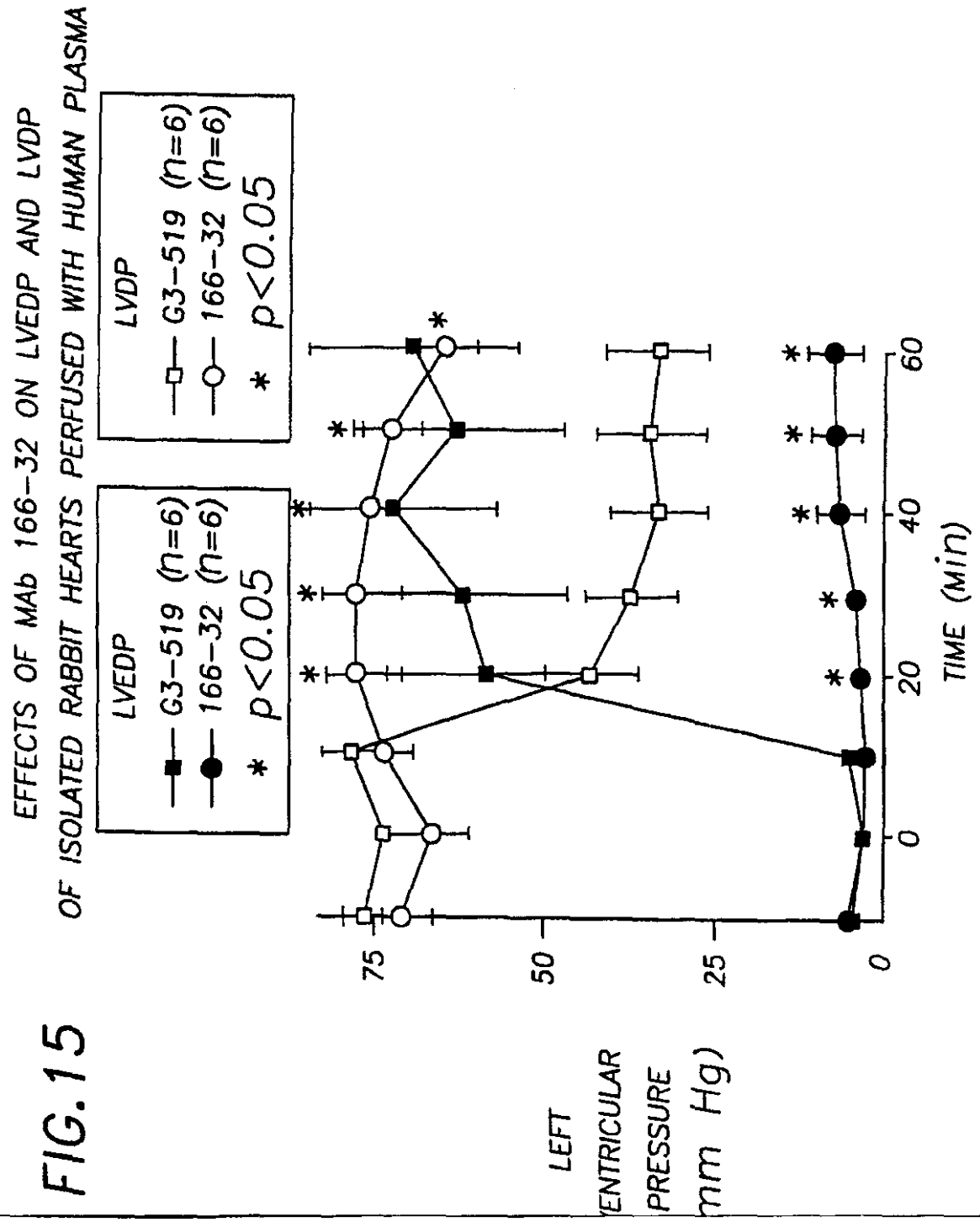

INHIBITION OF AP HEMOLYSIS BY CHIMERIC 166-32 IgG AND ITS Fab
CHIMERIC IgG
cFab/9aa
cFab
% INHIBITION
100
90
80
70
60
50
0,1
1
10
PROTEIN CONCENTRATION (nM)

FIG. 14

FIG. 15


EFFECTS OF MAb 166-32 ON LVEDP AND LVDP
OF ISOLATED RABBIT HEARTS PERFUSED WITH HUMAN PLASMA
LVEDP
G3-519 (n=6)
166-32 (n=6)
* p<0.05
LVDP
G3-519 (n=6)
166-32 (n=6)
* p<0.05
LEFT VENTRICULAR PRESSURE (mm Hg)
75
50
25
0
0
20
40
60
TIME (Min)

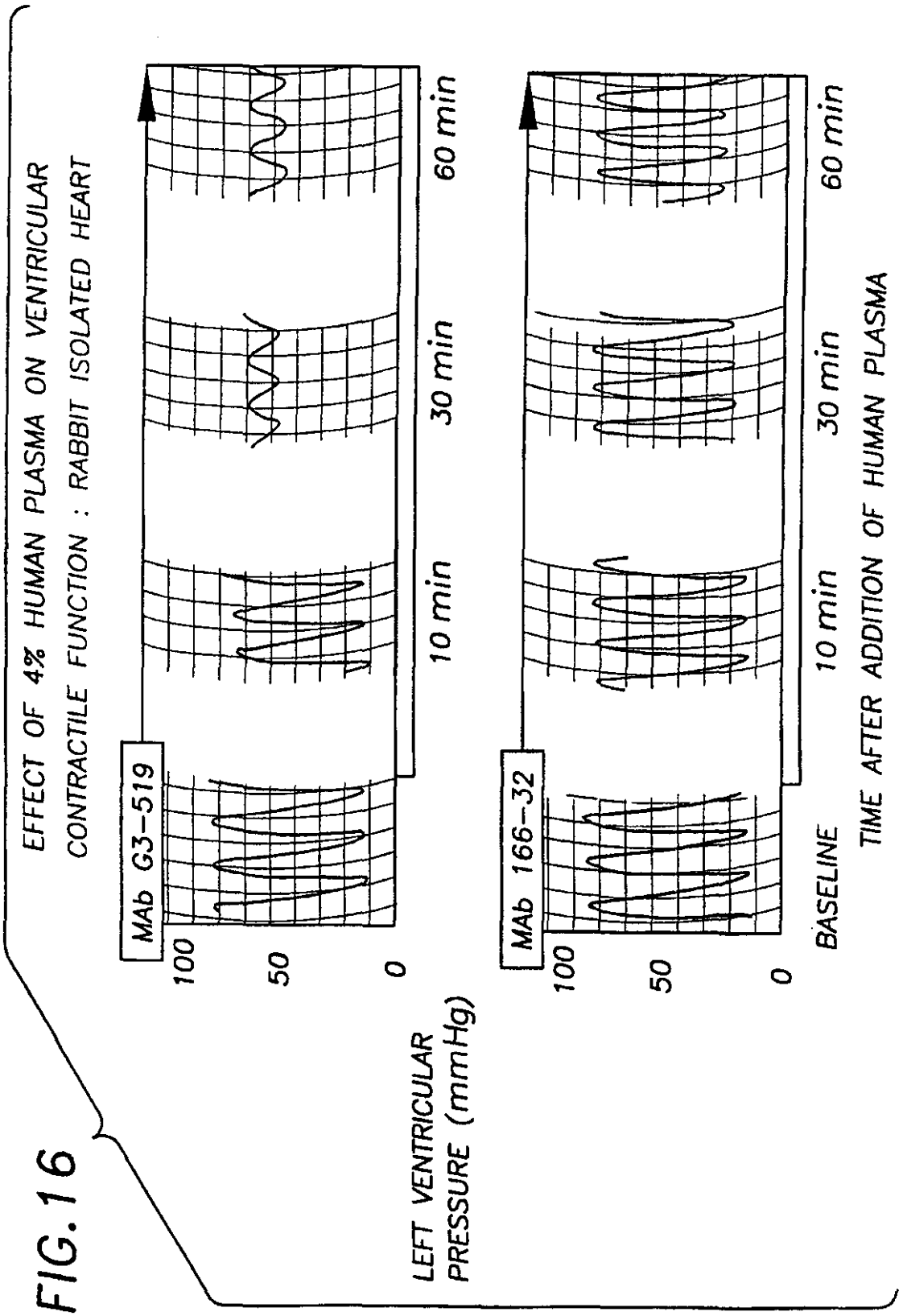
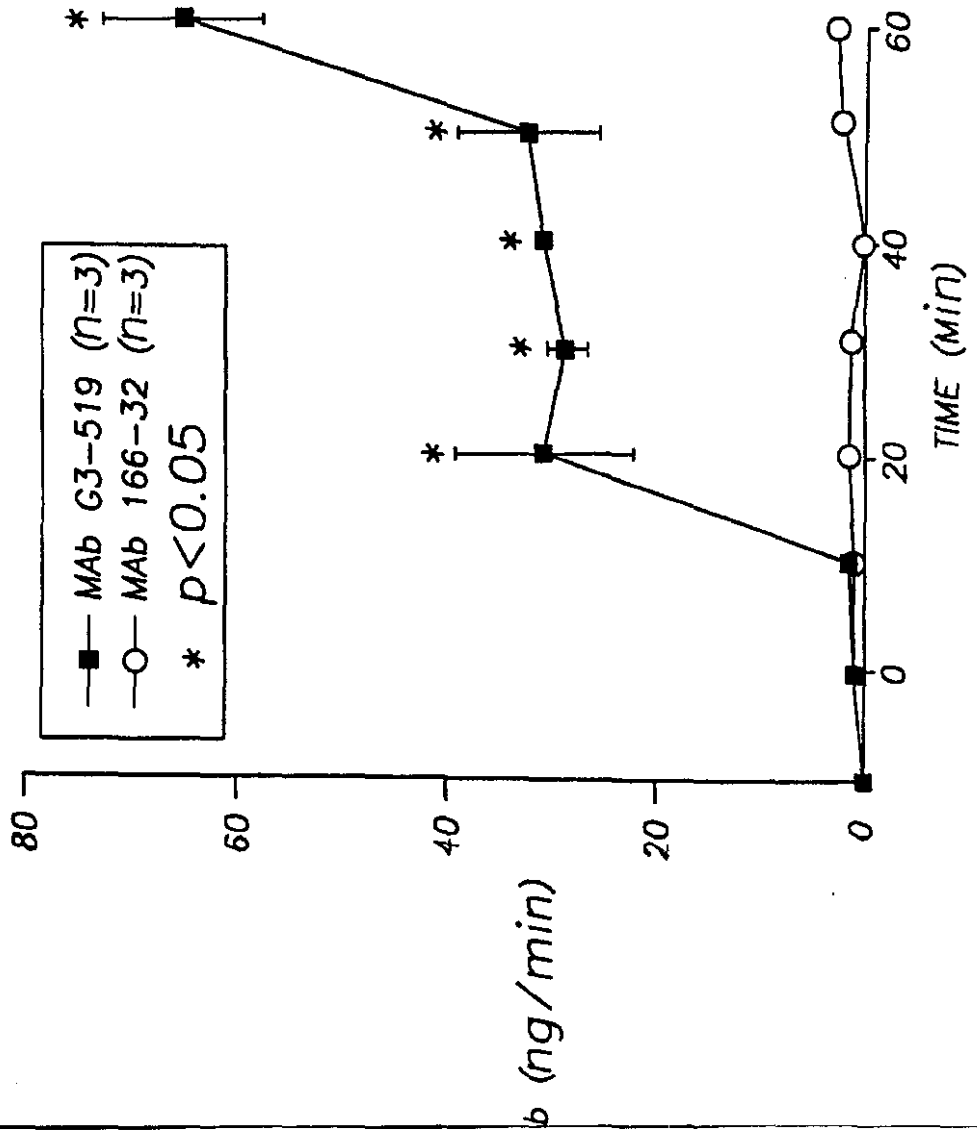

EFFECT OF 4% HUMAN PLASMA ON VENTRICULAR CONTRACTILE FUNCTION : RABBIT ISOLATED HEART
MAb G3-519
MAb 166-32
100
50
0
LEFT VENTRICULAR PRESSURE (mmHg)
BASELINE
10 min
30 min
60 min
TIME AFTER ADDITION OF HUMAN PLASMA

FIG. 16

FIG. 17

EFFECTS OF MAb 166-32 ON THE LEVELS OF Bb IN CARDIAC LYMPH OF ISOLATED RABBIT HEARTS PERFUSED WITH HUMAN PLASMA



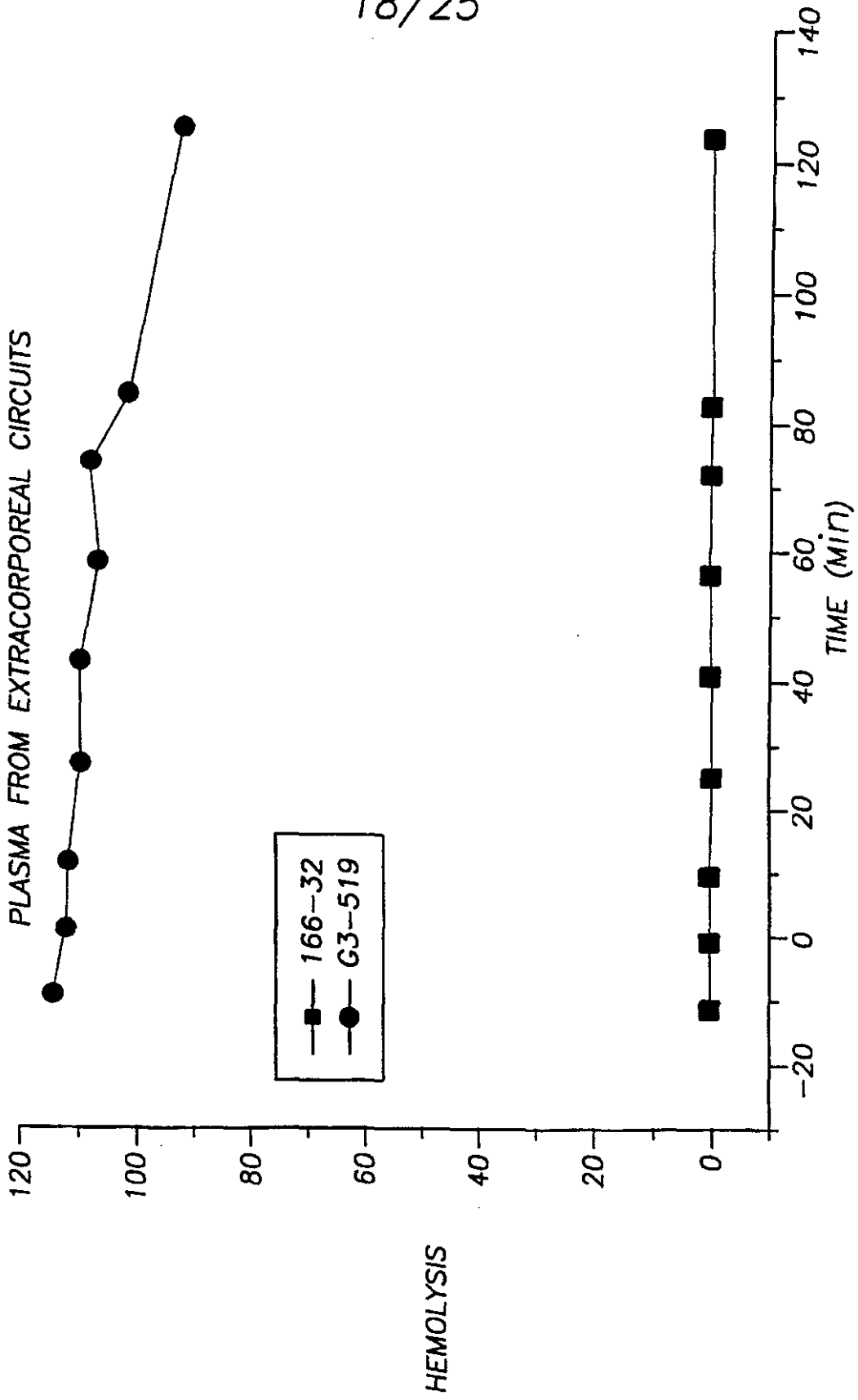

ALTERNATIVE PATHWAY HEMOLYTIC ACTIVITY OF
PLASMA FROM EXTRACORPOREAL CIRCUITS
166-32
G3-519
HEMOLYSIS
TIME (Min)
120
100
80
60
40
20
0
-20
0
20
40
60
80
100
120
140

FIG. 18

FIG. 19

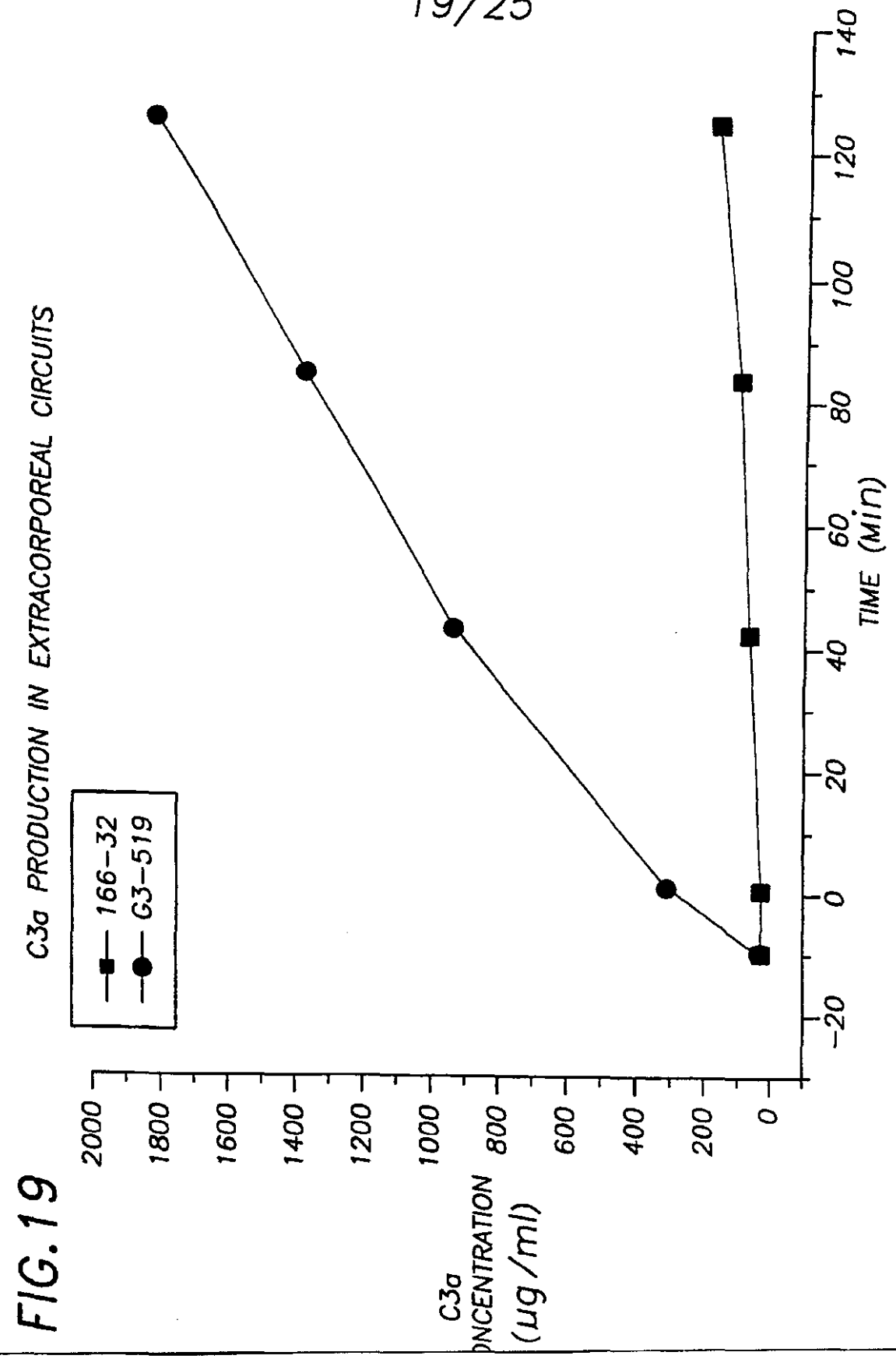
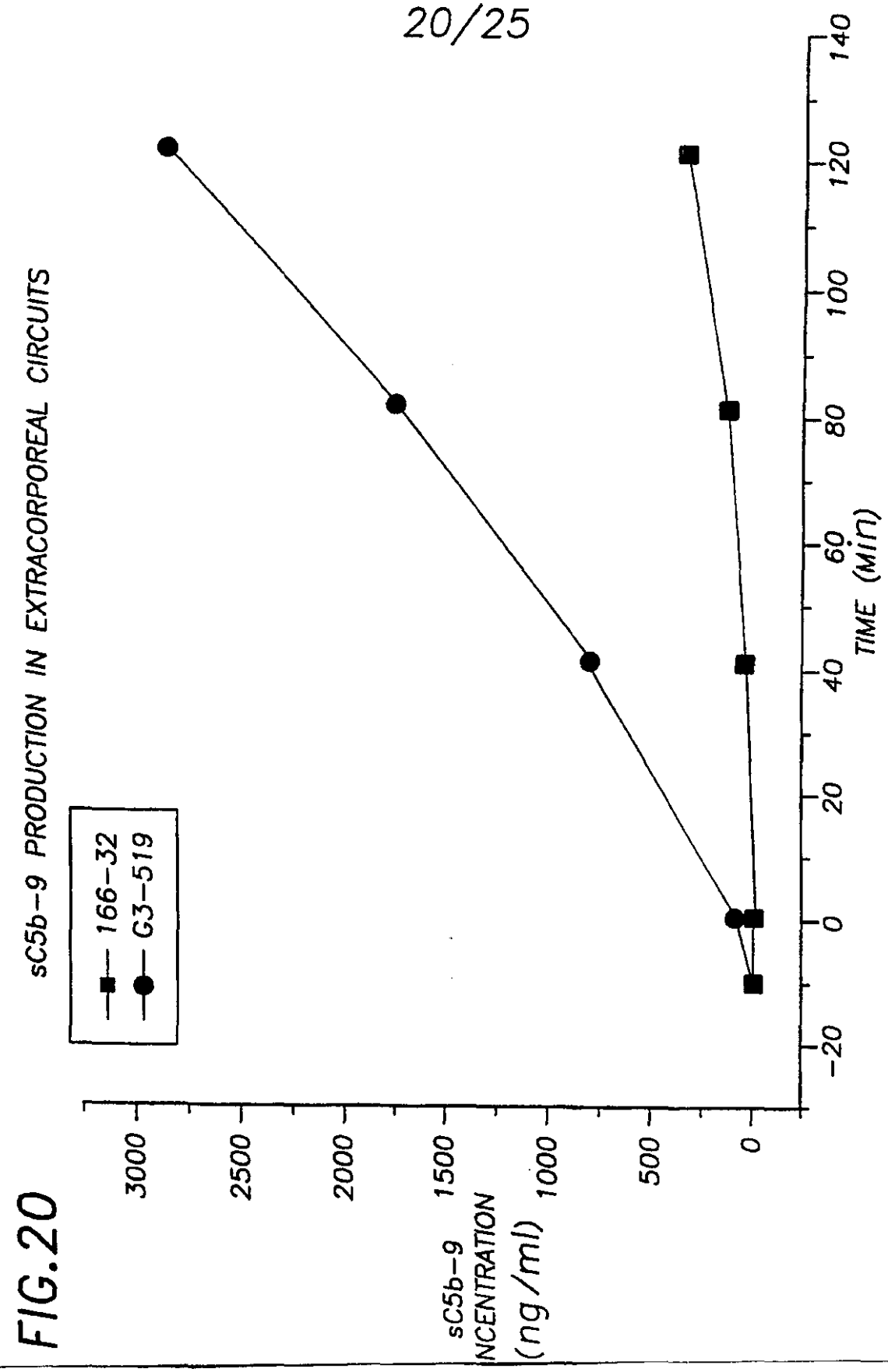

C3a PRODUCTION IN EXTRACORPOREAL CIRCUITS
166-32
G3-519
C3a CONCENTRATION (µg/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-20
0
20
40
60
80
100
120
140
TIME (Min)

FIG. 20


sC5b-9 PRODUCTION IN EXTRACORPOREAL CIRCUITS
166-32
G3-519
sC5b-9 CONCENTRATION (ng/ml)
3000
2500
2000
1500
1000
500
0
-20
0
20
40
60
80
100
120
140
TIME (Min)

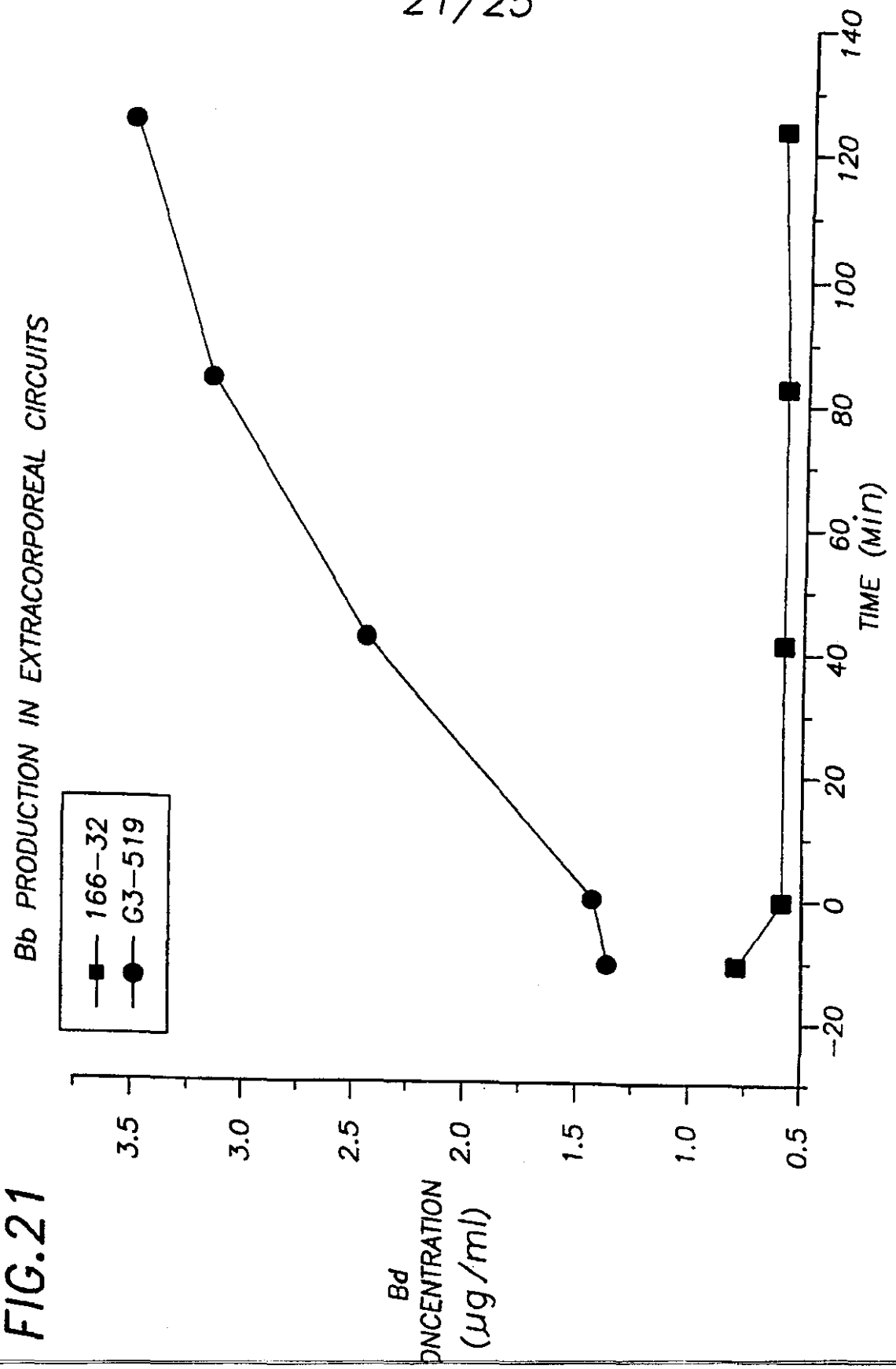

Bb PRODUCTION IN EXTRACORPOREAL CIRCUITS
166-32
G3-519
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Bd
ONCENTRATION
(µg/ml)
-20
0
20
40
60
80
100
120
140
TIME (MIN)

FIG.21

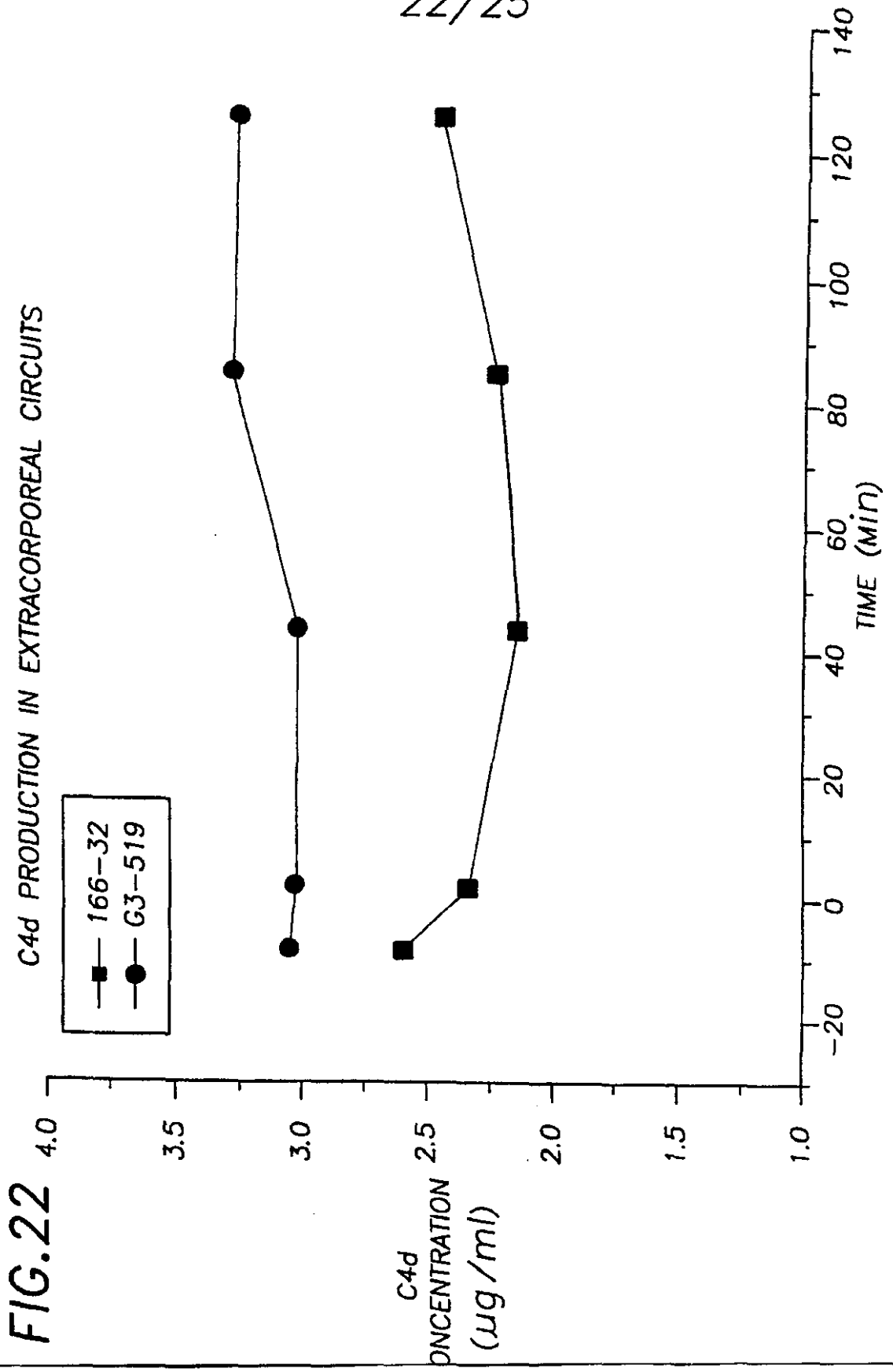

C4d PRODUCTION IN EXTRACORPOREAL CIRCUITS
166-32
G3-519
C4d CONCENTRATION (μg/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
-20
0
20
40
60
80
100
120
140
TIME (MIN)

FIG.22

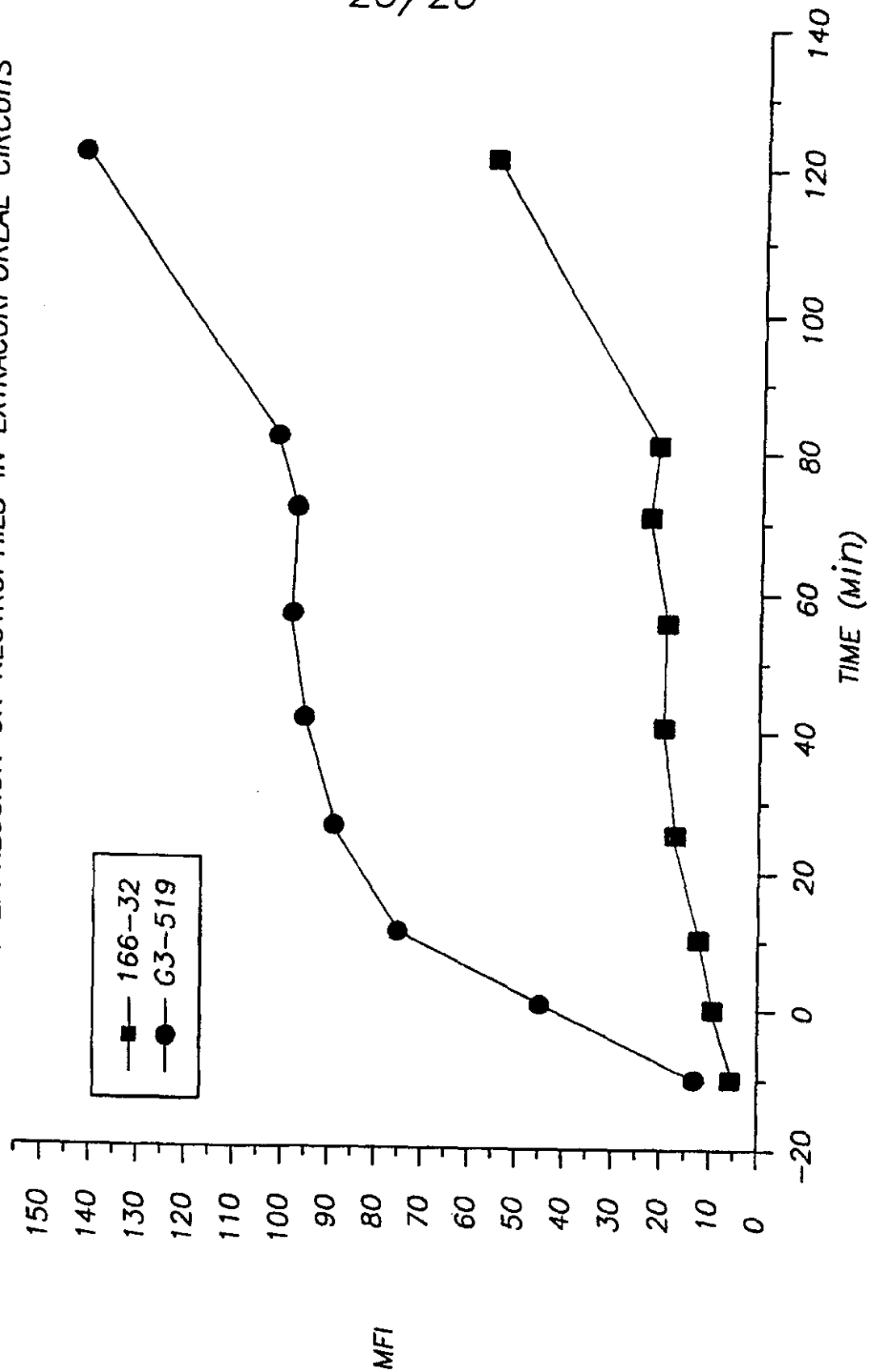

CD11b EXPRESSION ON NEUTROPHILS IN EXTRACORPOREAL CIRCUITS
166-32
G3-519
MFI
TIME (Min)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-20
0
20
40
60
80
100
120
140

FIG.23

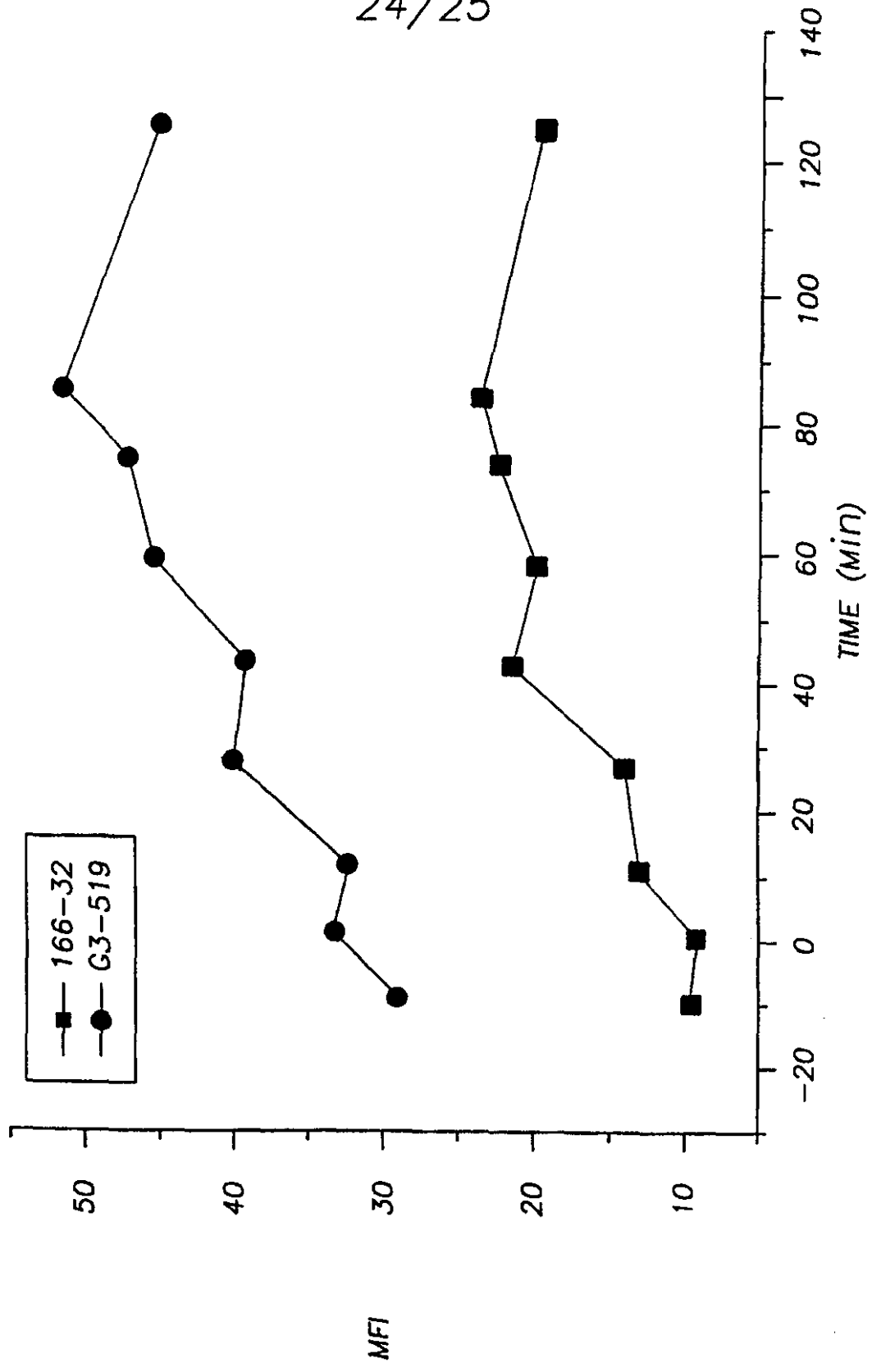

CD62P EXPRESSION ON PLATELETS IN EXTRACORPOREAL CIRCUITS
166-32
G3-519
MFI
50
40
30
20
10
-20
0
20
40
60
80
100
120
140
TIME (MIN)

FIG.24

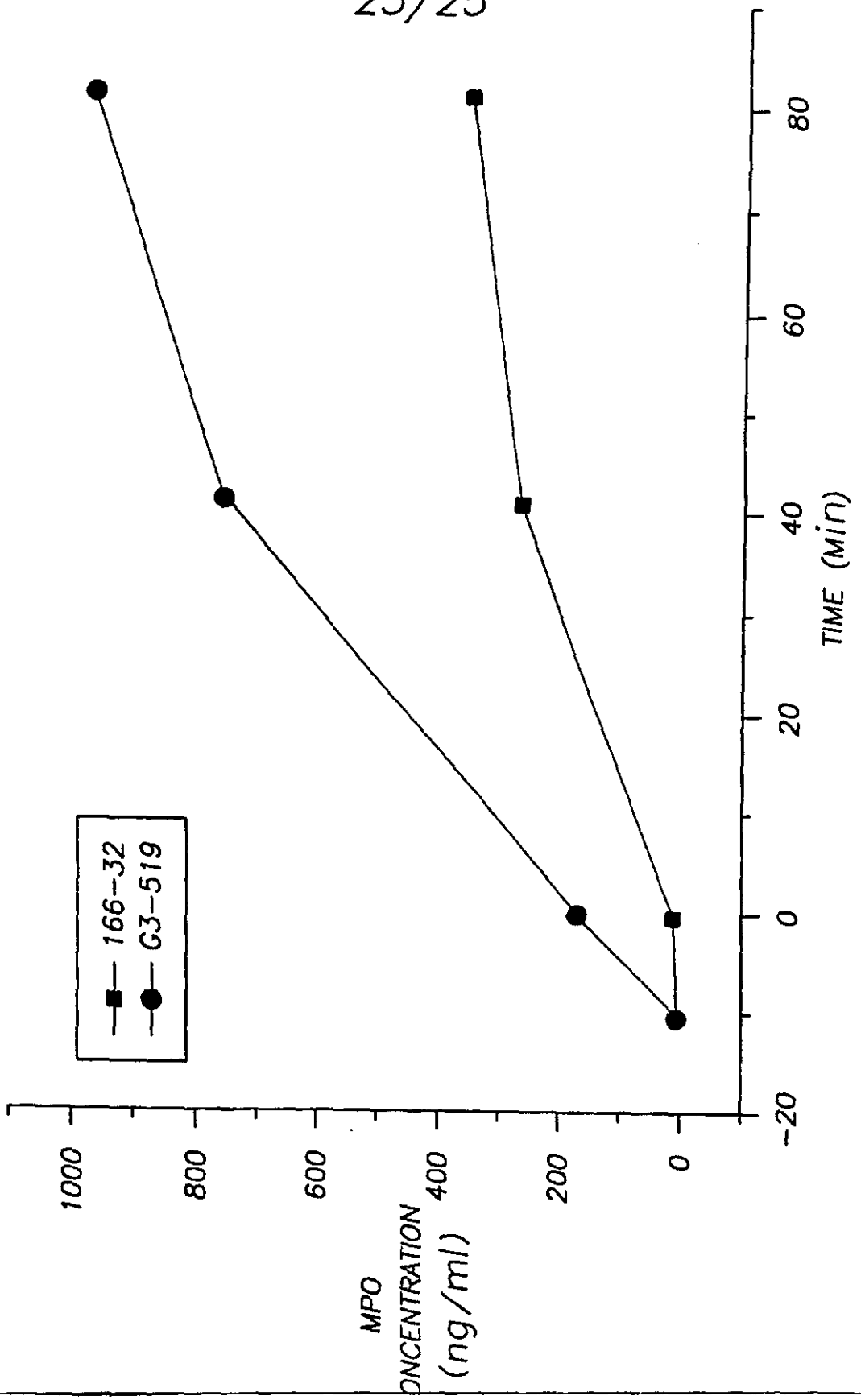

MYELOPEROXIDASE LEVEL IN EXTRACORPOREAL CIRCUITS
166-32
G3-519
MPO CONCENTRATION (ng/ml)
1000
800
600
400
200
0
-20
0
20
40
60
80
TIME (Min)

FIG.25

[19]中华人民共和国国家知识产权局

[51]Int. Cl[7]
A61K 39/395
C07K 16/36 C12N 5/12

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[71]申请人 泰诺士公司
地址 美国德克萨斯州
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B·N·C·孙

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代理人 张广育 王其灏

权利要求书2页 说明书42页 附图页数25页

[54]发明名称 补体活化的抑制剂

[57]摘要

本发明涉及因子D的抑制剂,它结合因子D并阻断因子D在补体活化中的功能活性。此抑制剂包括抗体分子,以及同源物、相似物及它们的修饰形式或衍生形式,包括免疫球蛋白片段如Fab、(Fab')$_2$及Fv;小分子包括肽、寡核苷酸、肽模拟物及有机化合物。获得了一株单克隆抗体,它能结合因子D并阻断其补体活化功能,该抗体被命名为166-32。产生此抗体的杂交瘤保藏于美国典型培养物保藏中心,10801 University Blvd.,Manassas,VA20110-2209,保藏号为HB-12476。

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权 利 要 求 书

1. 一种补体活化的抑制剂，它结合因子 D，并且在摩尔比约为 1.5∶1(抑制剂∶因子 D)时能抑制补体活化。

2. 一种补体活化的抑制剂，它结合因子 D，并且在摩尔比低于 80∶1(抑制剂∶因子 D)时能抑制补体活化。

3. 一种补体活化的抑制剂，它结合因子 D，并且在适于治疗使用的抑制剂∶因子 D 摩尔比时能抑制补体活化。

4. 权利要求 1、2 或 3 的抑制剂，其中补体活化的抑制作用在体外测定。

5. 权利要求 4 的抑制剂，其中补体活化的抑制在体外用体外分析进行测定。

6. 一种补体活化抑制剂，它结合人因子 D 中氨基酸残基号码 Cys154 及 Cys170 之间(且含)的区域。

7. 权利要求 6 的抑制剂，它在无氨基酸残基 Arg156、His159 及 Leu168 时不结合人因子 D。

8. 权利要求 1～3、6 或 7 中任一项的抑制剂，它是一种抗体或同源物、相似物或其片段、肽、寡核苷酸、肽模拟物或有机化合物。

9. 权利要求 8 的抑制剂，其中抗体片段为 Fab、$(Fab')_2$、Fv 或单链 Fv。

10. 权利要求 9 的抑制剂，其中抗体是一种嵌合的、去免疫的、人源化的、去免疫化的或人抗体。

11. 单克隆抗体 166-32。

12. 产生单克隆抗体 166-32 的杂交瘤，它保藏于美国典型培养物保藏中心，保藏号码为 HB-12476。

13. 一种单克隆抗体或片段、相似物或其同源物、或肽、寡核苷酸、肽模拟物或有机化合物，它与抗体 166-32 结合至因子 D 的相同表位。

14. 权利要求 13 的片段，它是一种 Fab、$(Fab')_2$、Fv 或单链 Fv。

15. 一种嵌合体形式，它含有权利要求 14 的 Fab 片段的小鼠可变区及人恒定区。

16. 产生单克隆抗体或其片段的细胞系，其中该抗体与抗体 166-32 结合至因子 D 相同表位。

17. 产生权利要求 14 的片段的细胞系。

18. 产生权利要求 15 的嵌合体 Fab 片段的细胞系。

19. 一种嵌合体形式，它含有单克隆抗体 166-32 的小鼠可变区及人恒定区。

20. 一种嵌合体形式，它含有单克隆抗体 166-32 的 Fab 片段的小鼠可变区及人恒定区。

21. 一种治疗由补体系统过度或失控活化介导的疾病或症状的方法，包括在体内或体外给药一种权利要求 1～3、6 及 7 中任一项的抑制剂。

22. 一种治疗由补体系统过度或失控活化介导的疾病或症状的方法，包括在体内或体外给药一种权利要求 8 的抑制剂。

23. 一种治疗由补体系统过度或失控活化介导的疾病或症状的方法，包括在体内或体外给药一种权利要求 9～11、13～15 或 19 及 20 中任一项的抑制剂。

24. 一种治疗与心肺分流相关的补体-介导的症状的方法，包括在体内或体外给药一种权利要求 1～3、6 及 7 中任一项的抑制剂。

25. 一种治疗与心肺分流相关的补体-介导的症状的方法，包括在体内或体外给药一种权利要求 8 的抑制剂。

26. 一种治疗与心肺分流相关的补体-介导的症状的方法，包括在体内或体外给药一种权利要求 9～11、13～15 或 19 及 20 中任一项的抑制剂。

说　明　书

补体活化的抑制剂

发明领域

本发明涉及因子 D 的专一抑制剂、以及使用此抑制剂来抑制补体系统活化和抑制补体旁路活化途径。

发明背景

补体系统在清除免疫复合物及对感染物、外来抗原、病毒感染细胞及肿瘤细胞的免疫应答中扮演中心角色。然而，补体也参与病理炎症及自身免疫疾病。因此，抑制过度或失控的补体级联反应活化可为有此疾病或症状的病人提供临床益处。

补体系统包含两个不同活化途径，被称为经典途径及旁路途径(V. M. Holers, In Clinical Immunology：Principles and Practice, ed. R. R. Rich, Mosby Press; 1996, 363-391)。经典途径为钙/镁依赖的级联反应，该途径一般由抗原-抗体复合物的形成而活化。旁路途径为镁依赖的级联反应，该途径通过 C3 在特定易感表面(如酵母菌、细菌及特定生物多聚物的细胞壁多糖)沉积和活化而活化。补体途径的活化产生补体蛋白的生物活性片段，如 C3a、C4a 及 C5a 过敏毒素及 C5b-9 膜攻击复合物(MAC)，它们介导炎症活性，包括白细胞趋化性、巨噬细胞、中性粒细胞、血小板、肥大细胞及内皮细胞的活化、血管通透性、细胞裂解及组织伤害。

因子 D 为补体活化的旁路途径所需的高度专一丝氨酸蛋白酶。它可切割与 C3b 结合的因子 B，产生 C3b/Bb 酶，这种酶是旁路途径 C3/C5 转换酶的活性成份。因子 D 可以是抑制的合适靶，因为它在人类中的血浆浓度非常低(1.8微克/毫升)，已经显示它是补体活化的旁路途径中的限制酶(P. H. Lesavre 及 H. J. Muller-Eberhard. J. Exp. Med., 1978; 148：1498-1510; J. E. Volanakis 等人, New Eng. J. Med., 1985; 312：395-401)。

补体活化的下调已经证明在动物模型中及体外研究中可有效治疗数个疾病，如系统性红斑性狼疮症及肾小球性肾炎(Y. Wang 等人, Proc. Natl. Acad. Sci.; 1996, 93： 8563-8568)、类风湿性关节炎(Y. Wang 等人, Proc. Natl. Acad. Sci, 1995; 92： 8955-8959)、心肺分流及溶血症(C.S. Rinder, J. Clin. Invest., 1995; 96：1564-1572)、器官移植超

急性排斥(T. J. Kroshus 等人, Transplantation, 1995; 60：II 94-1202)、心血管栓塞(J. W. Homeister 等人, J. Immunol, 1993; 150：1055-1064; H. F. Weisman 等人, Science, 1990; 249：146-151)、再灌流损伤(E. A. Amsterdam 等人, Am. J. Physiol, 1995; 268：H448-H457)、及成人呼吸困难综合征(R. Rabinovici 等人, J. Immunol, 1992; 149：1744-1750)。此外，其它炎症及自身免疫/免疫复合物疾病亦与补体活化密切相关(V. M. Holers, ibid., B. P. Morgan. Eur. J. Clin. Invest, 1994：24：219-228)，这包括热损伤、严重哮喘、过敏性休克、肠炎、荨麻疹、血管水肿、脉管炎、多发性栓塞、重症肌无力症、膜增生性肾小球性肾炎、及 Sjogren 氏症。

发明简述

本发明包括因子 D 的抑制剂，它与因子 D 结合并阻断因子 D 的补体活化的功能活性，包括补体活化的旁路途径。这种抑制剂包括抗体分子以及同源物、相似物及其修饰形式或衍生形式，这包括免疫球蛋白片段如 Fab、$F(ab')_2$及 Fv。小分子包括肽、寡核苷酸、肽模拟物，能结合因子 D 并阻断其功能活性的有机化合物也包含在本发明内。

已经制备了一种单克隆抗体，它能与因子 D 结合并阻碍其补体活化功能，它被命名为 166-32。产生此抗体的杂交瘤保藏于美国典型培养物保藏中心，10801 University Blvd., Manassas, VA 20110-2209，保藏号码为 HB-12476。

附图简述

图 1 显示在 ELISA 中抗因子 D 的单克隆抗体(MAbs)与纯化的人因子 D 结合。实心圆形标记的线代表 MAb 166-11。实心三角形标记的线代表 MAb 166-32。实心菱形标记的线代表 MAb 166-188。实心正方形标记的线代表 MAb 166-222。Y-轴表示 MAbs 与因子 D 的反应性，它用 450 nm 处的光密度(OD)表示，X-轴代表 MAbs 的浓度。

图 2 显示 MAb 166-32 在存在 10％人血清的情况下，对未致敏的兔血红细胞(RBCs)的旁路(AP)溶血的抑制作用。实心正方形标记的线代表 MAb 166-32。实心圆形标记的线代表无关的同种型匹配的对照 MAb G3-519，后者对 HIV 包膜糖蛋白 gp120 有专一性。如文中的进一步介绍，Y-轴表示％

的溶血抑制作用。X-轴表示 MAbs 的浓度。

图 3 显示 MAb 166-32 在存在 90％人血清的情况下，对未致敏的兔血红细胞（RBCs）的旁路(AP)溶血的抑制作用。实心正方形标记的线代表 MAb 166-32。实心圆形标记的线代表无关的同种型匹配的对照 MAb G3-519，后者对 HIV 包膜糖蛋白 gp120 有专一性。如文中的进一步介绍，Y-轴表示％的溶血抑制作用。X-轴表示 MAbs 的浓度。

图 4 显示 MAb 166-32 不能抑制已致敏的鸡 RBCs 的经典途径(CP)溶血，但阳性对照抗人 C5 MAb 137-76 能够抑制。实心圆形标记的线代表 MAb 137-76。实心菱形及实心正方形标记的线分别代表 MAb 166-32 及阴性对照 MAb G3-519。Y-轴表示％溶血抑制作用。X-轴表示 MAbs 的浓度。

图 5 显示 MAb 166-32 对旁路途径(AP)溶血的抑制作用。溶血可通过在已使用抗因子 D MAb 166-222 亲合性层析清除其因子 D 的人血清中加入不同浓度的纯化人因子 D 来增强。此分析在存在或无 0.3 微克/毫升的试验 MAbs 的条件下进行。实心正方形标记的线代表不加抗体。实心圆形标记的线代表 MAb 166-32。实心三角形标记的线代表无关同种型匹配的对照 MAb G3-519。Y-轴代表％溶血抑制作用。X-轴代表因子 D 的浓度。

图 6 显示 MAb 166-32 对依赖因子的 EAC3b 细胞裂解的抑制作用。此旁路 C3 转换酶通过与因子 B、因子 P 及因子 D 温育装配于 EAC3b 细胞。在温育缓冲液中加入不同浓度的 MAb 166-32 以抑制因子 D 活性。实心正方形标记的线代表 MAb 166-32。实心圆形标记的线代表 MAb G3-519。Y-轴代表％溶血抑制作用。X-轴代表 MAbs 的浓度。

图 7 显示 MAb 166-32 可抑制 C3a 由酵母聚糖生成。酵母聚糖可在存在于人血清中时活化补体旁路途径。C3a 的生成可使用 ELISA 试剂盒测量。实心正方形标记的线代表 MAb 166-32。实心圆形标记的线代表无关同种型匹配的对照 MAb G3-519。Y-轴代表 C3a 产生的％抑制作用。X-轴代表 MAbs 的浓度。

图 8 显示 MAb 166-32 可抑制 sC5b-9 由酵母聚糖生成。酵母聚糖可在存在于人血清中时活化补体旁路途径。sC5b-9 的生成可使用 ELISA 试剂盒测量。实心正方形标记的线代表 MAb 166-32。实心圆形标记线代表无关同种型匹配的对照 MAb G3-519。Y-轴代表 sC5b-9 生成的％抑制作用。X-轴代表 MAbs 的浓度。

图 9 显示由 MAb 166-32 及其 Fab 对未致敏的兔 RBCs 的旁路途径溶血

的抑制作用。实心圆形标记的线代表 MAb 166-32 (全 IgG)。实心正方形标记的线代表 MAb 166-32 的 Fab。Y-轴代表%溶血抑制作用。X-轴代表 MAbs 的浓度。

图 10 显示 MAb 166-32 对于来自不同动物种属的血清中的因子 D 对未致敏的兔 RBCs 的旁路途径溶血的抑制作用。实心正方形标记的线代表人血清。实心圆形标记的线代表黑猩猩血清。实心三角形标记的线代表恒河猴血清。实心倒三角形标记的线代表狒狒血清。实心菱形标记的线代表 cynomolgus 猴血清。空圆形标记的线代表羊血清。空三角形标记的线代表狗血清。Y-轴代表%溶血抑制作用。X-轴代表 MAb 166-32 的浓度。

图 11 显示 MAb 166-32 与不同的杆状病毒表达的因子 D("FD")突变体及杂合体在 ELISA 中的反应力。实心正方形标记的线代表人因子 D---FD/Hu。实心圆形标记的线代表猪因子 D---FD/Pig。实心三角形标记的线代表 FD/Pighu。实心倒三角形标记的线代表融合蛋白 FD/Hupig。实心菱形标记的线代表突变蛋白 FDNDA。空圆形标记的线代表突变蛋白 FD/L。空正方形标记的线代表突变蛋白 FD/RH。空菱形标记的线代表无包被抗原的空白对照。重组蛋白将在文中进一步介绍。

图 12 显示用于嵌合体 166-32 Fab 的表达载体质粒的示意图：(A)pSV2dhfrFd 及(B)pSV2neo。实心盒代表编码 Fd 或μ基因的外显子。斜线片段如下文所示代表源自 HCMV 的增强子及启动子元件(E-P)。空心盒如其标记代表二氢叶酸还原酶(dhfr)及 neo 基因。pSV2 质粒含有来自以下不同来源的 DNA 片段：pBR322 DNA(窄线)包含 pBR322 DNA 的复制起始区域(pBR ori)及内酰胺酶---氨苄青霉素抗性基因(Amp)；SV40 DNA 用宽斜线代表及标明，包含 SV40 DNA 复制起始区域(SV40 ori)、早期启动子(dhfr 及 neo 基因的 5')、及聚腺苷酸化信号(dhfr 及 neo 基因的 3')。源自 SV40 的聚腺苷酸化信号(pA)也置于 Fd 基因的 3'.

图 13 显示未致敏的兔 RBCs 的旁路途径(AP)溶血的抑制作用。实心正方形标记的线代表小鼠 MAb 166-32。实心圆形标记的线代表嵌合体 MAb 166-32。实心三角形标记的线代表同种型匹配的的阴性对照抗体 G3-519。Y-轴代表溶血抑制作用(%)。X-轴代表抗体的浓度。

图 14 显示未致敏的兔 RBCs 的旁路途径(AP)溶血的抑制作用。实心正方形标记的线代表嵌合体 166-32 IgG。实心圆形标记的线代表 cFab/9aa。实心三角形标记的线代表 cFab。Y-轴代表溶血抑制作用(%)。X-轴代表

IgG 及 Fab 的蛋白浓度。

图 15 显示抗因子 D MAb 166-32 在治疗人血浆灌输的分离兔心脏的血动态功能中的效果。左心室终舒张压(LVEDP)用实心圆形(MAb 166-32)及实心正方形(MAb G3-519)表示。左心室压(LVDP)用空圆形(MAb 166-32)及空方形(MAb G3-519)表示。MAb G3-519 为无关的同种型匹配的对照。

图 16 是 2 个抗体群在分离的兔心脏研究中产生的左心室压(LVDP)的典型代表。上图代表用阴性对照抗体 MAb G3-519 处理的心脏，而下图代表用 MAb 166-32 处理的心脏。用 MAb G3-519 处理的心脏在用 4% 人血浆处理后，不能维持 LVDP，而用 MAb 166-32 处理的心脏在灌以 4% 人血浆 60 分钟后仍维持近基线的 LVDP。

图 17 显示在用 4% 人血浆灌输分离的兔心脏的过程中，在选择的各时间点淋巴管流出液中的 Bb 浓度。MAb 166-32 处理的心脏样品(空圆形)含有的 Bb 明显比 MAb G3-519 处理的心脏(实心正方形)要低，$p<0.05$。

图 18 显示血浆样品的旁路途径溶血活性，它们于不同时间点由 MAb 166-32(实心正方形)或 MAb G3-519(实心圆形)处理的体外循环系统收集。

图 19 显示血浆样品的 C3a 浓度，它们于不同时间点由 MAb 166-32(实心正方形)或 MAb G3-519(实心圆形)处理的体外循环系统收集。

图 20 显示血浆样品的 sC5b-9 浓度，它们于不同时间点由 MAb 166-32(实心正方形)或 MAb G3-519(实心圆形)处理的体外循环系统收集。

图 21 显示血浆样品的 Bb 浓度，它们于不同时间点由 MAb 166-32(实心正方形)或 MAb G3-519(实心圆形)处理的体外循环系统收集。

图 22 显示血浆样品的 C4d 浓度，它们于不同时间点由 MAb 166-32(实心正方形)或 MAb G3-519(实心圆形)处理的体外循环系统收集。

图 23 显示 CD11b 在中性粒细胞表面的表达水平，这些粒细胞于不同时间点由 MAb 166-32(实心正方形)或 MAb G3-519(实心圆形)处理的体外循环系统收集。CD11b 的表达量以由免疫细胞计数器分析测得的平均荧光强度(MFI)而表示。

图 24 显示血小板表面的 CD62P 的表达水平，它们于不同时间点由 MAb 166-32(实心正方形)或 MAb G3-519(实心圆形)处理的体外循环系统收集。CD62P 的表达量以由免疫细胞计数器分析测得的平均荧光强度(MFI)而表示。

图 25 显示血浆样品中中性粒细胞特异的髓过氧化物酶(MPO)浓度，它

们于不同时间点由 MAb 166-32(实心正方形)或 MAb G3-519(实心圆形)处理的体外循环系统收集。

序列表说明

SEQ ID NO：1 为人因子 D 的核苷酸序列。
SEO ID NO：2 为人因子 D 的氨基酸序列。
SEQ ID NO：3 为猪因子 D 的核苷酸序列。
SEQ ID NO：4 为猪因子 D 的氨基酸序列。
SEQ ID NO：5 为用以克隆 MAb 166-32 V_K基因的引物。
SEQ ID NO：6 为如下述用以克隆 MAb 166-32 V_K基因的退火接头(adaptor)，并用作引物。
SEO ID NO：7 为如下述用以克隆 MAb 166-32 V_K基因的退火接头。
SEQ ID NO：8 为如下述用以克隆 MAb 166-32 V_H基因的 3'引物。
SEQ ID NO：9 为用以克隆 MAb 166-32 V_H基因的引物。
SEQ ID NO：10 为用以克隆 MAb 166-32 V_H基因的引物。
SEQ ID NO：11 为用以 PCR 扩增 MAb 166-32 Fd 基因的 5'引物。
SEQ ID NO：12 为用以 PCR 扩增 MAb 166-32 Fd 基因的 3'引物。
SEQ ID NO：13 为用以 PCR 扩增 MAb 166-32 Fd 基因的 5'引物。
SEQ ID NO：14 为用以 PCR 扩增 MAb 166-32 Fd 基因的 3'引物。

本发明的完成及其用途

A. 抗人因子 D 的单克隆抗体(MAbs)的制备

在本发明的一个具体实施方案中，抗-因子 D 的 MAbs 可通过用纯化自人血浆或尿的天然因子 D 免疫鼠类(例如小鼠，大鼠，仓鼠及豚鼠)或用真核或原核系统表达重组因子 D 或其片段来制备。亦可使用其它动物进行免疫，例如非人灵长类、表达人免疫球蛋白的转基因小鼠及移植了人 B 淋巴细胞的严重结合免疫缺陷(SCID)小鼠。杂交瘤可用传统方法制备，通过来自已免疫动物的 B 淋巴细胞与黑瘤细胞(例如 Sp2/0 及 NSO)的融合而获得，如 G. Kohler 及 C. Milstein(Nature，1975：256：495-497)所述。此外，抗因子 D 抗体亦可以通过在噬菌体展示系统中从人 B 淋巴细胞筛选重组单链 Fv 或 Fab 文库来获得。MAbs 对人因子 D 的专一性可由 ELISA、Western 印迹、或其它免疫化学技术进行检测。抗体对补体活化的抑制活

性，对于旁路途径可使用未致敏的兔或豚鼠红血球细胞(RBCs)的溶血分析进行评价，对于经典途径可使用未致敏的鸡或羊 RBCs 进行评价。阳性对照孔中的杂交瘤用限制稀释进行克隆。纯化抗体用于上面介绍的试验以分析对人因子 D 的专一性。

如果用于治疗人的炎症或自身免疫，抗因子 D 抗体优选以嵌合体、去免疫化、人源化或人抗体的形式使用。这样的抗体可降低免疫性，因而避免人抗鼠抗体(HAMA)反应。抗体优选为 IgG4、IgG2、或其它遗传突变的 IgG 或 IgM，它们不会增强依赖抗体的细胞毒性(S.M. Canfield 及 S.L. Morrison, J. Exp. Med., 1991：173：1483-1491)及补体介导的细胞裂解(Y.Xu 等人, J. BioL Chem., 1994：269：3468-3474; V. L. Pulito 等人, J. Immunol., 1996; 156：2840-2850)。

嵌合抗体由本领域熟知的重组技术制备，它含有动物可变区及人恒定区。人源化抗体比嵌合抗体具有更高程度的人肽序列。在人源化抗体中，只有负责抗原结合及专一性的补体决定区域(CDRs)源自动物并含有动物抗体相应的氨基酸序列，而基本上分子的所有剩余部份(除在某些例子中可变区内框架区的小部份)源自人并相当于人抗体的氨基酸序列。参见 L. Riechmann 等人, Nature, 1988; 332：323-327; G. Winter, 美国专利 5225539; C.Queen 等人, 美国专利 5530101.

去免疫化抗体为已除去 T 及 B 细胞表位的抗体，如国际专利申请 PCT/GB98/01473 所述。当其应用于体内时，没有或具有降低的免疫性。

人抗体可由数种方法制备，包括使用人免疫球蛋白表达文库(Stratagene Corp., La Jolla, California)产生人抗体的片段(VH、VL、Fv、Fd、Fab 或$(Fab')_2$，并使用类似产生嵌合抗体的技术用这些片段构建完整的人抗体。人抗体亦可在携带了人免疫球蛋白基因组的转基因小鼠中制备。此小鼠可从 Abgenix, Inc., Fremont, California, 及 Medarex, Inc., Annandale, New Jersey 获得。

亦可构建单肽链结合分子，其中重及轻链 Fv 区域相连。单链抗体("ScFv")及其构建方法述于美国专利 4946778。此外，Fab 亦可以相似方法构建及表达(M.J. Evans 等人, J. ImmunoL Meth., 1995; 184：123-138)。所有完整及部份的人抗体都比完整的小鼠 MAbs 免疫性低，而且片段及单链抗体也具有较低的免疫性。因此所有这些类型的抗体相比较而言不会引发免疫或过敏反应。同时，它们比完整的动物抗体都更适于对人进行体内给

药，尤其在需要重复或长期给药时。此外，抗体片段的较小的大小亦可促进组织的生物利用，这对于急性病中的较佳剂量累积可能非常重要。

基于抗因子 D 抗体可变区的分子结构，可使用分子模型及合理分子设计来制备和筛选能模拟抗体结合区域的分子结构并抑制因子 D 活性的小分子。这些小分子可以是肽、肽模拟物、寡核苷酸或有机化合物。这种模拟分子可以在炎症及自身免疫疾病中用作补体活化的抑制剂。此外，亦可使用本领域常用的大规模筛选方法来从组合化合物文库中分离适当的小分子。

本发明的一个优选实施方案中，使用一具有动物(小鼠)可变区及人恒定区的嵌合体 Fab 来治疗疾病，Fab 之所以优选是因为：

1. 它比完整的免疫球蛋白小，并且可提供更好的组织穿透性；
2. 作为单价分子，它具有较小的形成免疫复合物和聚合物的可能性；
3. 它可在微生物系统中制备，微生物系统比真核系统更容易规模化。

B. 抗因子 D 分子的运用

抗因子 D 结合分子、抗体、及本发明片段可以适当制剂通过各种途径给药至病人，包括但不限于静脉灌注、静脉大量注射、及腹膜、皮肤、肌肉、皮下、鼻内、支气管、椎管内、颅内及经口给药。这种给药可使它们结合至内源性因子 D，并因而抑制 C3b、C3a 及 C5a 过敏毒素以及 C5b-9 的生成。

这种抗体及分子的估计优选剂量为 10 及 500 微克/毫升血清之间。实际剂量可依临床常用的测量最佳剂量的方法测得，即给予各种剂量并确定最有效的剂量。

抗因子 D 分子可抑制体内补体活化及／或补体旁路途径以及伴随的炎症，如巨噬细胞、中性粒细胞、血小板以及肥大细胞的吸引及活化、水肿及组织损伤。可使用这些抑制剂来治疗因补体系统过度或不加控制地活化而介导的疾病或情况。它们包括但不限于：(1)因心血管栓塞后缺血再灌流的组织伤害、动脉瘤、中风、出血休克、撞击伤害、多处器官衰竭、血容积不足休克及肠缺血；(2)炎症性失调如烧伤、内毒素血症及败血性休克、成人呼吸困难症、心肺分流、血液透析、过敏性休克、严重哮喘、血管水

肿、局限性回肠炎、镰刀性细胞贫血、后链球菌肾小球肾炎及胰炎；(3)移植排斥如超急性移植排斥；及(4)不良药物反应如药物过敏、IL-2 引发的血管渗漏症及放射性造影介质过敏。自身免疫异常包括但不限于：系统性红斑狼疮、重症肌无力、类风湿性关节炎、早老性痴呆及多发性硬化，它们也可用本发明的抑制剂进行治疗。

抗因子 D 分子亦可用于诊断以确定因子 D 在组织或体液样品如血清、血浆、尿或骨髓中的存在或进行定量。此申请书中，可使用熟知分析形式分别如免疫组织化学或 ELISA。这种诊断试验可用于确定特定的个体是否缺乏或过度产生因子 D。

C. 因子 D 抑制剂的治疗有效性的动物模型

上面介绍的因子 D 抑制剂在各种疾病中的治疗活性可使用能够得到的患有各种炎症及自身免疫的各种动物进行证明。下面实施例中介绍的体外试验足以阐明其有效性。

与人的各种补体相关临床疾病相当的动物模型亦可用于证明因子 D 抑制剂的体内有效性。它们包括但不限于：心血管缺血再灌流损伤(H. F. Weisman 等人，Science，1990；249：146-151)、心血管栓塞(J. W. Homeister 等人，J. ImmunoL 1993；150：1055-1064)、系统性红斑狼疮及肾小球肾炎(S. K. Datta. Meth. EnzymoL，1988；162：385-442；D. J. Salvant 及 A. V. Cybulsky，Meth. EnzymoL，1988；162：421-461)、类风湿性关节炎(Y. Wang 等人，Proc. Natl. Acad. Sci.，1995；92：8955-8959)、成人呼吸困难症(R. Rabinovici 等人，J. ImmunoL，1992；149：1744-1750)、超急性器官移植排斥(T. J. Kroshus 等人，Transplantation，1995；60：1194-1202)、灼伤(M. S. Mulligan 等人，J. ImmunoL，1992；148：1479-1485)、以及心肺分流(C. S. Rinder 等人，J. Clin. Invest，1995；96：1564-1572)。

下面介绍如何完成和使用本发明，以及证实其用途。

实施例 1：抗因子 D MAbs 的制备

将 8-12 周龄的雄 AIJ 小鼠(Harlan，Houston，TX)皮下注射以 25 克纯化自人血清(Advanced Research Technologies，San Diego，CA)的因子 D，该因子 D 溶于 Freund 氏完全佐剂(Difco Laboratories，Detroit，

Michigan)并溶于 200 微升磷酸盐缓冲盐水(PBS)pH7.4 中。此因子 D 制备物用 + 二硫烷基苯磺酸钠(SDS)-聚丙烯酰胺凝胶电泳(PAGE)检测为纯度 >95%。此因子 D 按下面的介绍进行试验并发现其在溶血中具有生物活性。按 2 周的间隔对小鼠皮下注射以 Freund 氏不完全佐剂中的 25 微克人因子 D 两次。然后在 2 周后和杀死前 3 天，对小鼠再由腹膜注射以 PBS 中的 25 微克相同抗原。在每次融合中，从每只免疫小鼠的脾脏制备单细胞悬液，并用来与 Sp2/0 黑瘤细胞融合。在含 50% 聚乙二醇(M.W. 1450)(Kodak, Rochester, NY)及 5% 二甲基亚砜(Sigma Chemical Co., St. Louis, MO)的培养液中将 5 x 10^8 的 Sp2/0 及 5 x 10^8 脾细胞融合。然后在补充了 10% 小牛血清、100 单位/毫升青霉素、100 微克/毫升链霉素、0.1 毫摩尔次黄嘌呤、0.4 摩尔氨基喋呤以及 16 摩尔胸腺嘧啶的 Iscove 培养液中(Gibco, Grand Island, NY)将细胞调至 1.5 x 10^5 脾细胞/200 微升悬浮液的浓度。将 200 毫升细胞悬浮液加至约 20 个 96 孔微培养平板的孔中。约 10 天后取出培养上清液，用于在 ELISA 中筛选能与纯化的因子 D 反应者。

将 Immulon 2(Dynatech Laboratories, Chantilly, VA)微测试平板的孔通过加入 50 微升纯化的人因子(50 微毫克/毫升)在室温下过夜进行包被。低浓度的包被用因子 D 可筛选具高亲合性的抗体。拍打平板除去包被溶液后，加入 200 微升 PBS 中的 BLOTTO(脱脂奶粉)至各孔中 1 小时以封闭非专一性位点。1 小时后，用缓冲液 PBST(含 0.05% Tween 20 的 PBS)清洗孔。从各融合孔收集 50 微升的培养上清液，与 50 微升 BLOTTO 混合，再加至微测试平板各孔中。温育 1 小时后，用 PBST 清洗孔。然后通过与用 BLOTTO 按 1：2000 稀释的辣根过氧化物酶(HRP)偶连的羊抗小鼠 IgG(Fc 专一性)(Jackson Immunoresearch Laboratories, West Grove, PA)反应来检测结合的鼠抗体。将含 0.1% 的 3, 3, 5, 5 四甲基联苯胺(Sigma, St. Louis, MO)和 0.0003% 过氧化氢(sigma)的过氧化物酶底物溶液加至各孔中显色 30 分钟。加入 50 微升的 2M H_2SO_4 至各孔中以中止反应。使用 BioTek ELISA 计数器(BioTek Instruments, Winooski, VM) 在 450 nm 处读取反应液 OD.

然后将阳性对照孔的培养上清液用 2 个试验进行检测：i)按下面介绍的方法，检测其在预测定浓度的人血清对未致敏的兔 RBC 的旁路途径溶血中的抑制作用；及 ii)按下面的方法，检测其在用人血清处理的酵母聚糖诱导的 C3a 生成中的抑制作用。将阳性孔中的细胞通过限制稀释进行克

隆。再次检测 MAbs 在 ELISA 中与因子 D 的反应性。将所选杂交瘤在转瓶中培养，收集培养上清液，用蛋白 A 亲合性柱层析纯化抗体。用 ELISA 试验得到 4 个对人因子 D 具强烈反应的 MAbs。这些 MAbs 分别被命名为 166-11、166-32、166-188、及 166-222(图 1)。其中，MAb 166-32(IgG1)按下面的介绍可强烈抑制未致敏的兔 RBCs 旁路途径溶血。

实施例 2：用表面细胞质基因组共振方法测定抗因子 D MAbs 的动力常数

MAbs 166-11、166-32、166-188、及 166-22 结合至人因子 D 的动力常数可用基于表面细胞质基因组共振的测量方法，使用 BIAcore 仪器(Pharmacia Biosensor AB, Uppsala, Sweden)测得。所有结合测量方法都在 HEPES-缓冲盐水(HBS)(10 毫摩尔 HEPES、pH 7.4、150 毫摩尔 NaCl、3.4 毫摩尔 EDTA，0.005%表面活化剂 P20)中在 25℃进行。为测量因子 D 结合至 MAbs 的结合率常数，将兔抗小鼠 IgG(H+L)固定至 CM5 侦测板(sensorchip)，固定使用 N-羟基琥珀酰亚胺及 N-乙基-N'-(3-二乙胺丙基)碳二亚胺通过胺偶联来进行。然后将每种 MAb 先捕捉至已包被的侦测板，再注射以不同浓度的因子 D。为测量缔合率常数(K_{assoc})，按厂商指示的浓度制备因子 D 的 5 个稀释浓度(2.5 纳摩尔，5 纳摩尔，10 纳摩尔，15 纳摩尔及 20 纳摩尔)，以 5 微升/分的速率注射至孔中。为测量解离率常数(k_{dissoc})，以 5 微升/分的速率注射 100 纳摩尔因子 D 至孔中。感测图形式的数据可使用 BIAcore 系统中数据嵌合程序分析。因为 MAb 166-32 有极快的 K_{assoc}，由于大量输入效应的限制，它已超出该试验程序的置信度限制，因此可使用另一结合程序来测量其动力速率常数。将因子 D 按上面的介绍通过胺偶联固定至侦测板，同时将不同稀释浓度的 MAb 166-32(5 纳摩尔，10 纳摩尔，15 纳摩尔，20 纳摩尔及 25 纳摩尔用于测量 K_{assoc} 及 200 纳摩尔用于测量 K_{dissoc})以 5 微升/分的速率流至侦测板。感测图形式的数据可按上面的介绍进行分析。BIAcore 上因子 D 结合至 MAbs 的动力常数列于如下表 1。MAbs 166-32 及 166-222 对因子 D 具极高亲合性，其平衡解离常数(K_D)小于 0.1 纳摩尔。

表 1 BIAcore 上因子 D 结合至 MAbs 的动力常数

MAbs	K_{assoc} (X 10^5 $M^{-1}S^{-1}$)	K_{dissoc} (X 10^4S^{-1})	K_D (X 10^{-10}M) [c]
166-32[a]	>10	1.1	<1

166-32[b]	4.6	0.76	1.6
166-188[a]	8.75	2.1	2.4
166-11[a]	8.0	1.0	1.24
166-222[a]	>10	0.8	<1

[a]在测定过程中，使用因子 D 为分析物，将其流至包被了抗因子 D MAb 的侦测板，抗因子 D MAb 用兔抗小鼠 IgG 俘获。

[b]使用 MAb 166-32 为分析物，因子 D 通过胺偶联方法交联至侦测板。

[c]K_D，平衡解离常数，$=k_{dissoc}/k_{assoc}$

实施例 3：补体活化的溶血作用的抑制

为研究抗因子 D MAbs 在体外抑制补体活化的功能活性，使用了 2 个溶血试验。

对于旁路途径，将未致敏的兔 RBCs 用明胶/巴比妥钠缓冲盐水(GVB/Mg-EGTA)清洗 3 次，该缓冲盐水中含 2 毫摩尔 $MgCl_2$ 及 1.6 毫摩尔 EGTA。10 毫摩尔的 EGTA 被用来抑制经典途径 (K. Whaley 等人，于 A.W. Dodds (Ed.), Complement. A Practical Approach. Oxford University Press, Oxford, 1997, pp. 19-47)。将清洗细胞按 1.7 x 10^8 细胞/毫升再悬浮于相同缓冲液。在圆底 96 孔微测试平板的各孔中，将 50 微升正常人血清(20%)与 50 微升 GVB/Mg-EGTA 或系列稀释的试验 MAb 混合，然后将 30 微升已清洗的兔 RBCs 悬浮液加至含有混合物的孔中。将 50 毫升正常人血清(20%)与 80 微升 GVB/Mg-EGTA 混合，以得到血清显色背景。阴性对照可使用同种型匹配的抗 HIV-1 gp120 MAb---G3-519。最终混合物在 37℃温育 30 分钟。然后将平板在微测试平板摇晃器上摇晃 15 秒。然后将平板以 300 x g 离心 3 分钟。收集上清液(80 微升)，移至平底 96 孔微测试平板的孔中以测量 405 nm 处的 OD。溶血的抑制百分比定义为 100 x [(无 MAb 的 OD-血清显色背景 OD)-(有 MAb 的 OD-血清显色背景 OD)]/(无 MAb 的 OD-血清显色背景 OD)。

图 2 显示了如下数据，MAb 166-32 以依赖剂量方式，强烈抑制未致敏的兔 RBCs 在存在 10%人血清时的旁路途径溶血，而无关的同种型匹配对照(MAb G3-519)无此抑制作用。MAb G3-519 对 HIV 包膜糖蛋白 gp120 有专一性。

在检测 MAb 166-32 在 90%人血清中的抑制活性的试验中，将冷冻人

血清融化，以终浓度为 10 毫摩尔的 EGTA 预处理。将 10 毫升系列稀释的 MAb 166-32 或 G3-519 加至 90 微升用 EGTA 处理的人血清，并在 96 孔微测试平板中进行两个重复的孔，在室温下放置 15 分钟。将 30 毫升已清洗的兔 RBCs 加至各孔。将平板在 37℃温育 30 分钟。然后将平板在平板摇晃器上摇晃 15 秒，在 300 x g 下离心 3 分钟。收集上清液（80 微升），移至平底 96 孔微测试平板以测量 405nm 处的 OD。各平板均有 2 个含 100 微升的 90％人血清及 30 微升缓冲液作为血清显色背景的孔，以及 2 个含有用 100 微升 90％人血清在不存在单克隆抗体时裂解 RBCs 以代表完全裂解的孔。图 3 显示了如下数据，MAb 166-32 可以依赖剂量方式强烈抑制未致敏的兔 RBCs 在存在 90％人血清时的旁路途径溶血。

对于经典途径，将含有 0.5 毫摩尔 $MgCl_2$ 及 0.15 毫摩尔 $CaCl_2$ 的明胶/巴比妥钠缓冲盐水(GVB++)中的鸡 RBCs(5 x107 细胞/毫升)用 8 微克/毫升的纯化兔抗鸡 RBC 免疫球蛋白(Inter-Cell Technologies, Hopewell, NJ)在 4℃下致敏 15 分钟。然后用 GVB++清洗细胞。所用人血清的终浓度为 2％。

图 4 显示了以下数据，MAb 166-32 及无关同种型匹配的对照(G3-519)不会抑制致敏鸡 RBCs 的经典途径溶血，但阳性对照抗人 C5 MAb 137-76 会抑制。图 2、3 及 4 的数据指出，MAb 166-32 可专一地抑制补体活化的旁路途径。

实施例 4：MAb 166-32 对因子 D 的专一性

按下面的介绍，使用 2 个溶血试验来证明 MAb 166-32 对人因子 D 的专一性。

(1)使用未致敏兔 RBCs 的因子 D 依赖的溶血试验抑制

首先将人血清样品去除因子 D，这通过将其通过填充了偶联有抗因子 D MAb 166-222 的 3M Emphaze Biosupport Medium (Pierce, Rockford, IL)的亲合性柱来完成。流经的血清经检测因完全去除因子 D 而无法活化旁路途径溶血。此试验的过程类似上面实施例 3 中介绍的方法，除了将各浓度的纯化因子 D 加至除去了因子 D 的血清以重组其溶血活性。在此情况下，兔 RBCs 的溶血依赖于因子 D。重组溶血活性显示对补充的因子 D 浓度呈线性比例(由 0.01 微克/毫升至 2 微克/毫升)(图 5)。图 5 数据亦显示，0.3 微克/毫升的 MAb 166-32 可在存在 0.1 微克/毫升补充的因子 D 情况下完

全抑制未致敏兔RBCs的溶血，而阴性对照MAb G3-519对依赖因子D的溶血无影响。这些数据说明，在摩尔比率为1：2时（MAb 166-32对因子D），MAb 166-32可有效抑制人因子D的生物活性。因此MAb 166-32对因子D来说是强效的高亲合性抗体。这种抗体具有用于临床治疗由补体旁路途径引起的疾病或情况的潜力。

(2)EAC3b细胞上旁路C3转化酶形成的抑制

EAC3b细胞为包被了人C3b(购自National Jewish Center of Immunology and Respiratory Medicine，Denver，CO)的绵羊RBCs。在本试验中，通过加入因子B、因子P(备解素)及因子D，旁路C3转换酶装配于EAC3b细胞表面。然后EAC3b细胞(5×10^8)用$DGVB^{++}$液(50%巴比妥钠缓冲盐水、pH7.2，包含0.075毫摩尔$CaCl_2$、0.25毫摩尔$MgCl_2$、0.1%明胶、2.5%(w/v)葡聚糖，及0.01%叠氮钠)清洗3次。将清洗后的细胞悬浮于1.5毫升的$DGVB^{++}$、因子P(30微克)及因子B(20微克)中。因子P及因子B浓度预先测定为过量。将50毫升细胞悬浮液加至圆底96孔微测试平板各孔中。然后将50微升因子D（1.2微毫克/毫升)与系列稀释的MAb 166-32或MAb G3-519的混合物加至含细胞的孔中，在30℃温育15分钟。因子D的浓度(1.2微毫克/毫升)在此情况下预测能够产生高于90%的溶血。温育后，将细胞在GVB-EDTA培养液(含10毫摩尔EDTA的明胶/巴比妥钠缓冲盐水)中清洗2次。然后将细胞悬浮于30微升的GVB-EDTA培养液。为起始溶血，将100微升豚鼠血清(Sigma)(1：10稀释于GVB-EDTA)加至各孔中。然后将混合物在37℃温育30分钟。然后将微测试平板于300 x g离心3分钟。收集上清液，测量405nm处的OD。

图6显示了MAb 166-32抑制EAC3b细胞裂解的实验结果，而无关的MAb G3-519不产生抑制。MAb 166-32抑制因子D对因子B的切割，因而避免C3转换酶在EAC3b细胞表面的形成。

实施例5：MAb 166-32对通过补体活化的酵母聚糖生成C3a的抑制

为进一步确定MAb 166-32对因子D的功能专一性，检测了MAb对酵母聚糖(活化的酵母颗粒)上的补体旁路活化作用的影响。将酵母聚糖A(来自啤酒酵母，Sigma)(1毫克/毫升)在GVB/Mg-EGTA中清洗3次，然后按1毫克/毫升悬浮于相同培养液。将25微升不同浓度的MAb 166-32或G3-519与25微升人血清(1：5稀释于GVB/Mg-EGTA)在微试管中混合，在室温下

温育 15 分钟。对照组不含抗体，但含培养液及血清。温育后，将 50 微升清洗的酵母聚糖悬浮液加至各管中，在 37℃温育 30 分钟。将微管于 2000 x g 离心 5 分钟，收集上清液，与等量的样品稳定液(Quidel，San Diego，CA)混合。将样品冷冻于-25℃直至分析。样品中 C3a 及 sC5b-9 的浓度可用定量型 ELISA 试剂盒(Quidel)依厂商说明书进行测定。

图 7 显示，MAb 166-32 可抑制 C3a 自补体活化酵母聚糖的产生，而无关的 MAb G3-519 则无此效果。这些数据表明，MAb 166-32 可抑制因子 D 的 C3 转换酶生成。MAb 166-32 对因子 D 的完全抑制可有效的阻碍 C3 转换酶的产生，这可通过其不能产生 C3a 来显示。这将导致补体级联反应随后步骤中的 C5 转换酶的抑制，这可由 sC5b-9(MAC)形成的抑制证明(图 8)。

实施例 6：MAb 166-32 的 Fab 对补体活化的溶血的抑制

为检测 MAb 166-32 单价形式可否如亲本双价体 MAb 166-32 一样有效抑制补体旁路途径，MAb 166-32 的 Fab 使用商品化的试剂盒(Pierce)通过木瓜蛋白酶消化进行制备。然后按上面的介绍使用未致敏的兔 RBCs 来检测 Fab 对旁路途径溶血的抑制活性。

图 9 显示了实验的数据，全 IgG 及 Fab 都显示相似的阻断旁路补体活化的能力。这些结果表明，MAb 166-32 的单价形式具有活性，它保留了与其亲本双价抗体相似的拮抗因子 D 的能力。此性质对于考虑使用 Fab 或单链 Fv 作为替代产品来说非常重要。使用后一单价形式的一个优点是，因为其形体小而具有更好的组织渗透性。鉴于 MAb 166-32 的 Fab 有活性，该 MAb 在因子 D 上识别的结合表位可能是其重要功能部份。

实施例 7：MAb 166-32 对不同种类动物血清的旁路途径溶血的影响

为研究 MAb 166-32 与来自不同种类动物的因子 D 的交叉反应，使用不同种类动物的血清进行旁路途径溶血分析。首先使用不同种类动物的新鲜血清(人、恒河猴、黑猩猩、狒狒、cynomolgus 猴、羊、狗、小鼠、田鼠、大鼠、兔、豚鼠及猪)检测其 CH50 值，CH50 值定义为能使未致敏的兔 RBCs 达到 50％溶血的血清稀释倍数。然后检测并比较 MAb 166-32 对每种血清的相同溶血活性(CH50)的抑制活性。

图 10 显示 MAb 166-32 对人、恒河猴及黑猩猩的血清具有强烈抑制活性，并对 cynomlgus 猴、狒狒、羊及狗的血清具有中度抑制活性。该抗体

不能抑制小鼠、田鼠、大鼠、兔、豚鼠及猪的血清。这些数据表明，MAb 166-32可结合人、恒河猴、黑猩猩、狒狒、cynomolgus 猴、羊及狗的因子 D 的一个共有表位。

实施例 8：构建人因子 D 突变体对 MAb 166-32 进行表位作图

为描绘 MAb 166-32 所识别的人因子 D 的结合表位，首先检测抗体与人因子 D 在 Western 印迹上的反应性。MAb 166-32 不与固定在硝纤维素膜上的 SDS 变性的人因子 D(还原或不还原)反应。这个结果表明 MAb 166-32 结合天然的而不是变性的因子 D。

因为 MAb 166-32 如实施例 7 所示不抑制小鼠及猪因子 D 的溶血活性，因此 MAb 166-32 极可能结合于人因子 D 中与小鼠及猪因子 D 具有高氨基酸序列差异的位点。根据这一点，按照下面的介绍，通过用猪的因子 D 的相应氨基酸残基取代人因子 D 中的氨基酸残基制备各种因子 D 突变体及杂合体，用来进行 MAb 166-32 结合表位作图。

(1)构建因子 D 突变体及杂合体

人因子 D 基因片段通过聚合酶链反应(PCR)使用人脂细胞 cDNA(Clontech, San Francisco, CA)为模板和适当的寡核苷酸引物获得。扩增的 DNA 片段用 BamHI 及 EcoRI 限制性切割，并将切割产物插入杆状病毒转移载体 pVL1393(Pharmingen, San Diego, CA)的 BamHI 及 EcoRI 位点，得到野生型 pVL1393-因子 D/Hu。人因子 D 命名为因子 D/Hu。成熟的人因子 D 蛋白的核苷酸序列及推导的氨基酸序列示于 SEQ ID NOS：1 及 2(R.T. White 等人，J. Biol. Chem.，1992;267:9210-9213; GenBank 读取号码：M84526)。

猪因子 D 的 cDNA 克隆 pMon24909 由内布拉斯加大学 J.L. Miner 赠送(GenBank 读取号码：U29948)。将 pMon24909 的 BamHI-EcoRI 片段克隆至 PVL1393，得到 pVL1393-因子 D/pig。猪因子 D 命名为因子 D/pig。成熟的猪因子 D 蛋白的核苷酸序列及推导的氨基酸序列示于 SEQ ID NOS：3 及 4。

三个人因子 D 的突变体使用适当引物和重叠 PCR 进行构建。氨基酸突变被设计为用猪序列中的相应氨基酸残基取代人序列中的氨基酸基残基，这种相应是指人和猪的因子 D 的氨基酸序列对应排列进行同源比较时。第一个突变体为因子 D/VDA，它具有三个氨基酸突变：V113E、D116E 及 A118P。(这是命名突变的一种简写方法，例如其中 V113E 指人因子 D 第 113

位氨基酸基的缬氨酸变为猪因子 D 的谷氨酸)。第二个突变体为因子 D/RH，它具有两个氨基酸突变：R156L 及 H159Y。第三个突变体为因子 D/L，它具有单一一个氨基酸突变：L168M。编码这些突变体的 DNA 序列用 DNA 测序进行确认。用适当的酶切割后，将 DNA 片段嵌入杆状病毒转移载体 pVL1393 的 BamHI 及 EcoRI 位点，分别得到 pVL1393-因子 D/VDA、pVL1393-因子 D/RH 及 PVL1393-因子 D/L。

两个嵌合的人-猪因子 D 杂合体也由适当引物以重叠 PCR 构建得。第一个杂合体为因子 D/Hupig，它含有位于于 N-端的 52 个来源于人因子 D 的氨基酸，其余氨基酸来源于猪因子 D。另一个杂合体为因子 D/Pighu，它含有位于 N-端的 52 个来源于猪因子 D 的氨基酸，其余氨基酸来源于人因子 D。将 BamHI 及 EcoRI 消化的 DNA 片段嵌入杆状病毒转移载体 pVL1393 的 BamHI 及 EcoRI 位点，得到 PVL 1393-因子 D/Hupig 及 PVL 1393-因子 D/Pighu。

(2)因子 D 突变体及杂合体的表达

质粒转染、重组杆状病毒制备以及重组因子 D 蛋白在昆虫细胞 Sf9 中制备的步骤都依厂商说明书施行(杆状病毒表达载体系统，Pharmingen)。

(3)因子 D 突变体及杂合体的纯化

来自感染的 Sf9 细胞培养上清的因子 D 突变体和杂合体蛋白，使用纯化的羊抗人因子 D 多克隆抗体(The Binding Site Limited，San Diego，CA)以亲合性柱层析进行纯化。将 3 毫升羊抗人因子 D 抗体(13.2 毫克/毫升)在偶联缓冲液(0.1 M 硼酸盐及 0.75M Na_2SO_4，pH 9.0)中平衡，并与 4 毫升 Ultralink Biosupport Medium(Pierce) 在室温下偶联 2 小时。胶粒先用 50 毫摩尔二乙胺(pH 11.5)清洗用以将所有残余的未反应部位饱和，再以含 10 毫摩尔 Tris、0.15 M NaCl、5 毫摩尔 EDTA、1% Triton X-100 及 0.02% NaN_3(pH 8.0)的缓冲液清洗。将胶粒在缓冲液中于 4℃下保存。

将收集自 100 毫升旋转培养的各杆状病毒突变株感染的 Sf9 细胞的培养上清液，通过羊抗因子 D 亲合性柱，其中羊抗因子 D 亲合性柱事先已用 PBS 预平衡以去除保存缓冲液。结合的因子 D 蛋白用 50 毫摩尔二乙胺，pH 11.5 洗脱。收集的级份立即用 1 M Hepes 缓冲液中和至 pH 7.0。残留的盐使用 Millipore 膜超滤(M.W. cut-off：3,000)(Millipore Corp.，Bedford，MA)用 PBS 进行缓冲液置换来去除。蛋白浓度由 BCA 法(Pierce)测定。

(4)因子 D 的 ELISA

MAb 166-32 与不同因子 D 突变体及杂合体的反应活性可用 ELISA 试验进行检测。将 96 孔微测试平板的不同孔用蛋白(因子 D/Hu、因子 D/pig、因子 D/Hupig、因子 D/Pighu、因子 D/VDH、因子 D/RH、及因子 D/L)包被，包被通过加入 100 微升的 PBS 溶液中的各种蛋白(0.5 微克/毫升)来完成。室温温育过夜后，将孔用 PBSTB (PBST 含 2%BSA)处理，以饱和残留的结合位点。将孔用 PBST 清洗。将 100 毫升系列稀释的 MAb 166-32(1 微克/毫升~0.5 微毫克/毫升)加至孔中，置于室温 1 小时。然后将孔用 PBST 清洗。结合抗体通过与稀释的 HRP-羊抗小鼠 IgG(Fc)(Jackson Immunoresearch)在室温温育 1 小时进行检测。然后按上面的介绍将过氧化物酶受体溶液加入进行显色反应。使用 ELISA 读取仪测定 450 nm 处的 OD。

图 11 显示，MAb 166-32 与因子 D/Hu、因子 D/Pighu 及因子 D/VDA 反应，但不与因子 D/pig、因子 D/Hupig、因子 D/RH 及因子 D/L 反应。ELISA 结果显示,人因子 D 的氨基酸残基 Arg156、His159 及 Leu168 为 MAb 166-32 结合所必需。这与 MAb 166-32 在人因子 D 的 C-端为猪的相应部位取代时不与因子 D/Hupig 结合的事实相一致。氨基酸残基 Arg156、His159 及 Leu168 位于所谓"甲硫氨酸环"，该环由 Cys154 及 Cys170 之间的二硫键以及位于 169 位的甲硫氨酸组成(J.E. Volanakis 等人，于 The Human Complement System in Health and Disease，J.E. Volanakis 及 M.M. Franks，eds.，Marcel Dekker，1998，pp. 49-81)。就结构言，"甲硫氨酸环"为一种紧密的 1 型 β 转角。根据 X 光晶体衍射图谱研究的数据，人们发现它暴露于因子 D 分子表面(S.V.L. Narayana 等人，J. Mol. Biol.，1994，235：695-708)。但"甲硫氨酸环"对受体专一性及因子 D 催化活性的贡献则尚未有研究(J.E. Volanakis 等人，Protein Sci.，1996;5：553-564)。此数据首次证明，"甲硫氨酸环"在因子 D 功能活性中扮演重要角色。MAb 166-32 及其 Fab 在结合至因子 D 的该区域时，可有效地抑制因子 B 的催化活性。

实施例 9：抗因子 D MAb 166-32 可变区基因的克隆以及嵌合体 166-32 IgG 及其 Fab 的构建及表达

为减低在人体中使用时 MAb 166-32 的免疫原性，通过用人 IgG1 的恒

定区取代小鼠恒定区制备了 MAb 166-32 的嵌合体. 抗体的两种 Fab 嵌合体形式也用人的相应区域取代小鼠恒定区进行制备. MAb 166-32 可变区基因的克隆以及嵌合型 166-32 抗体及其 Fab 的构建及表达详述于后.

(1)抗因子 D 的 MAb 可变区基因的克隆

从分泌抗因子 D 的 MAb 166-32 的杂交瘤细胞分离总 RNA, 使用 RNAzol 按厂商说明书进行分离(Biotech, Houston, TX). cDNA 的第一链以寡聚 dT 为引物从总 RNA 合成. PCR 使用免疫球蛋白恒定(C)区衍生的 3'引物以及衍生自前导肽或鼠 V_H 或 V_K 基因第一架构区域的简并(degenerated)引物组作为 5'引物来进行. 虽然扩增的 DNA 标明为 V_H, 但对 V_K 来说, 没有扩增预期长度的 DNA 片段. V_H 及 V_K 基因都通过锚定 PCR 进行克隆.

嵌合锚定 PCR 可按 Chen 及 Platsucas(Scand. J. Immunol., 1992;35: 539-549)的介绍进行. 在克隆 V_K 基因时, 将双链 cDNA 使用 NotI-MAKI 引物(5'-TGCGGCCGCTGTAGGTGCTGTCTTT-3' SEQ ID NO: 5)进行制备. 将退火接头 AD1(5'-GGAATTCACTCGTTATTCTCGGA-3' SEQ ID NO: 6)及 AD2(5'-TCCGAGAATAACGAGTG-3' SEQ ID NO: 7)连接至双链 cDNA 的 5'及 3'端. 3'端的接头用 NotI 消化除去. 消化产物作为模板用于 PCR 中, 以 AD1 寡核苷酸为 5'引物, 以 MAK2(5'-CATTGAAAGCTTTGGGGTAGAAGTTGTTC-3' SEQ ID NO: 8)为 3'引物. 将约 500 bp 的 DNA 片段克隆至 pUC19. 挑选 12 个克隆用于进一步分析. 有 7 个克隆被发现含有对 Sp2/0 V 信号有专一性的 CDR3 序列, 并且很可能来自 166-32 杂交瘤细胞系融合部份的畸形 k 轻链信号. NotI-MAKI 及 MAK2 寡核苷酸衍生自鼠 C_k 区域, 并分别在 C_K 基因第一个 bp 下游的 182 及 84 bp. 用 DNA 测序对 3 个克隆进行分析, 得到的序列包括鼠 C_K 的一部分、全部 V_K 以及前导肽.

为克隆 V_H 基因, 使用 NotI-MAG1 引物(5'-CGCGGCCGCAGCTGCTCAGAGTGTAGA-3' SEQ ID NO: 9)制备双链 cDNA. 将退火接头 AD1 及 AD2 连接至双链 cDNA 的 5'及 3'端. 接头的 3'端用 NotI 消化除去. 将消化产物在 PCR 中用作模板, 以 AD1 寡核苷酸及 MAG2(5'-CGGTAAGCTTCACTGGCTCAGGGAAATA-31 SEQ IDNO: 10)为引物. 将长度为 500~600bp 的 DNA 片段克隆至 pUC19. NotI-MAG1 及 MAG2 寡核苷酸源自鼠 Cγ1 区域, 并且分别在 Cγ1 基因第一个 bp 下游的 180 及 93 bp. 用 DNA 测序对 3 个克隆进行分析, 得到的序列包含鼠的 Cγ1 的一部分、全部的 V_H 以及前导肽.

(2)构建嵌合型 166-32 IgG 及 Fab 的表达载体

在 PCR 中使用 V_H 及 Vκ基因为模板，以在 5'端加入 Kozak 序列和在 3'端加入剪接供体。在分析序列确定无 PCR 错误后，将 V_H 及 Vκ基因插入分别含有人 Cγ1 及 C 的表达载体盒(cassette)，得到 pSV2neoV_H-huCγ1 及 pSV2neoV-huCκ。将 CsCl 梯度-纯化的重链及轻链载体的质粒 DNAs 用电穿孔法感染 COS 细胞。48 小时后，ELISA 检测培养上清液中含有约 200 微毫克/毫升的嵌合体 IgG。收集细胞，制备总 RNA。使用寡聚 dT 为引物从总 RNA 合成第一链 cDNA。在 PCR 中将此 cDNA 用作模板，制备 Fd 及κ DNA 片段。对于 Fd 基因，使用(5'-AAGAAGCTTGCCGCCACCATGGATTGGCTGTGGAACT-3'SEQ ID NO：11)为 5'端引物及源自 CH1 的 3'端引物(5'-CGGGATCCTCAAACTTTCTTGTCCACCTTGG-3'SEQ ID NO：12)进行 PCR。DNA 序列被证明含有完整的 V_H 及人 IgG1 的 CH1 结构域。用适当酶消化后，将 Fd DNA 片段嵌入表达载体盒 pSV2dhfr-TUS 的 HindIII 及 BamHl 限制酶切位点，得到 pSV2dhfrFd(图 12A)。

对于κ基因，PCR 使用(5-AAGAAAGCTTGCCGCCACCATGTTCTCACTAGCTCT-3'SEQ ID NO：13)为 5'端引物及源自 Cκ的 3'端引物(5'-CGGGATCCTTCTCCCTCTAACACTCT-3' SEQ ID NO：14)来进行。DNA 序列被证明含有完整的 Vκ及人 Cκ区域。。用适当的限制酶消化后，将κ DNA 片段嵌入表达载体盒 pSV2neo-TUS 的 HindIII 及 BamHI 限制酶切位点，得到 pSV2neoκ(图 126)。Fd 及κ基因的表达可由源自 HCMV 的增强子及启动子驱动。因 Fd 基因不含参与链间二硫键的色氨酸残基，此重组嵌合体 Fab 含有非共价连接的重链及轻键。此嵌合体 Fab 名为 cFab。

为获得带有重链及轻键间二硫键的重组 Fab，扩展上述 Fd 基因，使其包含编码另外 9 个来源于人 IgG1 铰链区域的氨基酸的序列(EPKSCDKTH SEQ ID NO：12)。将 Fd 基因 3'端编码 30 个氨基酸的 BstEII-BamHI DNA 片段用编码扩展 Fd 的 DNA 片段进行取代。含有额外 9 个来源于人 IgG1 铰链区氨基酸的扩展 Fd 用 DNA 测序进行证实。将此 Fd/9aa 基因插入表达载体盒 pSV2dhfr-TUS，得到 pSV2dhfrFd/9aa。此嵌合体 Fab 命名为 cFab/9aa。

(3)嵌合体 166-32 IgG 及 Fab 的表达

为获得分泌嵌合体 166-32 IgG 的细胞系，将 NSO 细胞用 pSV2neoV_H-huCγ1 及 pSV2neoV-huCκ的纯化质粒 DNAs 通过电穿孔法进行转染。在存在 0.7 毫克/毫升 G418 下筛选转感染细胞。使用含血清培养液将细胞培养

于250-毫升旋转瓶。

为获得分泌嵌合体166-32 Fab的细胞系，将CHO细胞用pSV2dhfrFd(或pSV2dhfrFd/9aa)及pSV2neoκ的纯化质粒DNAs通过电穿孔法进行转染。在存在G418及氨甲喋呤下筛选转感染细胞。将所筛选细胞系在增加氨甲喋呤的条件下进行扩增。通过限制稀释对细胞进行单细胞亚克隆。然后使用含血清培养液将高产的单细胞亚克隆细胞系培养于100-毫升旋转培养液。

(4)嵌合体166-32 IgG的纯化

将100毫升旋转培养上清液上样至10毫升PROSEP-A柱(Bioprocessing, Inc., Princeton, NJ)。将柱用10倍床体积的PBS清洗。结合的抗体用50毫摩尔柠檬酸盐缓冲液、pH 3.0进行洗脱。将等量的1M Hepes、pH 8.0加至含纯化抗体的级份以将pH调至7.0。残留的盐通过用PBS进行缓冲液置换并使用Millipore膜超滤(M.W. cut-off：3000)来去除。纯化抗体的蛋白浓度用BCA法(Pierce)测定。

(5)嵌合体166-32 Fab的纯化

嵌合体166-32 Fab可通过亲合性层析使用小鼠抗MAb 166-32的抗独特型MAb进行纯化。此抗独特型MAb被命名为MAb 172-25-3。它通过用与匙孔蓝蛋白（KLH）偶联的MAb 166-32免疫小鼠，并筛选能与因子D竞争性结合MAb 166-32的特异性抗体来制备。

亲合性层析基质的制备按如下方法进行：将25毫克MAb 172-25-3与5毫升干燥的aziactone胶粒(UltraLink Biosupport Medium, Pierce)在缓冲液(0.1 M硼酸盐及0.75 M Na_2SO_4、pH 9.0)中及室温下混合2小时。然后将残余的未反应位点用1M乙醇胺(pH 9.0)在室温下封闭2.5小时。然后将胶粒在含10毫摩尔Tris、0.15M NaCl、5毫摩尔EDTA、1% Triton X-100及0.02% NaN_3、pH 8.0)的缓冲液中清洗，并在4℃下保存。

为进行纯化，将产cFab或cFab/9aa CHO细胞的旋转培养物的100毫升上清液上样至偶联了MAb 172-25-3的亲合柱。将柱用PBS彻底清洗，然后用50毫摩尔二乙胺、pH 11.5洗脱结合的Fab。残留的盐按上面的介绍通过缓冲液置换去除。纯化Fab的蛋白质浓度用BCA法(Pierce)测定。

(6)SDS-PAGE嵌合体166-32 IgG、cFab及cFab/9aa的SDS-PAGE

纯化的嵌合体166-32 IgG、cFab及cFab/9aa用SDS-PAGE分析其纯度及分子量。将蛋白质用含或不含巯基乙醇的样品缓冲液处理。然后将样品与预先染色的分子量标准(低分子量范围)(BIO-RAD Laboratories,

Hercules, CA)一起于预注胶(12.5%)(Amersham Pharmacia Biotech, Uppsala, Sweden)中电泳，电泳使用 PhastSystem (Amersham Pharmacia Biotech)。然后将胶在考马斯亮蓝液(BIO-RAD)中染色 5 分钟，再在含 40%甲醇及 10%乙酸的水溶液中脱色。

CFab、cFab/9aa 及嵌合体 IgG 在非还原及还原条件下的 SDS-PAGE 结果显示，嵌合体 IgG 具有 150 kD 的蛋白质条带以及两个分别约为 50 kD 及 29 kD 的重链(HC)及轻链(LC)蛋白质条带。与预期的一样，cFab/9aa 在非还原条件下仅有 1 个约为 40 kD 的蛋白质条带，这表明重链及轻链通过链间二硫键相连。另一方面，cFab 在非还原条件下有 2 个蛋白质条带，这表明重链及轻链不通过链间二硫键相连。

(7)测定嵌合体 166-32 IgG、cFab 及 cFab/9aa 的活性

嵌合体 166-32 IgG、cFab 及 cFab/9aa 的活性可按上面的介绍使用补体旁路溶血试验进行测定。图 13 显示，鼠及嵌合体形式的 MAb 166-32 具有相同的抑制因子 D 的效力。图 14 显示，cFab 及 cFab/9aa 具有基本上相同的抑制因子 D 的效力。更重要地是，两种形式的嵌合体 Fab 的效力与嵌合体 IgG 相同，虽然每个 IgG 分子有两个结合位点。总而言之，这些结果证明，嵌合体 IgG、cFab 及 cFab/9aa 保留了亲本鼠 MAb 166-32 的效力.

实施例 10：在用人血浆灌注的兔心脏体外模型中，MAb 166-32 对由补体介导的组织损伤的保护作用

补体系统的活化可造成超急性移植排斥。这可能是如下过程的结果:能够固定补体的抗体的结合、补体在外源细胞表面经由旁路途径直接活化、和/或外源器官不能调节补体(J.L. Platt 等人, Transplantation, 1991; 52：937-947)。根据特殊的种与种之间的相互作用，补体活化可经由占优势的经典或旁路途径，虽然有时这两个途径可同时运作(T. Takahashi 等人, ImmunoL Res., 1997; 16：273-297)。先前的研究显示，超急性排斥可在不存在抗供体抗体的情况下经由旁路活化途径发生(P.S. Johnston 等人, Transplant. Proc., 1991; 23：877-879)。

为证明旁路补体途径对组织伤害的重要性，使用一个体外模型来对抗因子 D 的 MAb 166-32 进行检测，在该模型中分离的兔心脏用稀释的人血浆灌注。此模型在先前的研究显示可因补体旁路活化途径对兔心肌造成伤害。(M.R. Gralinski 等人, Immunopharmacology, 1996：34：79-88)。

(1)由 Langendorff 灌注的兔心脏：

将雄性新西兰白兔(2.2-2.4 公斤)用断颈椎法致死。快速取出心脏，插管以通过主动脉灌注。灌注培养液含有循环体积(250 毫升)的修饰 Krebs-Henseleit(K-H)缓冲液(pH 7.4、37℃)，以 20-25 毫升/分的恒定速率给予。缓冲液培养液的组成(毫摩尔/L)为：NaCl，117；KCl，4.0；$CaCl_2.H_2O$，2.4；$MgCl_2.6H_2O$，1.2；$NaHCO_3$，25；KH_2PO_4，1.1；葡萄糖，5.0；L-谷氨酸单钠，5.0；丙酮酸钠，2.0；及 BSA，0.25%(w/v)。此 K-H 缓冲液通过一个气孔状"肺"，该"肺"由 SilasticTM Laboratory Grade Tubing(Dow Corning，Midland，MI)组成，长度为 55.49 米，内径为 1.47 毫米及外径为 1.96 毫米。将膜状"肺"持续暴露在 95% O_2 / 5% CO_2 下，使灌注培养液中氧分压为 500 毫米米汞柱。在整个试验中，心脏通过连于右肺动脉的电极进行搏动。由一台实验室方形波发生器(Grass SD-5，Quincy，MA)传送搏动刺激(3 Hz，4 msec 持续时间)。将肺动脉用聚乙烯管插管以便于肺动脉输出液的收集，后者代表冠状静脉回流。将腔静脉的外腔和内腔以及肺静脉结扎以防灌注液从被割断的管流出。通过左动脉插入一个左心室管、热敏电阻探针以及橡胶球，并且定位于左心室。将充满液体的橡胶球用硬管连接至压力传导器，以测量左心室的收缩压及终舒张压。左心室 developed 压定义为左心室收缩压及终舒张压之间的差异。心室间球用蒸馏水扩张，以达到 5 毫米汞柱的起始基线的左心终舒张压。冠状灌注压力用与心导管的侧臂连接的压力传导器进行测量。使用多频道计数器(Grass Polygraph 79D，Quincy，MA)连续监测所有的血动态变化。在整个试验过程中，使分离的心脏维持在 37℃，这通过将心脏置于调温的双层玻璃室，并通过加热器及传送器灌注培养液来实现。

(2)抗体处理：

使用两个治疗组来测定抗因子 D MAb 166-32 在以人血浆灌注的分离兔心脏中抑制补体活化的能力。第 1 组：同种型匹配的阴性对照，由在存在 0.3 微克/毫升对 HIV-1 gp120 有专一性的 MAb G3-519 的情况下用 4%的人血浆灌注的心脏组成(n=6)。第 2 组：治疗组，由在存在 0.3 微克/毫升 MAb 166-32 的情况下用 4%的人血浆灌注的心脏组成(n=6)。人血浆可由新鲜收集的全血中分离，并贮存于-80℃直到使用。使用此百分比的人血浆是因为它可在合理长度的时间内严重破坏心肌功能，并允许对治疗有效性进行评估。此系统中的高浓度人血浆可使心脏快速收缩，而低浓度下药物

的效用难以分析。初步研究已经测定，0.3 微克/毫升为能够从补体活化的影响保护分离心脏的最低有效浓度。在将任何一种抗体加入灌注培养液之前，所有心脏在 Langendorff 器中平衡 10-15 分钟。加入抗体 10 分钟后，将 4 %人血浆加入至灌注培养液(250 毫升，再循环)。血动态变化包括冠状动脉灌注压(CPP)、左心室收缩压(LVSP)、左心室终舒张压(LVEDP)以及左心室压(LVDP)在加入抗体前记录(基线)，在加入 4%人血浆前记录，此后每 10 分记录 1 次，共记录 60 分钟。

在接触 4%人血浆时，与用 MAb G3-519(0.3 微克/毫升)处理的心脏相比，MAb 166-32(0.3 微克/毫升)可减少冠状灌注压(CPP)的增加。CPP 的增加显示冠状血管抗性，该抗性通常与心肌组织伤害相关。用 MAb 166-32 灌注的分离兔心脏可维持左心室终舒张压(LVEDP)，这与用 MAb G3-519 获得的结果相反(图 15)。后一组心脏在接触 4%人血浆时可使 LVEDP 增加，这显示心室在舒张时无法舒缓(图 15)。与用 MAb G3-519 处理的心脏相比，在接触稀释的人血浆后，MAb 166-32 亦可减少左心室压(LVDP)的增加(图 15)。

图 16 描述了在存在 4%人血浆时的灌注之前及 10、30 及 60 分钟后心功能的代表性记录。10 分钟后，用 MAb G3-519 处理的兔心脏有明显的进程性的 LVEDP 增加及 LVDP 降低，并且在随后的 50 分内，心室功能进程性地恶化。另一方面，用 MAb 166-32 处理的心脏在 60 分钟后仍能保持心室功能。

总而言之，血动态数据表明，抗因子 D MAb 166-32 能保护分离的兔心脏，使之不受人补体-介导的损伤，这可用人血浆攻击后仍能维持完全的心肌功能来说明。

(3)补体 Bb 的 ELISA：

因子 D 催化结合状态的因子 B 的切割，产生 Ba 及 Bb 片段。Bb 存在的浓度可作为因子 D 活性的指标。从分离兔心脏收集的淋巴液中的活化成份 Bb 的浓度可使用商业化的 ELISA 试剂盒(Quidel)测量。此分析使用针对人补体 Bb 的 MAb 在存在人血浆的条件下灌注兔心脏的过程中测量人补体系统的活化。淋巴管流出淋巴液可从心脏尖收集，速冻于液态氮，并贮于-70℃直到分析。记录和说明淋巴管流出液的流速，以将 Bb 浓度正常化。

在用 4%人血浆灌注 10 分后以及在各时间点，用 MAb 166-32 处理的心脏与用 MAb G3-519 处理的心脏相比，淋巴管流出液存在明显($p< 0.05$)浓

度低的 Bb(图 17)。在用 MAb 166-32 处理的兔心脏中补体活化产物 Bb 的产生减少，证明了该抗体对因子 D 的抑制剂活性。

(4)C5b-9 沉积物的免疫组化定位：

完成上述步骤后，从 Langendorff 装置移出心脏，切成横向切片，冷冻于液态氮。丢弃心尖及动脉组织。将切片包埋于 O.C.T.化合物包埋培养液(Miles, Inc., Ekhart, IN)，切为 3 米，置于聚-L-缬氨酸包被的载玻片。用磷酸盐缓冲盐水(PBS)清洗后，将切片在室温下与 PBS 中的 4%多聚甲醛温育。将心脏切片用 PBS 清洗并与 1% BSA 温育 15 分钟以降低非专一染色。用 PBS 清洗后，将切片与 1：1000 稀释的鼠抗人 C5b-9 MAb(Quidel)一起在室温下温育 1 小时。将切片再用 PBS 清洗，在室温下与羊抗鼠 FITC 偶联抗体(Sigma, 1：320 稀释)温育 1 小时。最后用 PBS 清洗后，将切片用 Fluoromount-G(Electron Microscopy Sciences, Fort Washington, PA)固定，盖以盖玻片。对照包括不使用第一抗体处理的切片以及用同种型匹配的鼠抗体 IgG1(Sigma)取代抗 C5b-9 MAb 的切片。

使用免疫荧光染色检测用 MAb 166-32 及 MAb G3-519 处理的心脏的心脏切片中的人 MAC(或 C5b-9)沉积。用 MAb 166-32 处理的心脏与 MAb G3-519 处理的心脏相比，显示 MAC 沉积减少。

总而言之，兔心脏体外研究的数据证明了 MAb 166-32 通过抑制旁路补体途径在预防心组织损伤的效果。抑制补体活化已显示可延长移植存活率(S.C. Makrides, Pharmacological Rev., 1998, 50：59-87)。因此，MAb 166-32 有用作治疗药剂来保护移植物不受人血浆破坏的潜力。

实施例 11：在心肺分流的体外循环模型中，MAb166-32 对补体活化及炎症反应的抑制效应

进行心肺分流(CPB)的病人经常表现出系统性炎症应答症。在临床上，这些反应可表现为术后白细胞增多、发热、及血管外液体累积，这可导致恢复期延长，并偶而会造成严重的器官功能破坏(J.K. Kirklin 等人，J. Thorac. Cardiovasc. Surg., 1983; 86：845-857; L. Nilsson 等人，Scand. J. Thorac. Cardiovasc. Surg., 1988; 22：51-53; P.W. Weerwind 等人，J. Thorac. Cardiovasc. Surg., 1995; 110：1633-1641)。这种炎症反应包括体液及细胞的变化，而造成组织损伤及止血功能被破坏。补体活化已被认为是系统性炎症反应的重要原因(P. Haslam 等人，Anaesthesia,

1980; 25：22-26; A. Salama 等人, N. Eng. J. Med., 1980; 318：408-414; J. Steinberg 等人, J. Thorac. Cardiovasc. Surg., 1993; 106：1008-1016)。补体活化是引起血液与 CPB 体外循环机器的表面相互作用的原因(D. Royston, J. Cardiothorac. Vasc. Anesth., 1997; 11：341-354)。原炎症物质在补体系统活化后生成，它们包括过敏毒素 C3a 及 C5a、调理素 C3b、及膜攻击复合物 C5b-9。C5a 已显示可在体外在多形核细胞 PMN(主要含中性粒细胞)中上调 MAC-1 复合物中的 CD11b(整合素)及 CD18(整合素)(M.P. Fletcher 等人, Am. J. Physiol., 1993; 265：H1750-H1761)，并诱导 PMN 释放溶酶体酶。C5b-9 可诱导血小板上 P-选择素(CD62P)的表达(T. VViedmer 等人, Blood, 1991; 78：2880-2886)，而且 C5a 及 C5b-9 都诱导 P-选择素在内皮细胞的表面表达(K.E. Foreman 等人, J. Clin. Invest., 1994; 94：1147-1155)。C3a 及 C5a 可刺激人肥大细胞的趋化性(K. Hartmann 等人, Blood, 1997; 89：2868-2870)，并引发组织胺的释放(Y. Kubota, J. Dermatol., 1992; 19：19-26)，后者可诱导血管通透性(T.J. Williams, Agents Actions, 1983; 13：451-455)。

全血在体外分流循环系统中的体外再循环已被广泛用作在 CPB 中激活白细胞(J. Kappelamyer 等人, Circ. Res., 1993; 72：1075-1081; N. Moat 等人, Ann. Thorac. Surg., 1993; 56：1509-1514; C.S. Rinder 等人, J. Clin. Invest., 1995：96：1564-1572)和血小板(V.L. Jr. Hennesy 等人, Am. J. Physiol., 1977; 2132：H622-H628; Y. Wachtfogel 等人, J. Lab. Clin. Med., 1985; 105：601-607; C.S. Rinder 等人, ibid)变化以及补体活化(P.G. Loubser, Perfusion, 1987; 2：219-222; C.S. Rinder 等人, ibid; S.T. Baksaas 等人, Perfusion, 1998; 13：429-436)的模型。使用此 CPB 体外循环模型对抗因子 D 的 MAb 166-32 在人全血中抑制细胞及补体活化的有效性进行了研究。

(1)体外循环系统的制备：

体外循环系统使用如下部件进行装配：带有一个嵌入式热交换器的中空纤维儿科膜氧生成器 (D 901 LILLPUT 1; DIDECO, Mirandola(MO), Italy)、带有一个嵌入式开心术滤器的儿科静脉贮器 (D752 Venomidicard; DIDECO)、一个灌注管道装置(Sodrin Biomedical, Inc., Irvine, CA)以及多流旋转泵(Stockert Instruments GmbH, Munich,

Germany)。将血浆-Lyte A 液(Baxter Healthcare Corp., Deerfield, IL)注入氧生成器及循环器进行启动。将此启动液用冷热器回温至 32℃(Sarns; 3M Health Care, Ann Arbor, MI),并按 500 毫升/分钟进行循环,将通气流用 100%氧维持于 0.25 升/分钟。当加入血至循环系后,将通气流换成氧(95%)及 CO_2(5%)的混合气体。在整个再循环期间连续监测 pH、PCO_2、PO_2 以及灌注液温度。在需要时加入碳酸氢钠以将 pH 维持于 7.25-7.40。

(2)体外循环系统的操作及取样:

从健康无服药的自愿者在 5-10 分钟内抽 450 毫升血至转移袋中(Haemo-Pak; Chartermed, Inc., Lakewood, NJ),转移袋中含猪肝素(5 单位/毫升,终浓度; Elkins-Sinn, Cherry Hill, NJ)及抗因子 D 的 MAb 166-32 或同种型匹配的阴性对照 MAb G3-519(18 微克/ml; 终浓度)。抗体的这个浓度相当于血中因子 D 摩尔浓度的约 1.5 倍。在将血加入体外循环系统前,从袋中取一个血样品作为"循环前"样品,命名为"-10 分钟样品"。然后将血经由已启动的通道加入贮器。同时将启动液抽至氧生成器远端出口,产生 600 毫升的最终循环体积以及 25-28%的最终血细胞比容。将血与启动液一起循环,在 3 分钟内完成完全混合; 抽出基线样品,命名为 0 时间。为模拟体温过低下外科手术的一般过程,将循环液冷却至 27℃ 70 分钟,再回温至 37℃ 50 分钟(总共再循环 120 分)。

在再循环过程中,还在 10、25、40、55、70、80 及 120 分钟抽取血液样品。立即在 2000 x g 及 4℃下离心制备血浆样品。分装以用于旁路途径溶血分析及中性粒细胞-专一的髓过氧化物酶分析,将分装样品冷冻于干冰,贮于-80℃。立即将用于 ELISA 测量补体 C3a、C4d、sC5b-9、及 Bb 的分装样品与等体积的样品稳定培养基(Quidel)混合,冷冻于干冰,贮于-80℃。还收集全血样品用于对中性粒细胞及血小板上活化的细胞表面标记 CD11b 及 CD62P 进行免疫染色。为防止染色过程中全血样品的随后的补体活化,将 10 微升的 1 M EDTA 加入每毫升全血,使其终浓度为 10 毫摩尔。

(3)旁路途径溶血分析:

如上所述使用兔红血球细胞检测 MAb 166-32 处理的及 MAb G3-519 处理的循环在不同时间点时血浆样品中的旁路补体活性。将 50 微升的每种样品(20%)在加入 30 微升兔红血球细胞(1.7×10^8 细胞/毫升)之前与 50 微升 GVB/Mg-EGTA 缓冲液混合。在 37℃下温育 30 分钟后,收集上清液。使用

ELISA 平板计数器读取 405nm 处的 OD。

图 18 显示，用 MAb 166-32 处理的循环系统中旁路补体活性可完全被该抗体抑制，而 MAb G3-519 在用于相当的循环系统时对补体活性无影响。这些结果表明，MAb 166-32 为旁路补体途径的强力抑制剂。即使在摩尔比率仅为 1.5：1(MAb：因子 D)时，MAb 166-32 也可完全抑制旁路补体活性。

(4)补体活化产物的分析：

除了上面介绍的溶血分析，还可检测来自两个体外循环系统的血浆样品中的 C3a、sC5b-9、Bb 以及 C4d 的水平。这些物质可使用商业化的 ELISA 试剂盒(Quidel)按厂商说明书进行定量。与 C5a 一样，sC5b-9 是补体级联反应中 C5 转换酶活性的另一个标记。C5a 及 sC5b-9 皆为 C5 被 C5 转换酶切割的产物。补体 Bb 为补体旁路活化途径的专一标记，而 C4d 为经典活化途径的专一标记。

图 19 及 20 显示，MAb 166-32 可分别有效地抑制 C3a 及 sC5b-9 产生，但同种型匹配的阴性对照 MAb G3-519 则不能抑制它们的产生。MAb 166-32 的专一性及效力进一步在图 21 及 22 中阐明。由补体旁路途径产生的 Bb 可完全被 MAb 166-32 抑制，而 G3-519 循环中的 Bb 水平在再循环过程中随时间增加而增加。有趣地是，MAb 166-32 及 G3-519 循环中 C4d 的水平随时间不会显著变化。后面的有关 Bb 及 C4d 水平的结果强烈显示，体外循环系统中的补体活化主要由旁路途径介导。

总而言之，这些结果表明，MAb 166-32 为补体旁路途径的强力抑制剂。因子 D 的抑制可终止级联反应的后续步骤的补体活化，这可由 C3a 及 sC5b-9 形成的减少来表现。

(5)中性粒细胞及血小板活化的分析：

中性粒细胞及血小板的活化可分别通过测量中性粒细胞及血小板上 CD11b 及 CD62P 的细胞表面表达水平来定量。对于中性粒细胞的 CD11b 标记，将 100 微升收集自循环系统的血立即在微量离心管中在室温下与 20 微升的红藻素(PE)-抗 CD11b 抗体(克隆 D12, Becton Dickinson, San Jose, CA) 温育 10 分钟。在室温下加入 1.4 毫升的 FACS 裂解液(Becton Dickinson)10 分钟以裂解红细胞和固定白细胞。将微量离心管在 300 x g 离心 5 分钟。吸出上清液，将细胞悬浮于 PBS 进行清洗。将微量离心管再离心一次，吸出上清液，在使用 EPIC-XL 流式细胞仪(Coulter Corp., Miami, FL)进行分析前，将细胞悬浮于 0.5 毫升的 1%多聚甲醛过夜。为进

行双标记以同时鉴定中性粒细胞群，加入 5 微升的异硫氰酸荧光素(FITC)-抗 CD15 抗体(克隆 MMA，Becton Dickinson)以与 PE-抗 CD11b 抗体一起温育。

对于血小板的 CD62P 标记，将 40 微升收集自循环系统的血立即在微量离心管中在室温下与 20 微升 PE-抗 CD62P 抗体(克隆 ACI.2，Becton Dickinson)温育 10 分钟。将混合物按上面的介绍用 FACS 裂解液处理。将微量离心管在 2000 x g 离心 5 分钟。将血小板用 PBS 清洗，用 1 %多聚甲醛固定，再按上面的介绍进行分析。为进行双标记以同时鉴定血小板群，加入 5 微升的 FITC-抗 CD42a 抗体以与 PE-抗 CD62P 抗体一起温育。

为进行流式细胞测量，PMN(主要含中性粒细胞)及血小板群分别用基于前对侧散布参数的活动门以及用 FITC-抗 CD15 抗体及 FITC-抗 CD42a 抗体专一染色进行鉴定。背景染色使用同种型匹配的标记抗体设定门。CD11b 及 CD62P 的表达强度用平均荧光强度(MFI)表示。.

图 23 显示，来自 MAb 166-32 处理的体外循环系统的中性粒细胞，与来自 MAb G3-519 处理循环系统的中性粒细胞相比，显示明显低的 CD11b 表达。这些数据连同上述其他数据表明，MAb 166-32 对补体旁路活化的抑制可阻止中性粒细胞的活化。

与此类似，图 24 显示，来自 MAb 166-32 处理的体外循环系统的血小板，与来自 MAb G3-519 处理的循环系统的血小板相比，显示明显低的 CD62P 表达。同样，这些数据连同上述其他数据显示，MAb 166-32 对补体旁路活化的抑制可阻止血小板的活化。

(6)中性粒细胞-专一的髓过氧化物酶(MPO)的分析

中性粒细胞的活化程度亦可使用商业化的 ELISA 试剂盒(R & D Systems，Inc.，Minneapolis，MN)进行测量以测定来自体外循环系统的血浆样品中的中性粒细胞专一的髓过氧化物酶(MPO)的数量。MPO 主要贮于中性粒细胞的原粒(嗜苯胺蓝细胞)中。它在中性粒细胞因活化而脱粒时释放。因此 MPO 为中性粒细胞活化的可溶性标记。分析可按厂商说明书施行。简而言之，将样品于微平板的孔中温育，这些孔已用抗 MPO 的第一 MAb 包被。将此 MPO-MAB 复合物用从羊 MPO-抗血清中制备的一种与生物素偶联的多克隆抗体标记。此分析最后步骤基于生物素-亲和素偶联，其中亲和素已与碱性磷酸酶共价连接。在加入底物 4-硝苯基-磷酸盐(pNPP)后，通过读取 405 处的 OD，以酶学的方法测得各样品中 MPO 的量。

图 25 显示，MAb166-32 处理的循环系统中的 MPO 水平比 MAbG3-519 循环系统中的水平明显要低。这些结果与上面介绍的免疫荧光计数研究中的 CD11b 表达相吻合。

总而言之，有关补体、中性粒细胞及血小板的数据支持如下论点，抗因子 D MAb 166-32 对体外循环系统中补体旁路活化的有效抑制，可阻止炎症物质 C3a、C5a 及 sC5b-9 的形成，并因而降低中性粒细胞及血小板的活化。可以预测，MAb 166-32 及其片段、同源物、相似物及其小分子部份可有效预防或降低因 CPB 所致的临床炎症反应。

例 12：研究 MAb 166-32 在患有缺血及灌注损伤的狗模型中的效果

设计一研究以检测 MAb 166-32 是否可保护患有缺血及灌注狗的心肌组织不受伤害，虽然从一开始我们就认识到，狗可能不是研究 MAb 166-32 效用的理想动物模型。在溶血试验中，MAb 166-32 中和狗因子 D 的能力比中和人因子 D 的能力至少低 10 倍(参见例 7)。因为那时仅有有限量的 MAb 166-32，所以将 MAb 166-32 经由冠状血管给药至心脏。我们希望可在冠状血中累积至少 60 微克/毫升的抗体浓度以完全抑制心脏中的狗因子 D。计算剂量为 3.15 毫克/公斤/灌注，总共 6 次灌注。MAb G3-519 用作同种型匹配的对照。

简而言之，将实验用猎犬麻醉。在第四肋间左位进行胸廓切开术以露出心脏。分离近左围旋冠状动脉，结扎 90 分钟以诱导缺血，再灌注 6 小时。分别在缺血前 30 分钟、灌注前 10 分钟及灌注期间的 75、150、225、300 分钟给药抗体 6 次。在不同的时间点注入放射性微球以测量区域血流。在实验结束时，将心脏用伊凡蓝染料及三苯四锉灌注以分别测量危险面积及梗塞大小。在缺血前及实验结束时从颈静脉收集冠状淋巴液及血。使用这些样品来测量注射抗体的浓度以及旁路途径溶血活性。

结果显示，冠状淋巴液中 MAb 166-32 可达到的最高浓度约为 30 微克/毫升，这远低于在冠状循环中完全抑制狗因子 D 所需的浓度。在系统循环中也检测到抗体，这表明注射的抗体扩散到心脏之外。溶血分析数据显示，补体旁路活性没有降低；这与抗体浓度较低的事实吻合。因此，由在狗中所作的这些实验无法对 MAb 166-32 在灌注实验中的效应下结论。

上面的描述、术语、表达及例子仅为举例而不是限制。本发明包括前面实施方案的所有等价物，不管是已知的还是未知的。本发明仅受后面专

利要求的限制，并不受本文任何其他部分中的任何陈述或任何其他来源的限制。

00.10.20

序 列 表

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Bill N.C. Sun
Cecily R.Y. Sun

<120> 补体活化的抑制剂

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20 25 30

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Ala Glu Arg Trp Val Leu Ser Ala Ala His Cys Leu Glu Asp Ala Ala
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Arg Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro Leu Val Cys Gly Gly
180 185 190

gtg ctc gag ggc gtg gtc acc tcg ggc tcg cgc gtt tgc ggc aac cgc 624
Val Leu Glu Gly Val Val Thr Ser Gly Ser Arg Val Cys Gly Asn Arg
195 200 205

aag aag ccc ggg atc tac acc cgc gtg gcg agc tat gcg gcc tgg atc 672
Lys Lys Pro Gly Ile Tyr Thr Arg Val Ala Ser Tyr Ala Ala Trp Ile
210 215 220

gac agc gtc ctg gcc tagtaggaat tc 699
Asp Ser Val Leu Ala
225

<210> 2
<211> 228
<212> PRT
<213> 人

<400> 2

Ile Leu Gly Gly Arg Glu Ala Glu Ala His Ala Arg Pro Tyr Met Ala
1 5 10 15
Ser Val Gln Leu Asn Gly Ala His Leu Cys Ala Gly Val Leu Val Ala
20 25 30
Glu Arg Trp Val Leu Ser Ala Ala His Cys Leu Glu Asp Ala Ala Asp
35 40 45
Gly Lys Val Gln Val Leu Leu Gly Ala His Ser Leu Ser Gln Pro Glu
50 55 60
Pro Ser Lys Arg Leu Tyr Asp Val Leu Arg Ala Val Pro His Pro Asp
65 70 75 80
Ser Gln Pro Asp Thr Ile Asp His Asp Leu Leu Leu Leu Gln Leu Ser
85 90 95
Glu Lys Ala Thr Leu Gly Pro Ala Val Arg Pro Leu Pro Trp Gln Arg
100 105 110
Val Asp Arg Asp Val Ala Pro Gly Thr Leu Cys Asp Val Ala Gly Trp
115 120 125
Gly Ile Val Asn His Ala Gly Arg Arg Pro Asp Ser Leu Gln His Val
130 135 140
Leu Leu Pro Val Leu Asp Arg Ala Thr Cys Asn Arg Arg Thr His His
145 150 155 160
Asp Gly Ala Ile Thr Glu Arg Leu Met Cys Ala Glu Ser Asn Arg Arg
165 170 175
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro Leu Val Cys Gly Gly Val
180 185 190
Leu Glu Gly Val Val Thr Ser Gly Ser Arg Val Cys Gly Asn Arg Lys
195 200 205
Lys Pro Gly Ile Tyr Thr Arg Val Ala Ser Tyr Ala Ala Trp Ile Asp
210 215 220
Ser Val Leu Ala
225

<210> 3

<211> 714

<212> DNA

<213> 猪

<220>

<221> CDS

<222> (4)...(702)

<400> 3

cgg atc ctg ggt ggc cag gag gcc aag tcc cac gag aga ccc tac atg 48
Ile Leu Gly Gly Gln Glu Ala Lys Ser His Glu Arg Pro Tyr Met
1 5 10 15

gca tcg gtg cag gtg aac ggc aag cac gtg tgc gga ggc ttc ctg gtg 96
Ala Ser Val Gln Val Asn Gly Lys His Val Cys Gly Gly Phe Leu Val
20 25 30

tct gag cag tgg gtg ctg agt gca gca cac tgc ctg gag gac gtg gcc 144
Ser Glu Gln Trp Val Leu Ser Ala Ala His Cys Leu Glu Asp Val Ala
35 40 45

gag ggg aag ctg cag gtt ctc ctg ggt gcg cac tcc ctg tca cag ccc 192
Glu Gly Lys Leu Gln Val Leu Leu Gly Ala His Ser Leu Ser Gln Pro
50 55 60

gag ccc tcg aag cgc ctg tac gac gtg ctc cgc gcc gtg ccc cac cca 240
Glu Pro Ser Lys Arg Leu Tyr Asp Val Leu Arg Ala Val Pro His Pro
65 70 75

gac agc cag cct gac acc atc gac cat gat ctc ctc ctg ctg aag ctc 288

Asp Ser Gln Pro Asp Thr Ile Asp His Asp Leu Leu Leu Leu Lys Leu
80 85 90 95

tcc gag aag gcc gag ctg ggc cct gcc gtg cag ccc ctt gcc tgg caa 336
Ser Glu Lys Ala Glu Leu Gly Pro Ala Val Gln Pro Leu Ala Trp Gln
100 105 110

cga gag gac cac gag gtt ccg gca ggc acg ctc tgc gac gtg gcc ggc 384
Arg Glu Asp His Glu Val Pro Ala Gly Thr Leu Cys Asp Val Ala Gly
115 120 125

tgg gga gtg gtc agt cac act ggc cgc cgg ccc gac cgt ctg cag cac 432
Trp Gly Val Val Ser His Thr Gly Arg Arg Pro Asp Arg Leu Gln His
130 135 140

ctg ctc cta ccg gtg ctg gac cgc acc acc tgc aac ctg cgc aca tac 480
Leu Leu Leu Pro Val Leu Asp Arg Thr Thr Cys Asn Leu Arg Thr Tyr
145 150 155

cac gat ggc acc atc acc gag cgc atg atg tgc gcg gag agc aac cgt 528
His Asp Gly Thr Ile Thr Glu Arg Met Met Cys Ala Glu Ser Asn Arg
160 165 170 175

cgg gac agc tgc aag ggc gac tcc gga ggc ccg ctg gtg tgc ggg ggt 576
Arg Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro Leu Val Cys Gly Gly
180 185 190

gtg gcc gag gga gtg gtt acc tca ggc tcc cga gtc tgc ggc aac cgc 624
Val Ala Glu Gly Val Val Thr Ser Gly Ser Arg Val Cys Gly Asn Arg
195 200 205

aag aaa ccc ggc atc tac acg cgc ttg gcg agc tac gtg gcc tgg atc 672

Lys Lys Pro Gly Ile Tyr Thr Arg Leu Ala Ser Tyr Val Ala Trp Ile
210 215 220

gac gga gtg atg gct gac agc gca gcc gcc tagtaggaat tc 714
Asp Gly Val Met Ala Asp Ser Ala Ala Ala
225 230

<210> 4
<211> 233
<212> PRT
<213> 猪

<400> 4
Ile Leu Gly Gly Gln Glu Ala Lys Ser His Glu Arg Pro Tyr Met Ala
1 5 10 15
Ser Val Gln Val Asn Gly Lys His Val Cys Gly Gly Phe Leu Val Ser
20 25 30
Glu Gln Trp Val Leu Ser Ala Ala His Cys Leu Glu Asp Val Ala Glu
35 40 45
Gly Lys Leu Gln Val Leu Leu Gly Ala His Ser Leu Ser Gln Pro Glu
50 55 60
Pro Ser Lys Arg Leu Tyr Asp Val Leu Arg Ala Val Pro His Pro Asp
65 70 75 80
Ser Gln Pro Asp Thr Ile Asp His Asp Leu Leu Leu Leu Lys Leu Ser
85 90 95
Glu Lys Ala Glu Leu Gly Pro Ala Val Gln Pro Leu Ala Trp Gln Arg
100 105 110
Glu Asp His Glu Val Pro Ala Gly Thr Leu Cys Asp Val Ala Gly Trp
115 120 125
Gly Val Val Ser His Thr Gly Arg Arg Pro Asp Arg Leu Gln His Leu
130 135 140

Leu Leu Pro Val Leu Asp Arg Thr Thr Cys Asn Leu Arg Thr Tyr His
145 150 155 160
Asp Gly Thr Ile Thr Glu Arg Met Met Cys Ala Glu Ser Asn Arg Arg
165 170 175
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro Leu Val Cys Gly Gly Val
180 185 190
Ala Glu Gly Val Val Thr Ser Gly Ser Arg Val Cys Gly Asn Arg Lys
195 200 205
Lys Pro Gly Ile Tyr Thr Arg Leu Ala Ser Tyr Val Ala Trp Ile Asp
210 215 220
Gly Val Met Ala Asp Ser Ala Ala Ala
225 230

<210> 5
<211> 25
<212> DNA
<213> 人工序列

<220>
<223> 引物

<400> 5
tgcggccgct gtaggtgctg tcttt 25

<210> 6
<211> 23
<212> DNA
<213> 人工序列

<220>
<223> 引物

<400> 6

ggaattcact cgttattctc gga 23

<210> 7

<211> 17

<212> DNA

<213> 人工序列

<220>

<223> 引物

<400> 7

tccgagaata acgagtg 17

<210> 8

<211> 29

<212> DNA

<213> 人工序列

<220>

<223> 引物

<400> 8

cattgaaagc tttggggtag aagttgttc 29

<210> 9

<211> 27

<212> DNA

<213> 人工序列

<220>

<223> 引物

<400> 9

cgcggccgca gctgctcaga gtgtaga 27

<210> 10

<211> 28

<212> DNA

<213> 人工序列

<220>

<223> 引物

<400> 10

cggtaagctt cactggctca gggaaata 28

<210> 11

<211> 37

<212> DNA

<213> 人工序列

<220>

<223> 引物

<400> 11

aagaagcttg ccgccaccat ggattggctg tggaact 37

<210> 12

<211> 31

<212> DNA

<213> 人工序列

<220>

<223> 引物

<400> 12

cgggatcctc aaactttctt gtccaccttg g 31

<210> 13

<211> 36

<212> DNA

<213> 人工序列

<220>

<223> 引物

<400> 13

aagaaagctt gccgccacca tgttctcact agctct 36

<210> 14

<211> 26

<212> DNA

<213> 人工序列

<220>

<223> 引物

<400> 14

cgggatcctt ctccctctaa cactct 26

说 明 书 附 图

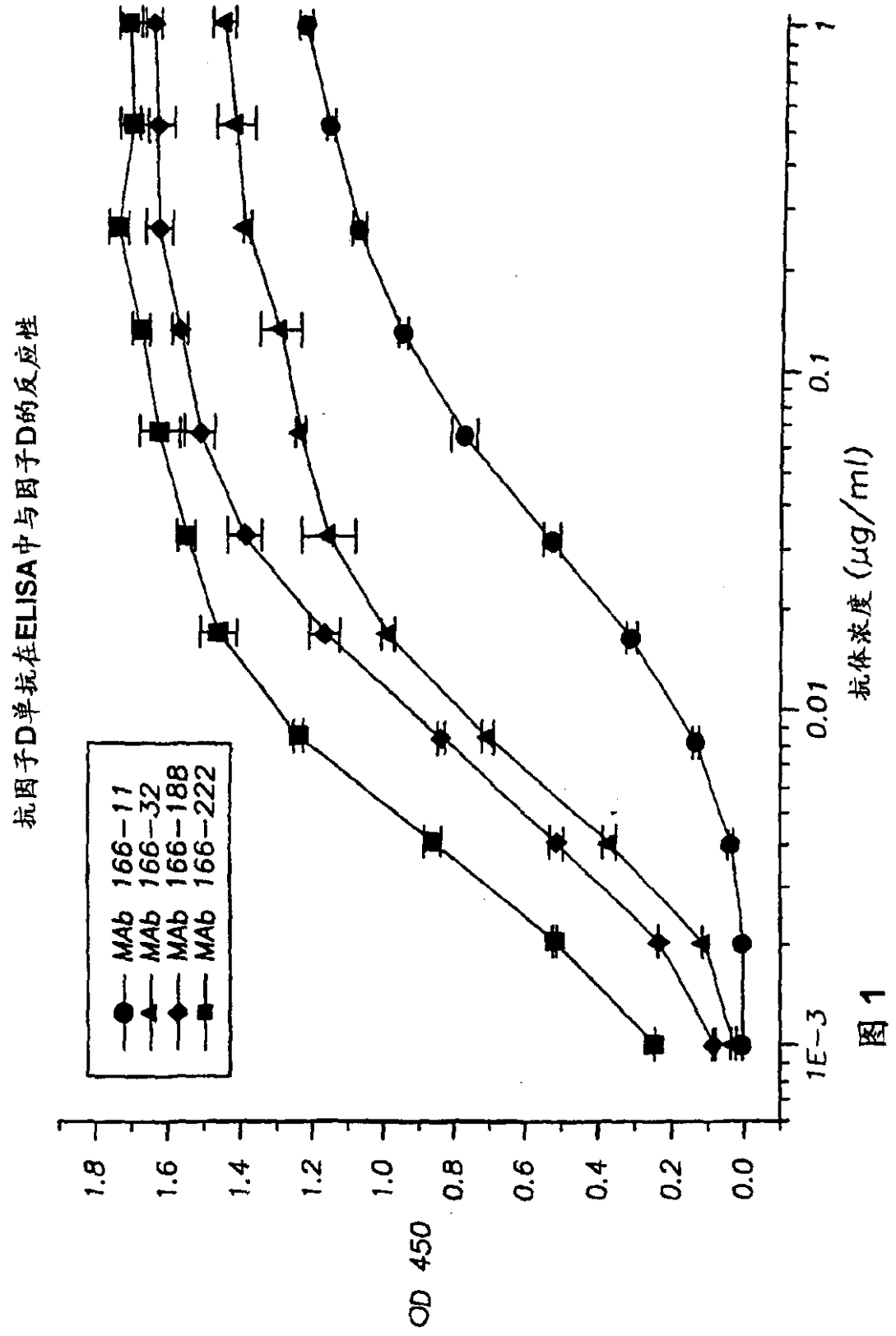

抗因子D单抗在ELISA中与因子D的反应性
MAb 166-11
MAb 166-32
MAb 166-188
MAb 166-222
OD 450
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1E-3
0.01
0.1
1
抗体浓度 (μg/ml)

图 1

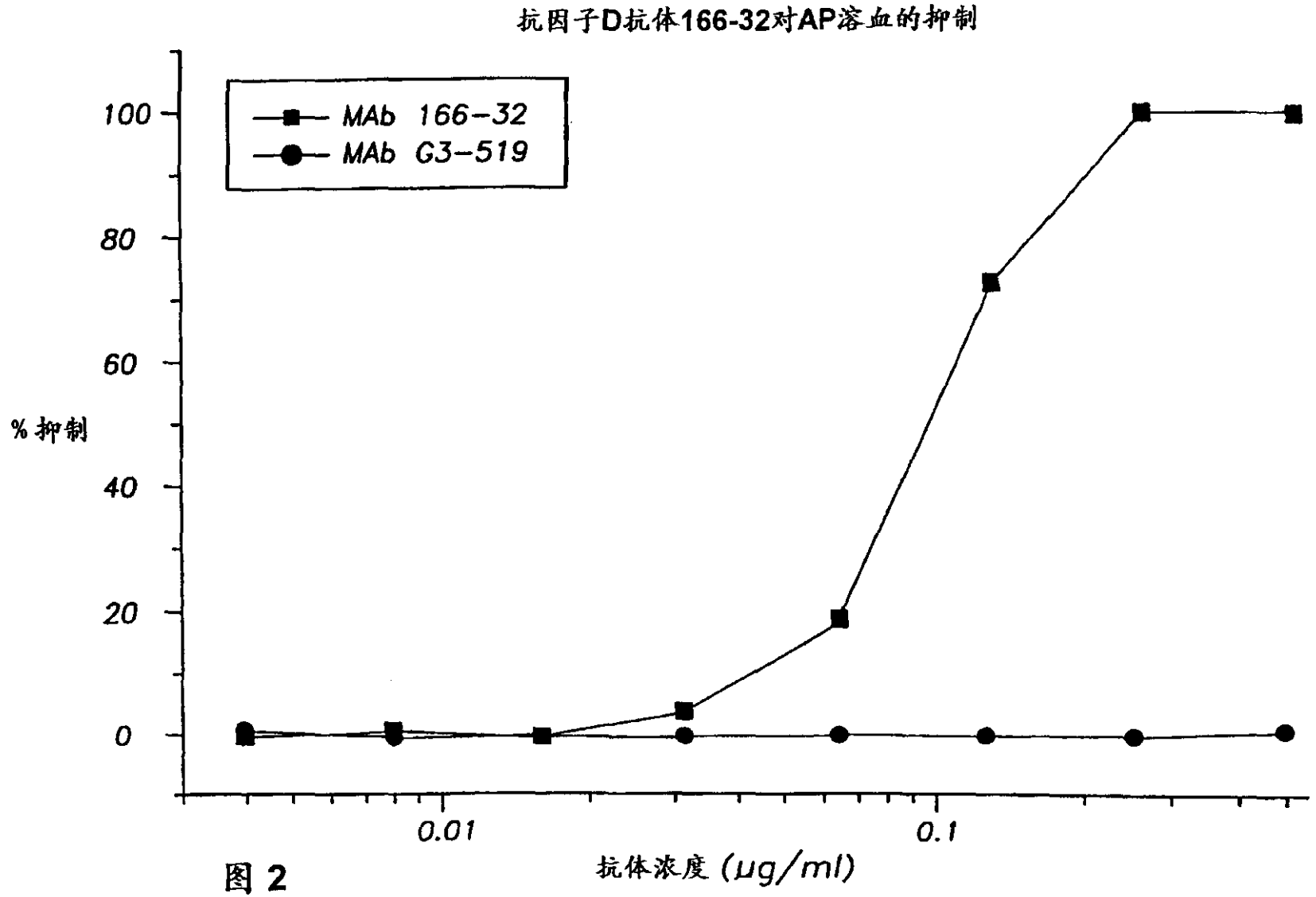

抗因子D抗体166-32对AP溶血的抑制
MAb 166-32
MAb G3-519
100
80
60
40
20
0
% 抑制
0.01
0.1
抗体浓度 (μg/ml)

图 2

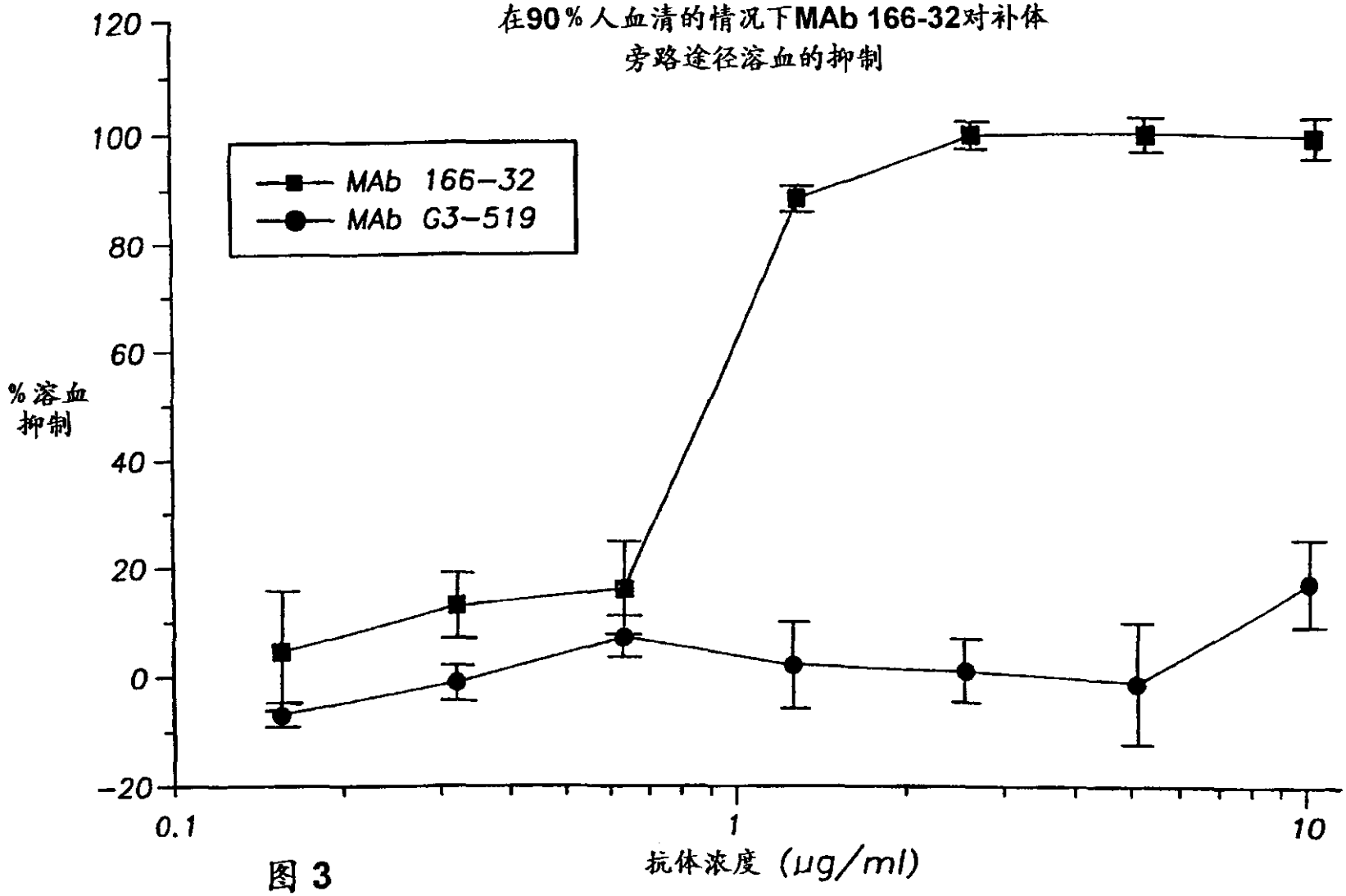

在90%人血清的情况下MAb 166-32对补体
旁路途径溶血的抑制
MAb 166-32
MAb G3-519
% 溶血
抑制
120
100
80
60
40
20
0
-20
0.1
1
10
抗体浓度 (μg/ml)

图 3

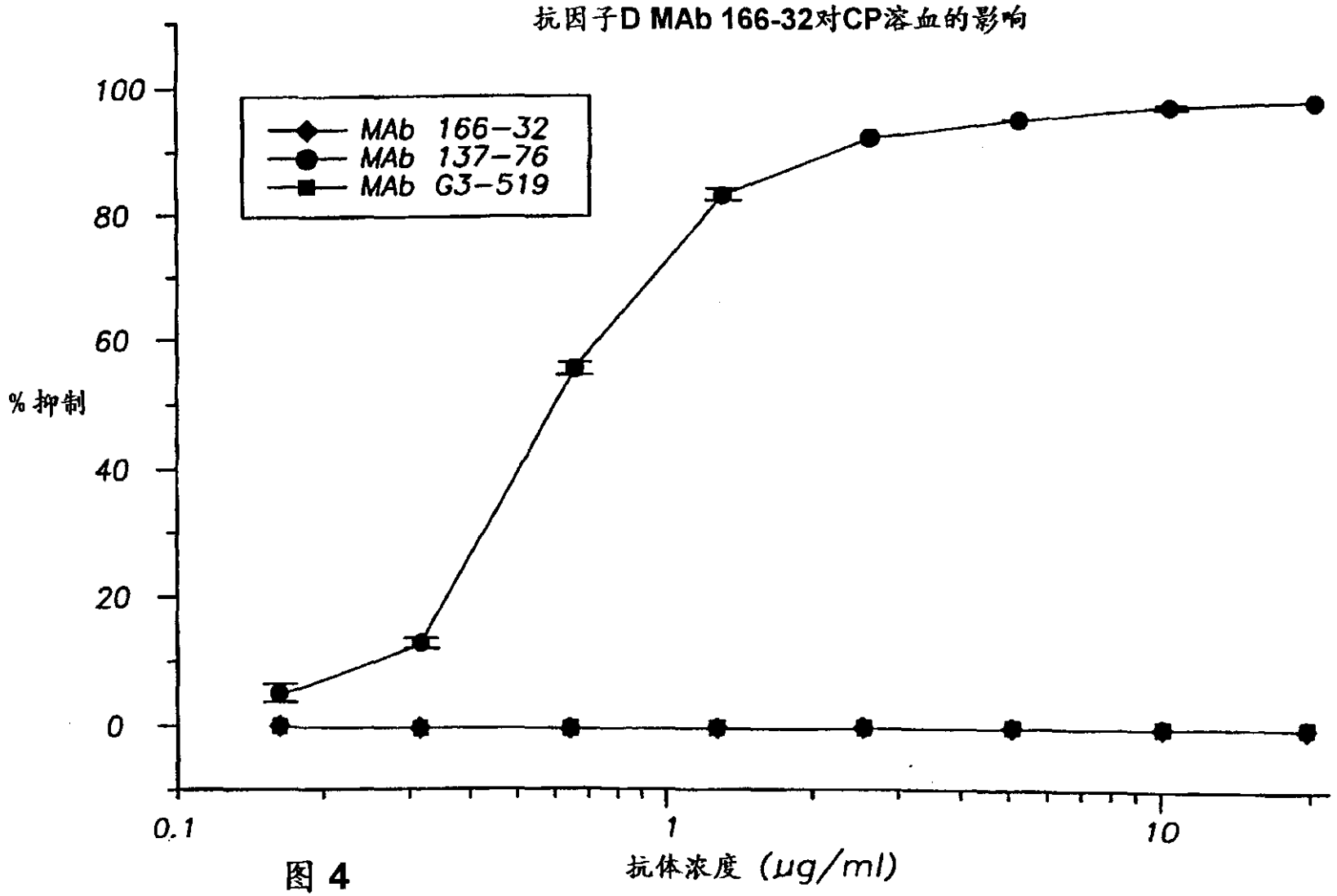

抗因子D MAb 166-32对CP溶血的影响
MAb 166-32
MAb 137-76
MAb G3-519
100
80
60
40
20
0
% 抑制
0.1
1
10
抗体浓度 (μg/ml)

图 4

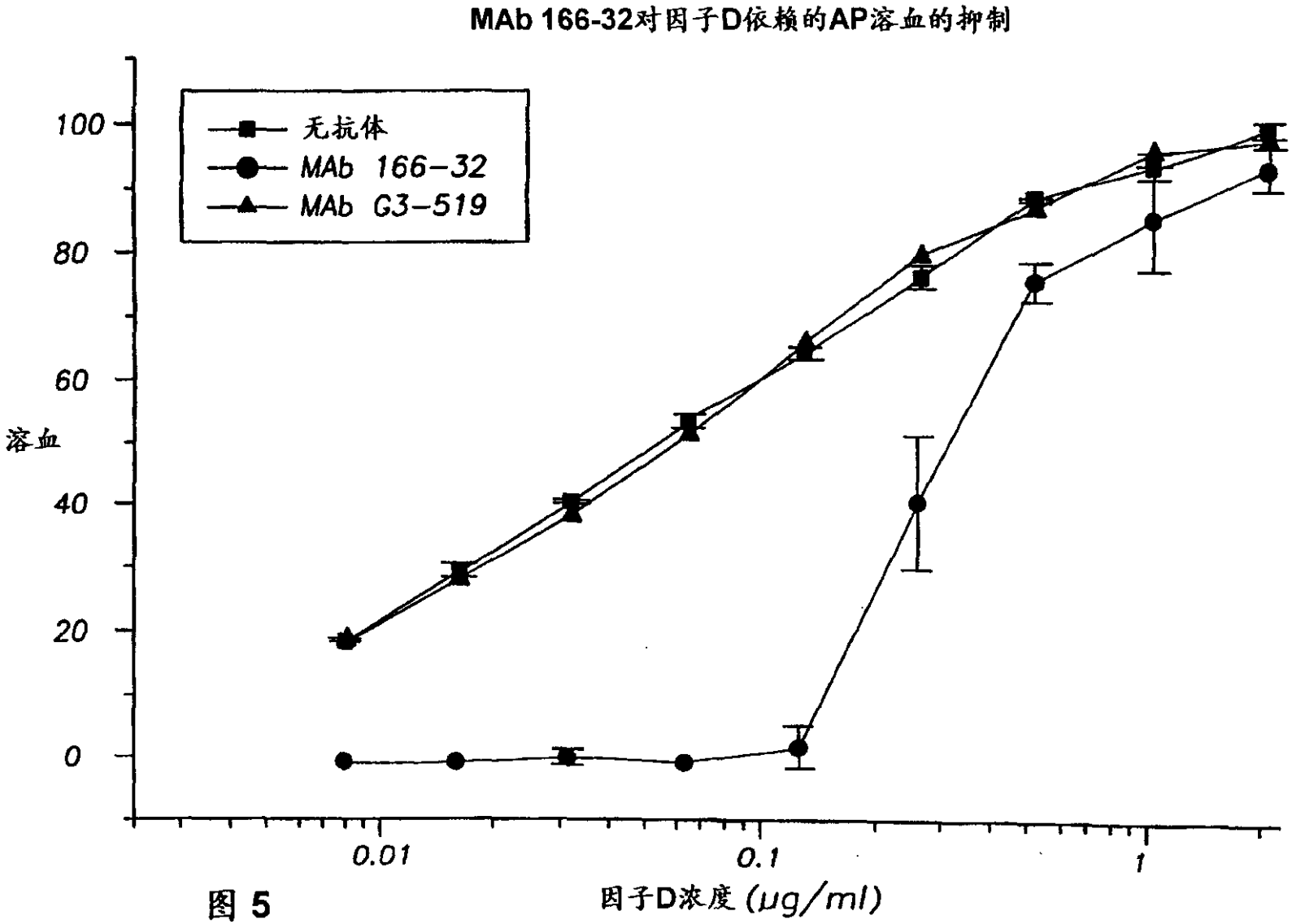

MAb 166-32对因子D依赖的AP溶血的抑制
无抗体
MAb 166-32
MAb G3-519
100
80
60
40
20
0
溶血
0.01
0.1
1
因子D浓度 (μg/ml)

图 5

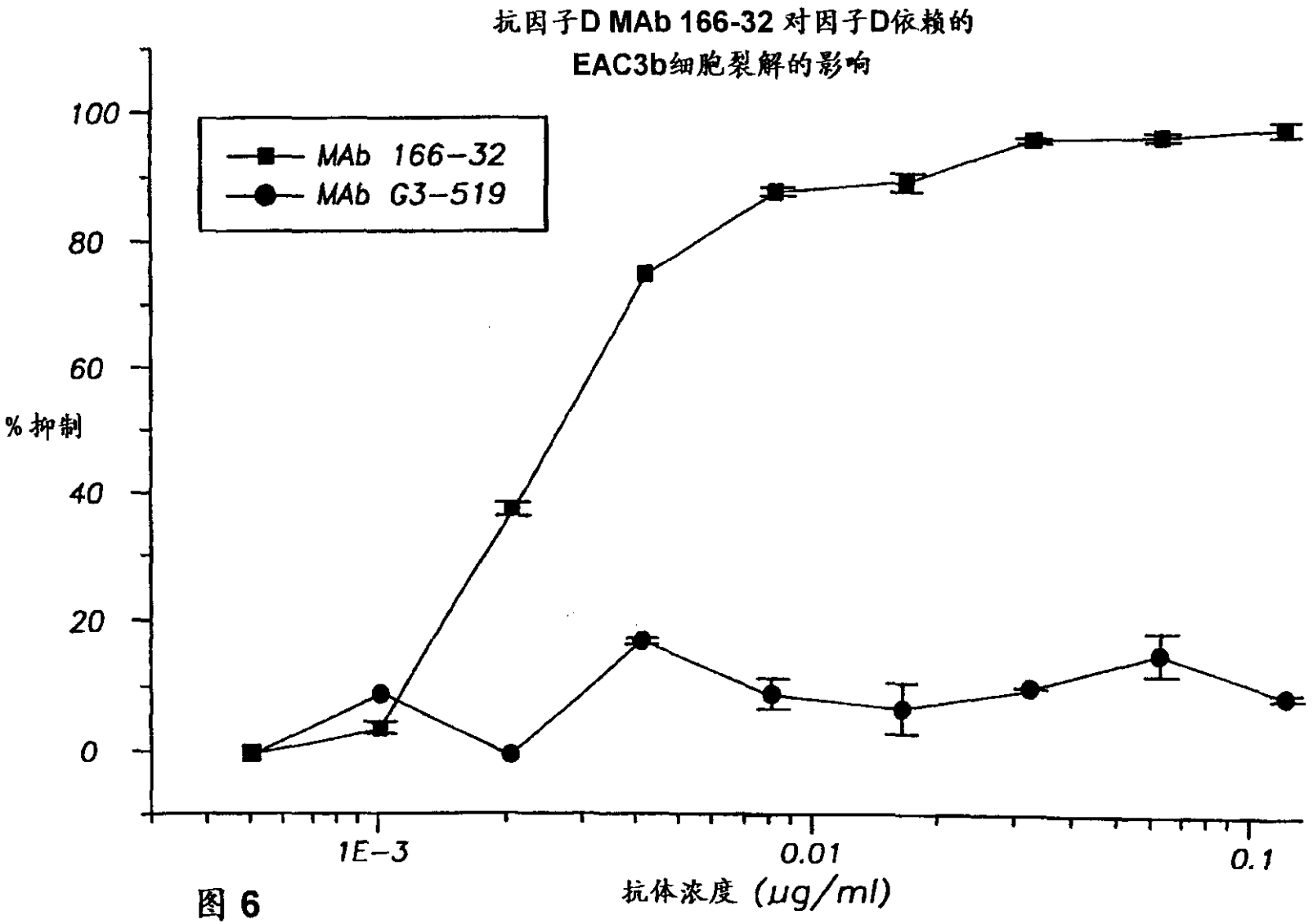

抗因子D MAb 166-32 对因子D依赖的
EAC3b细胞裂解的影响
MAb 166-32
MAb G3-519
100
80
60
40
20
0
% 抑制
1E-3
0.01
0.1
抗体浓度 (μg/ml)

图 6

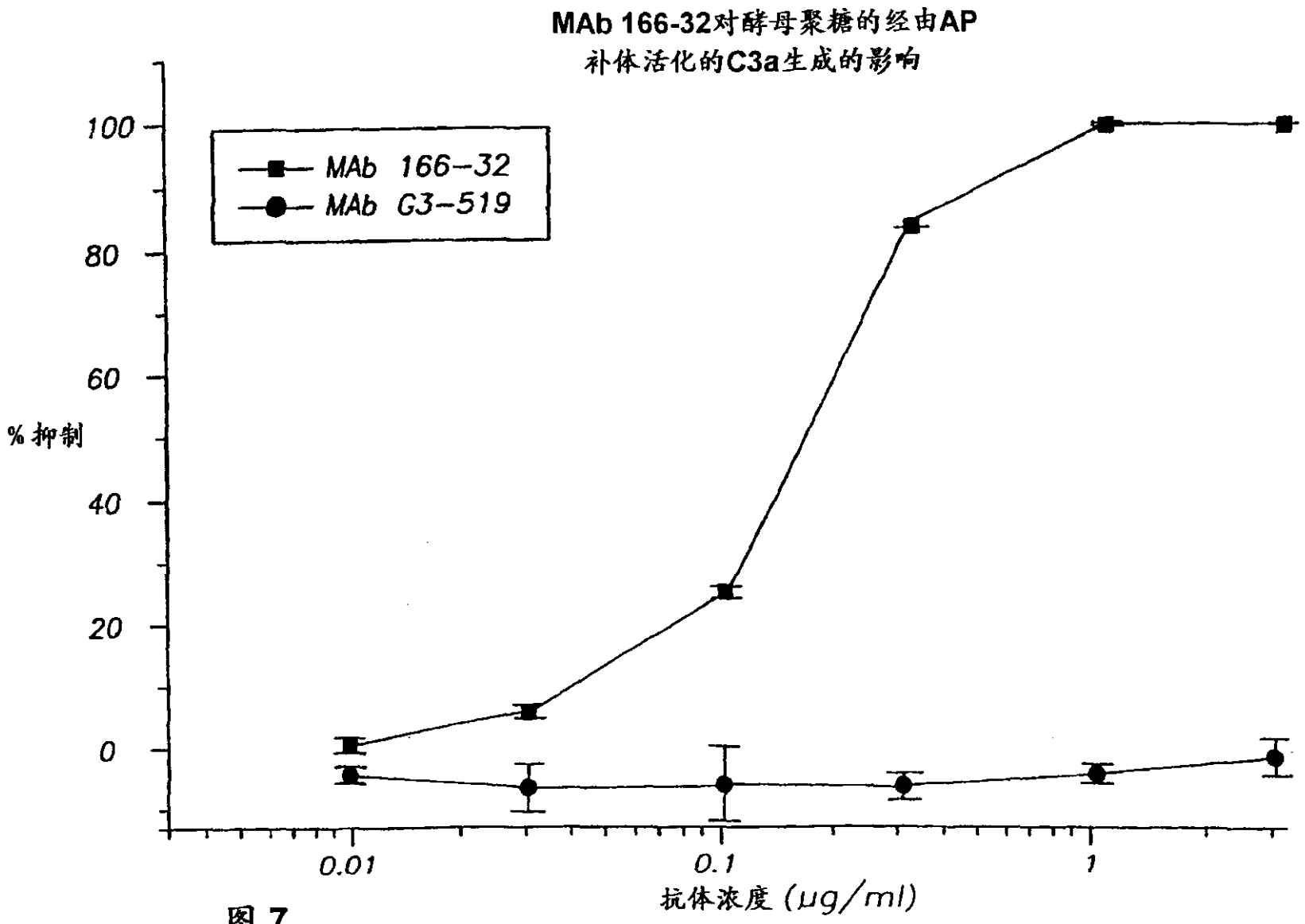

MAb 166-32对酵母聚糖的经由AP
补体活化的C3a生成的影响
MAb 166-32
MAb G3-519
100
80
60
40
20
0
% 抑制
0.01
0.1
1
抗体浓度 (µg/ml)

图 7

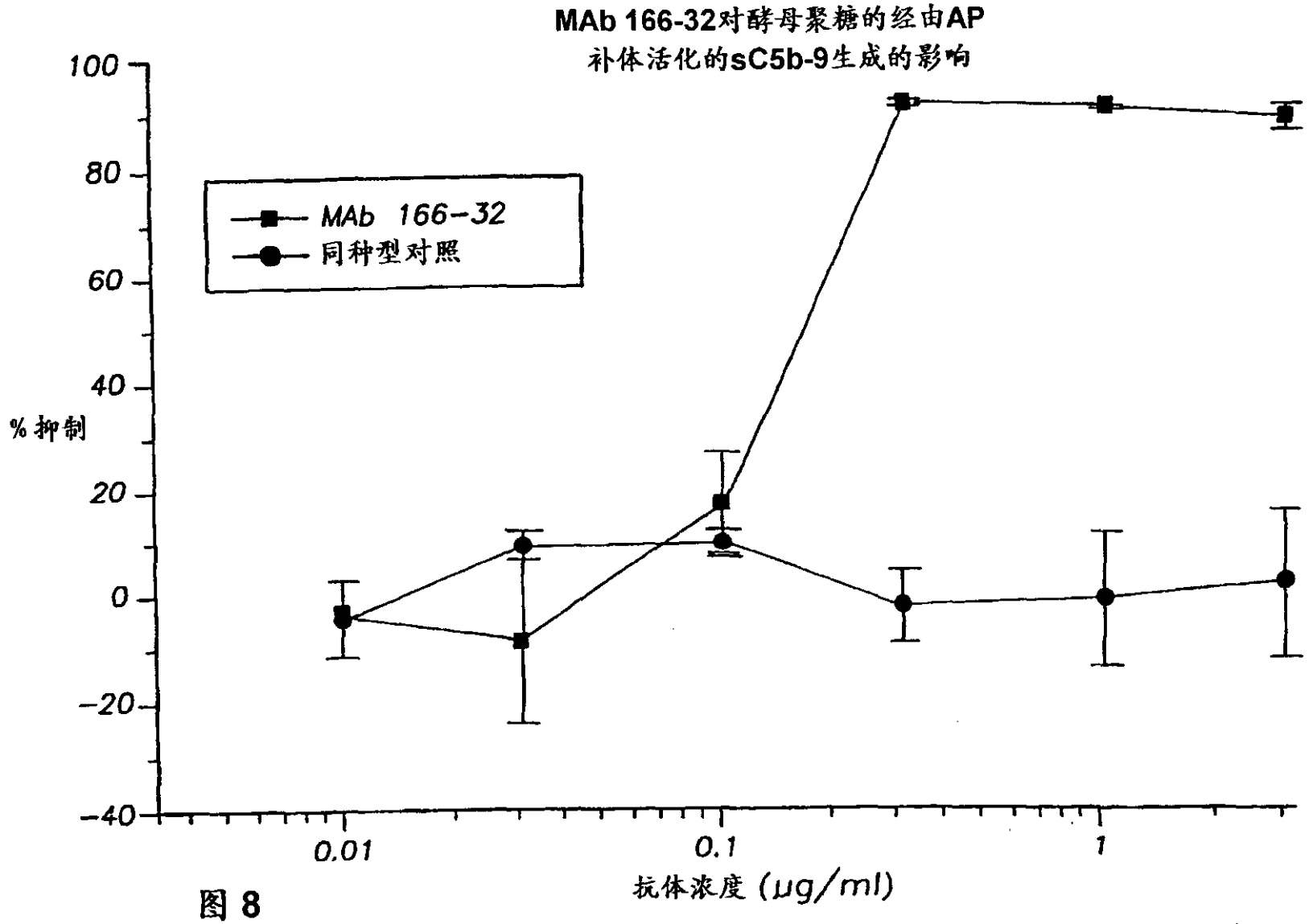

MAb 166-32对酵母聚糖的经由AP
补体活化的sC5b-9生成的影响
MAb 166-32
同种型对照
% 抑制
100
80
60
40
20
0
-20
-40
0.01
0.1
1
抗体浓度 (μg/ml)

图 8

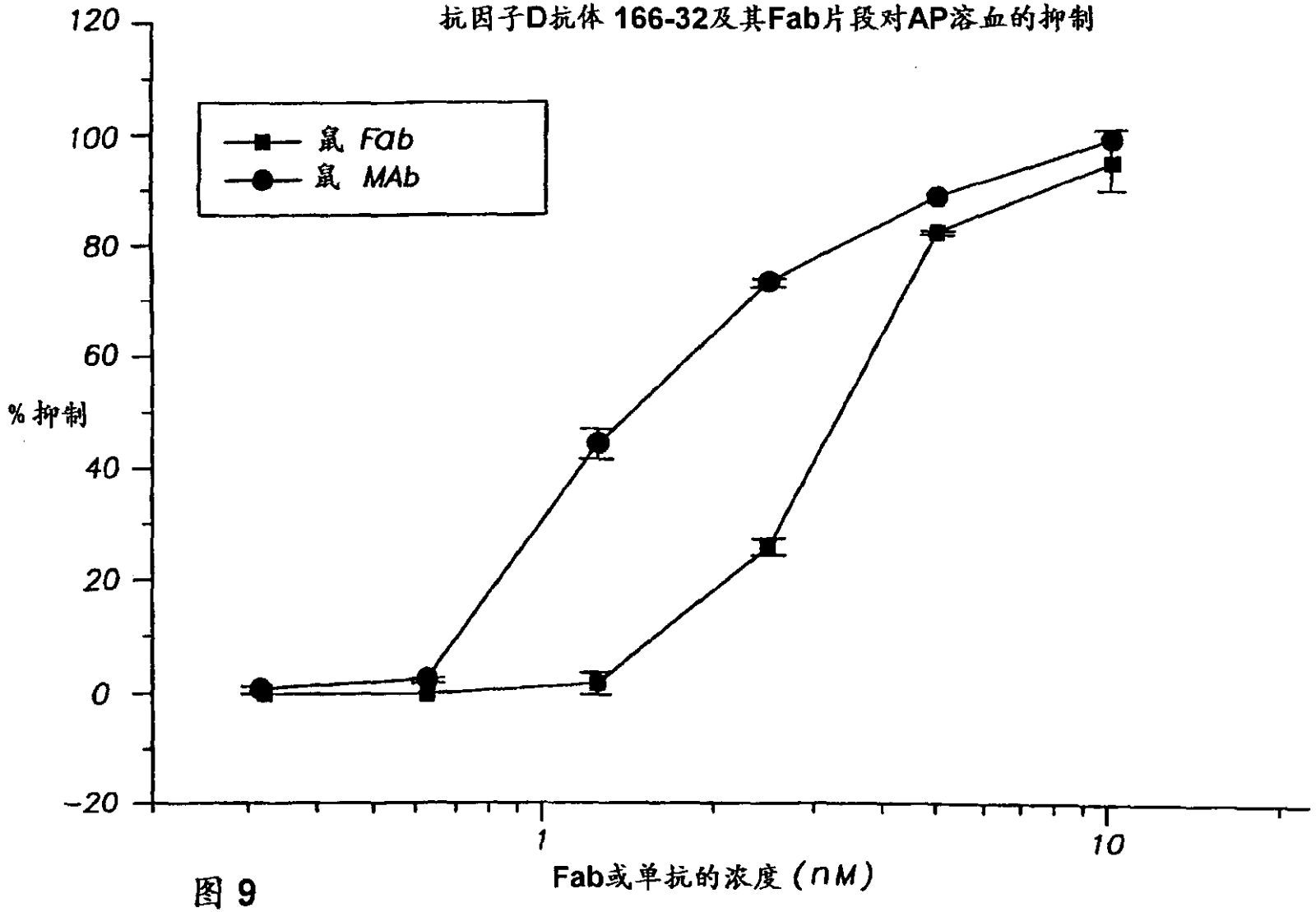

抗因子D抗体 166-32及其Fab片段对AP溶血的抑制
鼠 FAb
鼠 MAb
120
100
80
60
40
20
0
-20
% 抑制
1
10
Fab或单抗的浓度（nM）

图 9

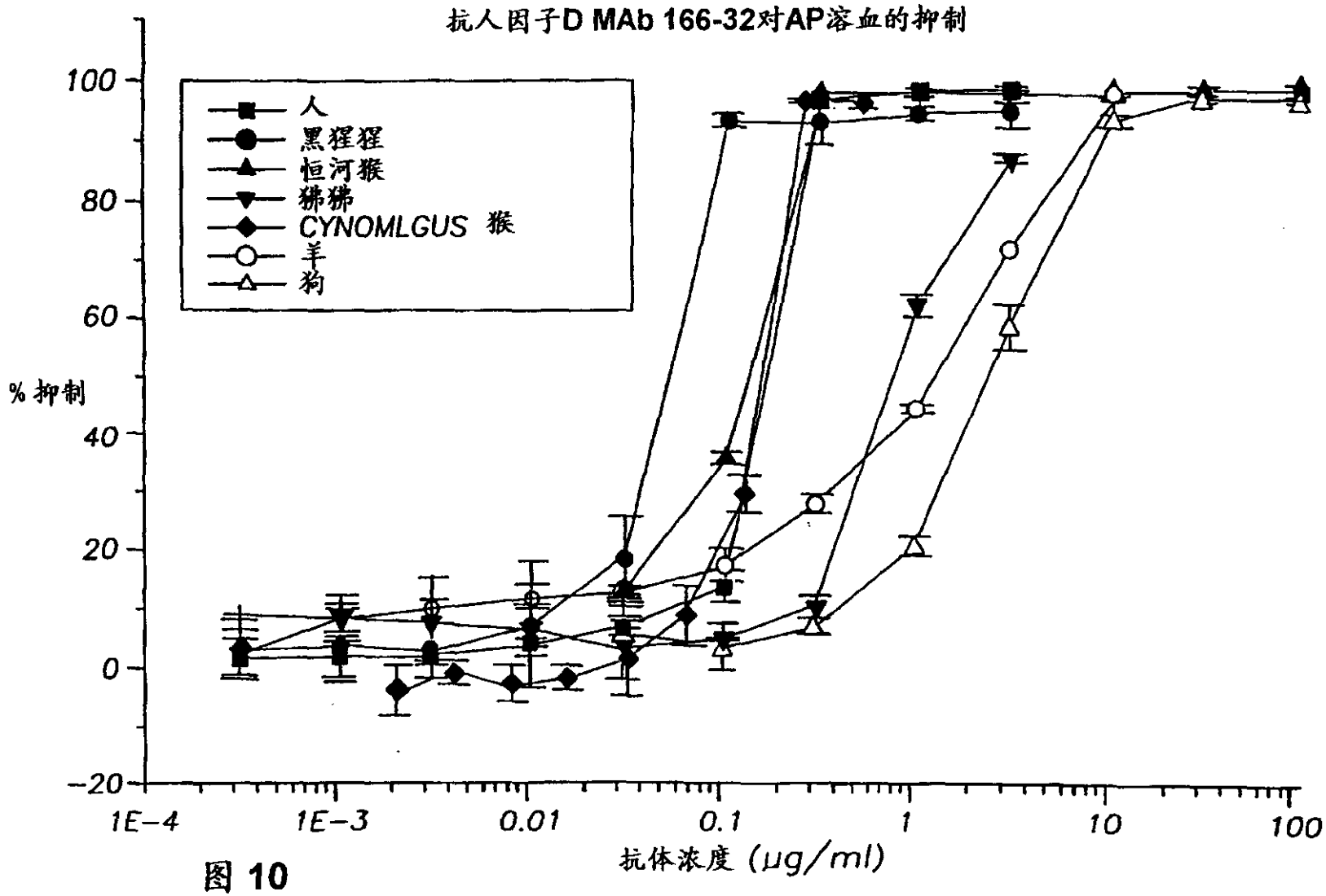

抗人因子D MAb 166-32对AP溶血的抑制
人
黑猩猩
恒河猴
狒狒
CYNOMLGUS 猴
羊
狗
% 抑制
100
80
60
40
20
0
-20
1E-4
1E-3
0.01
0.1
1
10
100
抗体浓度 (μg/ml)

图 10

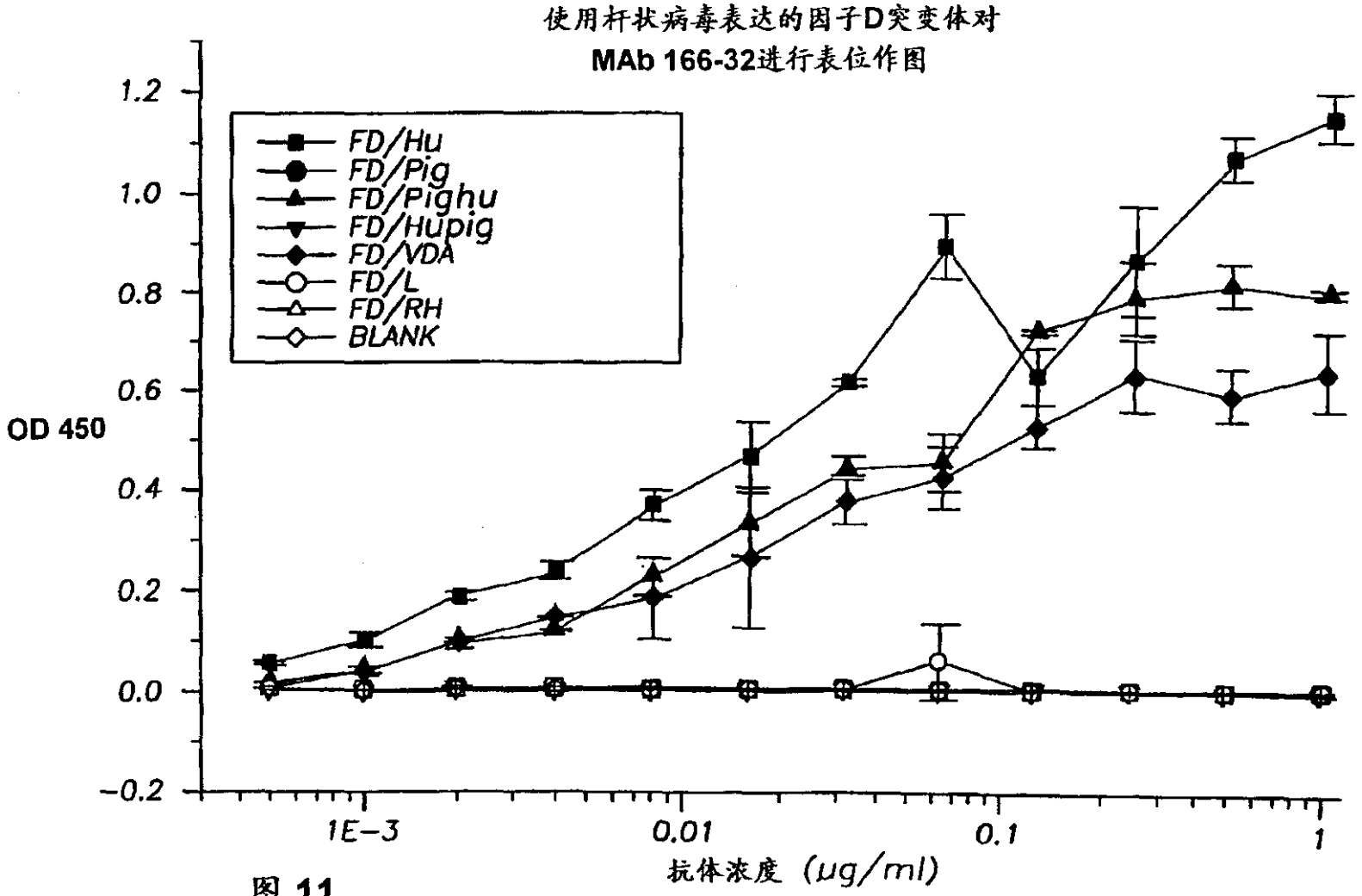
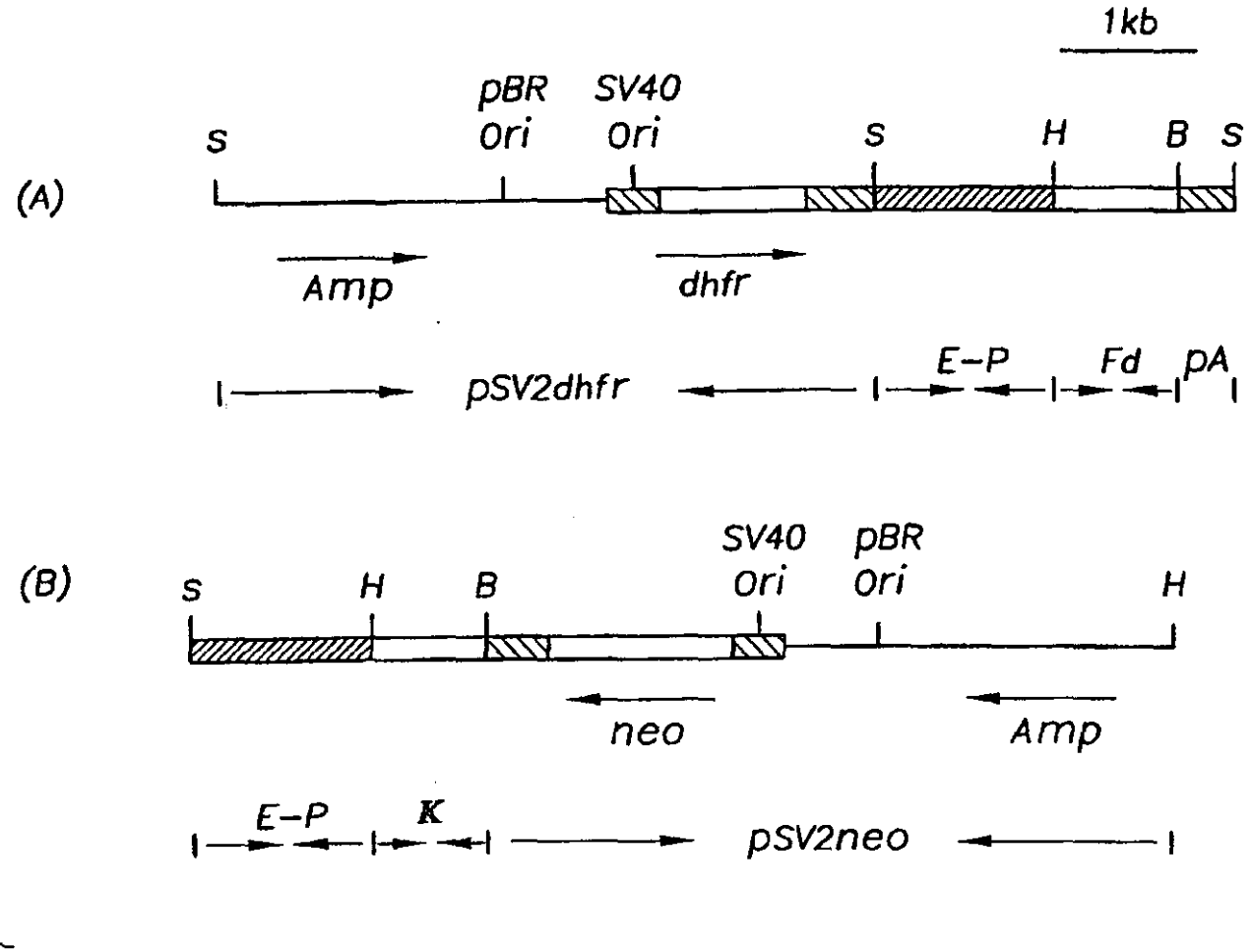

使用杆状病毒表达的因子D突变体对
MAb 166-32进行表位作图
FD/Hu
FD/Pig
FD/Pighu
FD/Hupig
FD/VDA
FD/L
FD/RH
BLANK
OD 450
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
1E-3
0.01
0.1
1
抗体浓度 (μg/ml)

图 11

图 12

嵌合型166-32 Fab的表达载体质粒的示意图:
(A) pSV2dhfrFd 和 (B) pSV2neoK



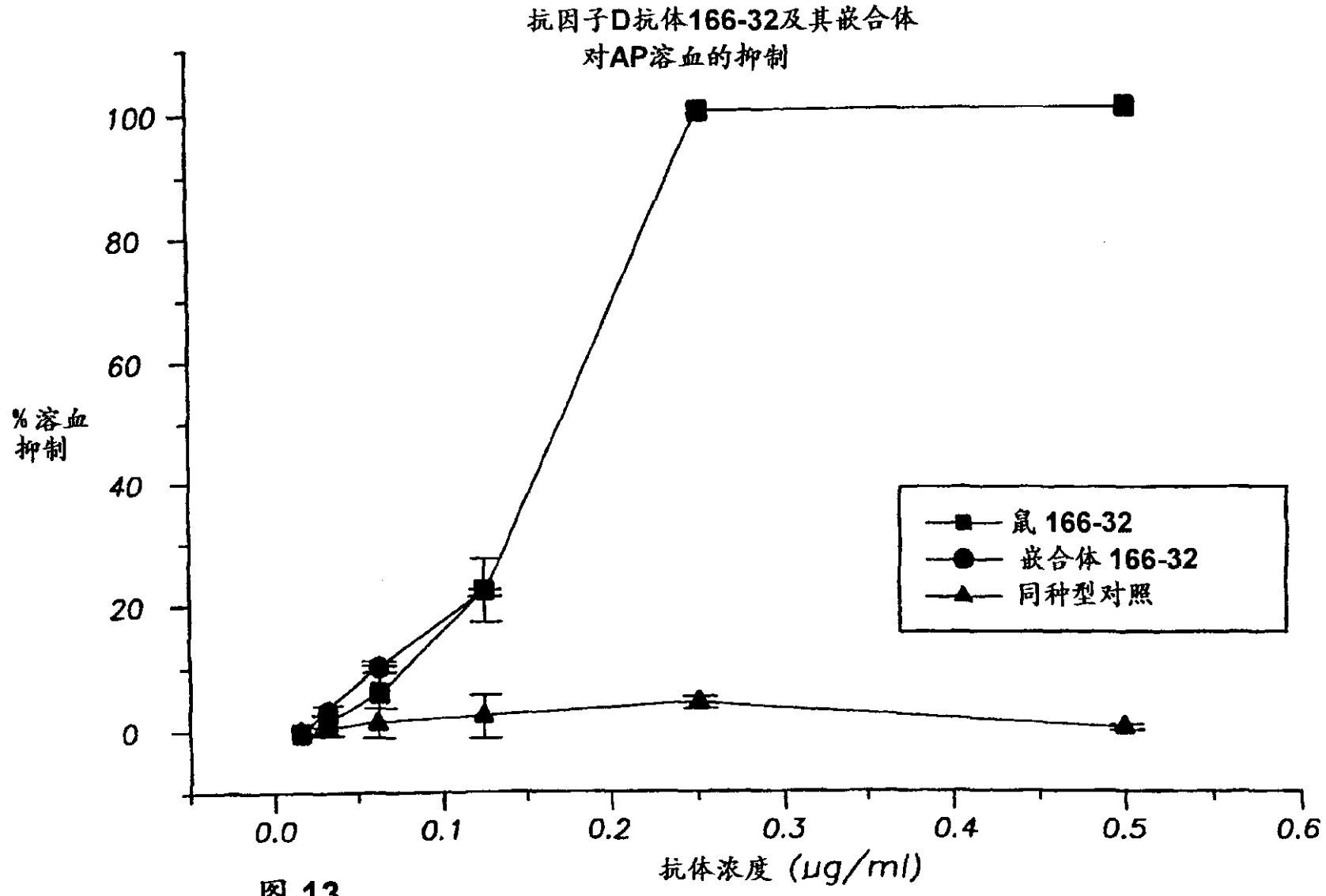

抗因子D抗体166-32及其嵌合体
对AP溶血的抑制
% 溶血
抑制
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
抗体浓度 (μg/ml)
鼠 166-32
嵌合体 166-32
同种型对照

图 13

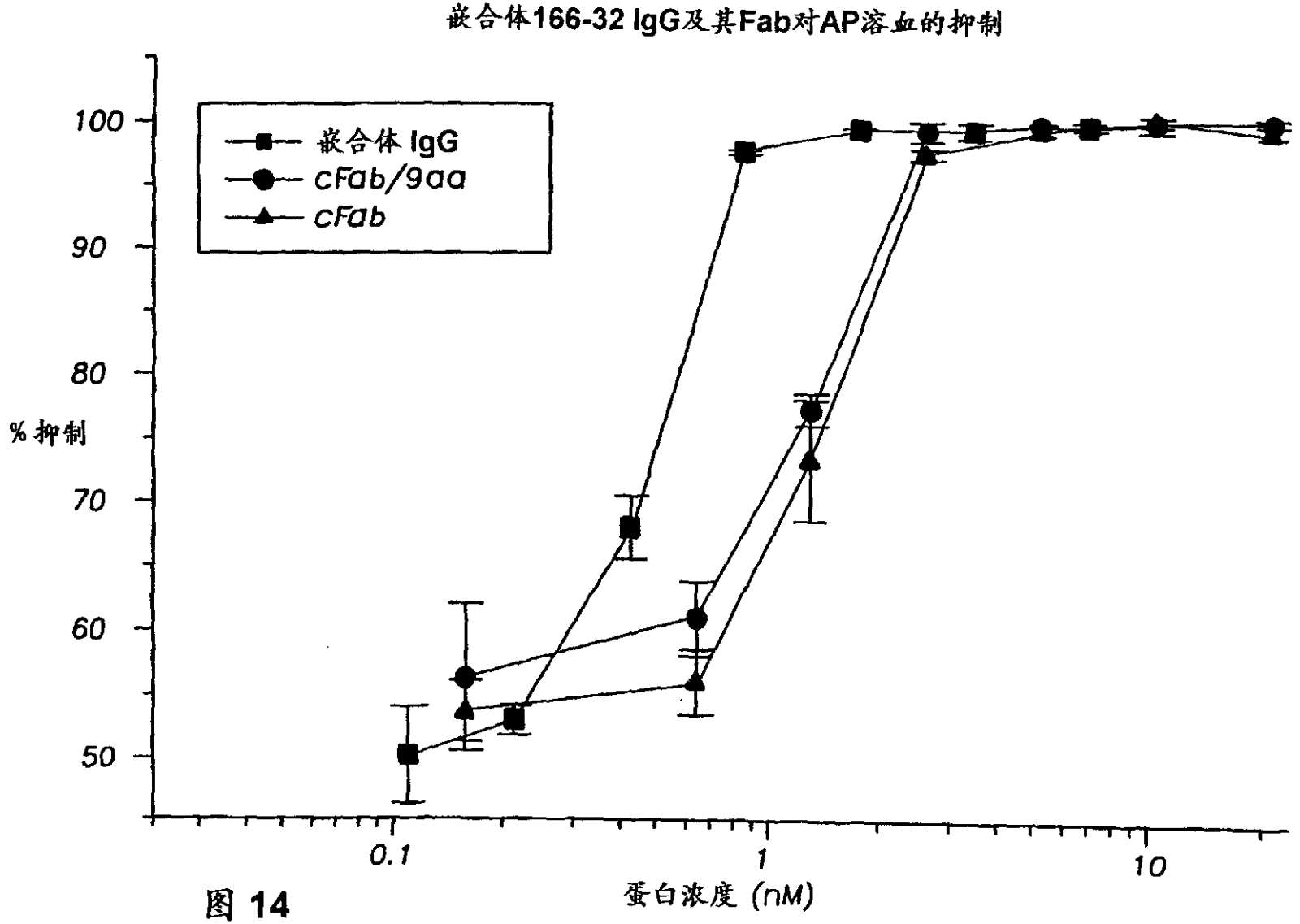

嵌合体166-32 IgG及其Fab对AP溶血的抑制
嵌合体 IgG
cFab/9aa
cFab
100
90
80
70
60
50
% 抑制
0.1
1
10
蛋白浓度 (nM)

图 14

MAb 166-32对用人血浆灌注的分离的兔心脏的LVDP和LVEDP的影响

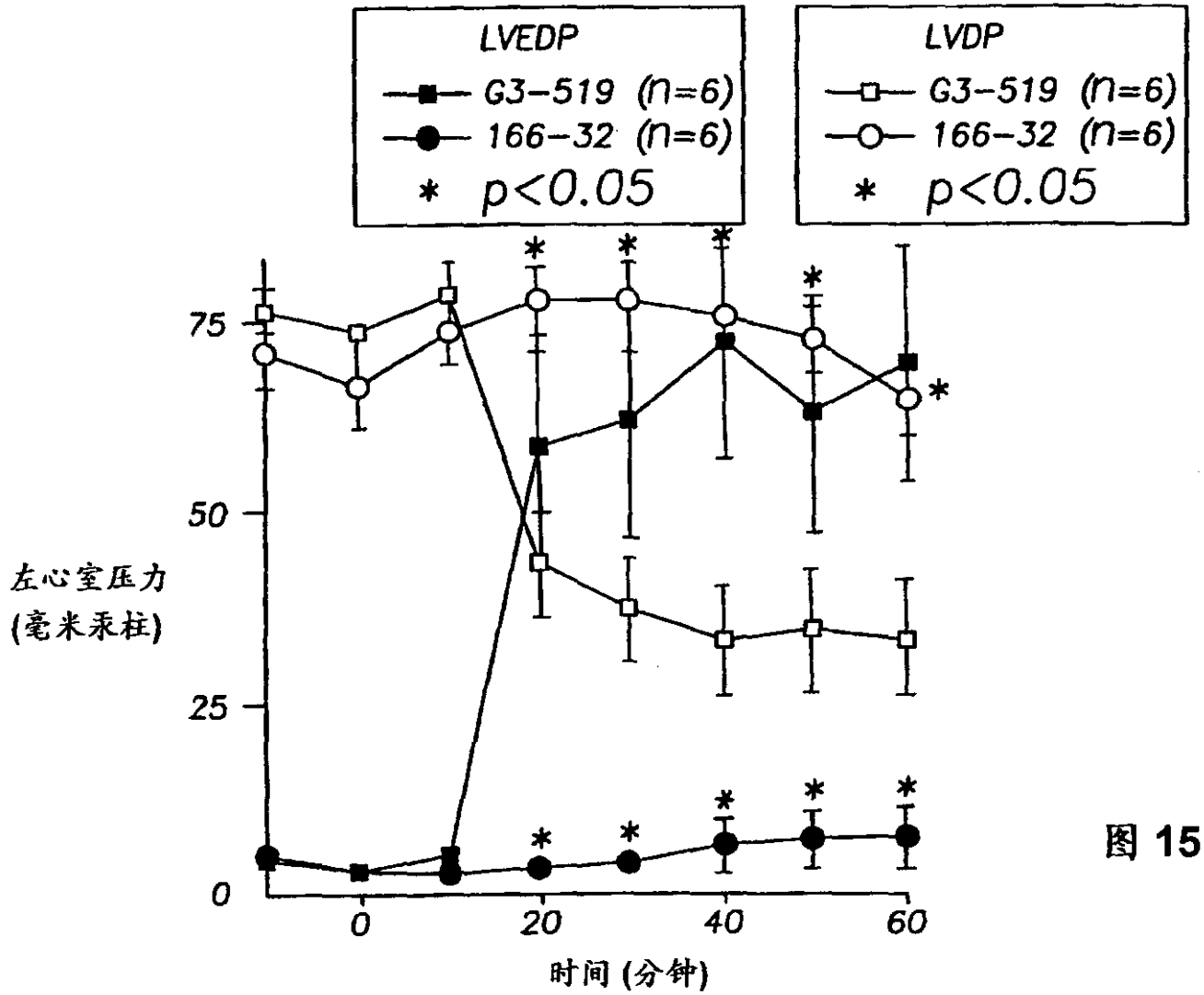
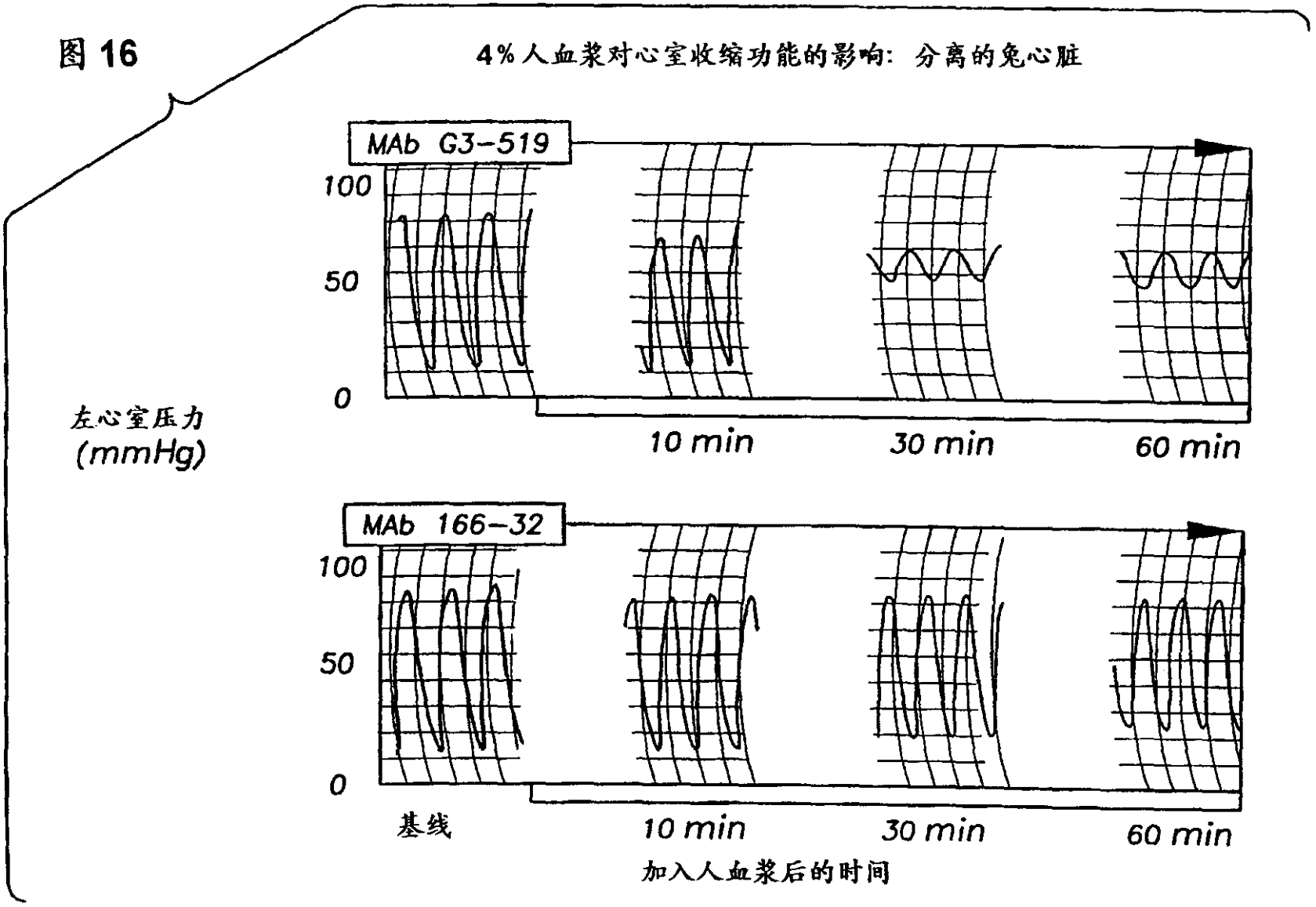


图 15

图 16

4%人血浆对心室收缩功能的影响：分离的兔心脏



MAb 166-32对用人血浆灌注的分离兔心脏中的Bb水平的影响

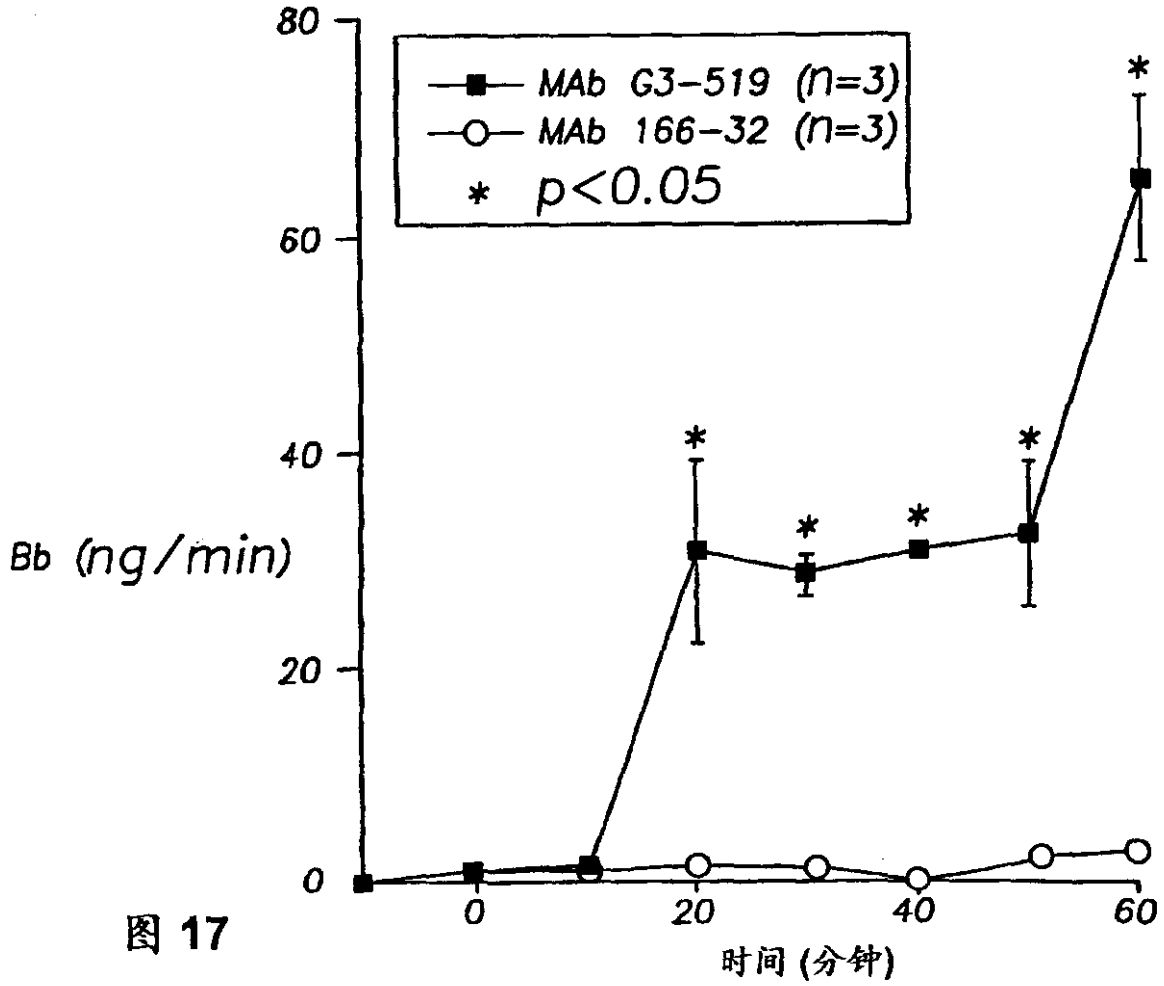


图 17

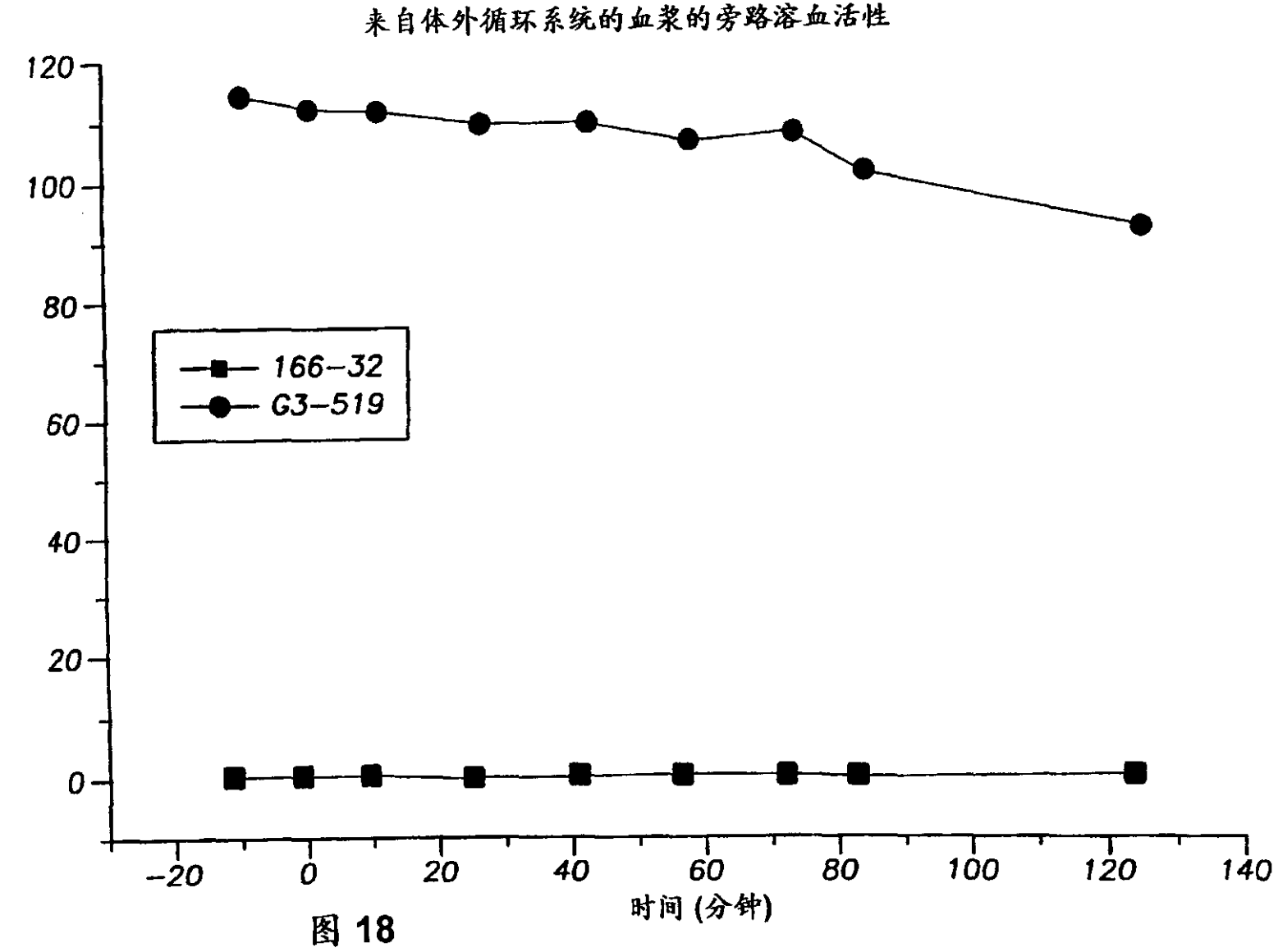

来自体外循环系统的血浆的旁路溶血活性
溶血
18
120
100
80
60
40
20
0
166-32
G3-519
-20
0
20
40
60
80
100
120
140
时间 (分钟)

图 18

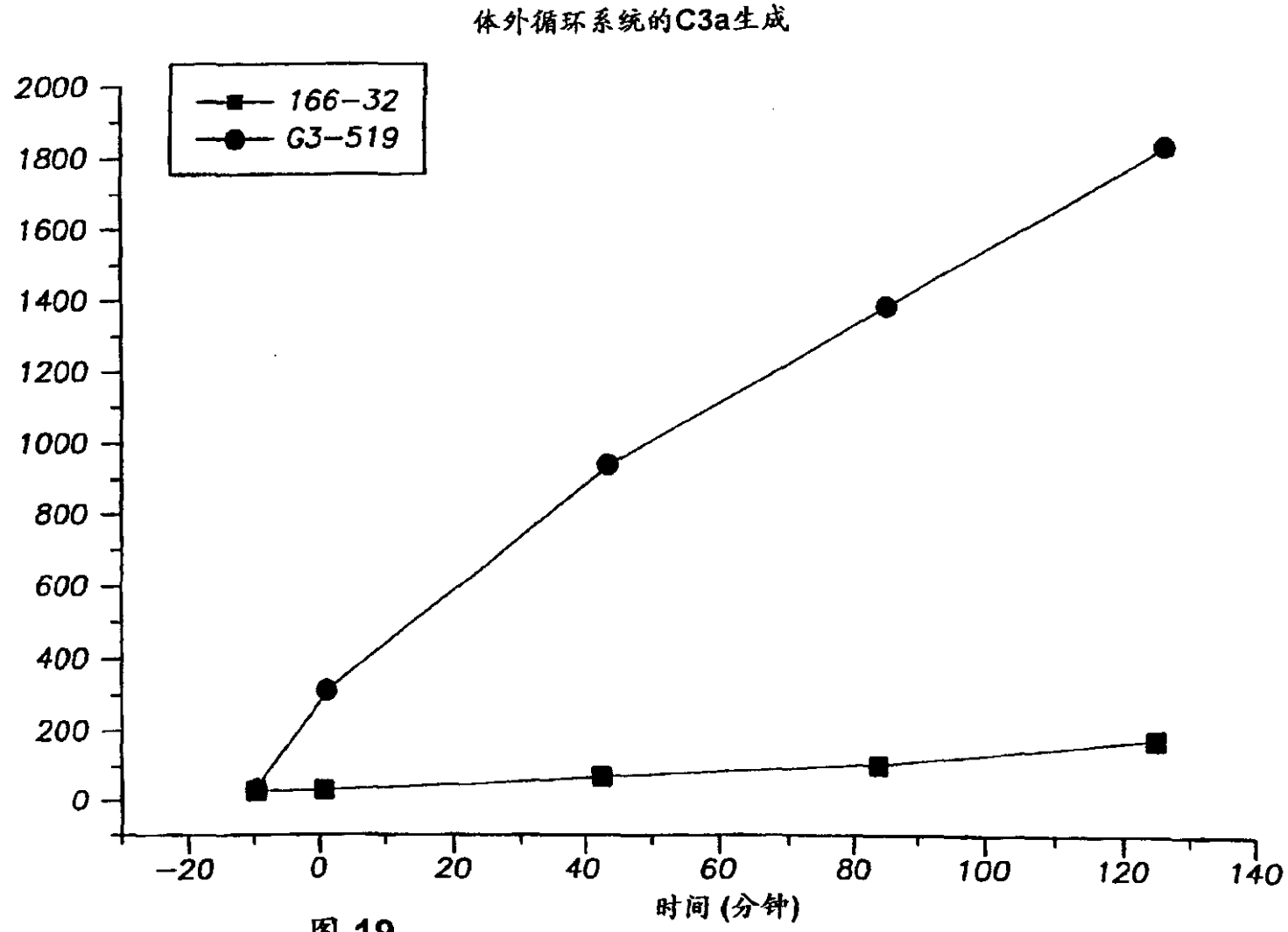

体外循环系统的C3a生成
166-32
G3-519
C3a 浓度
(μg/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-20
0
20
40
60
80
100
120
140
时间 (分钟)

图 19

体外循环系统中的sC5b-9生成
166-32
G3-519
3000
2500
2000
1500
1000
500
0
sC5b-9
浓度
(ng/ml)
-20
0
20
40
60
80
100
120
140
时间 (分钟)

图 20

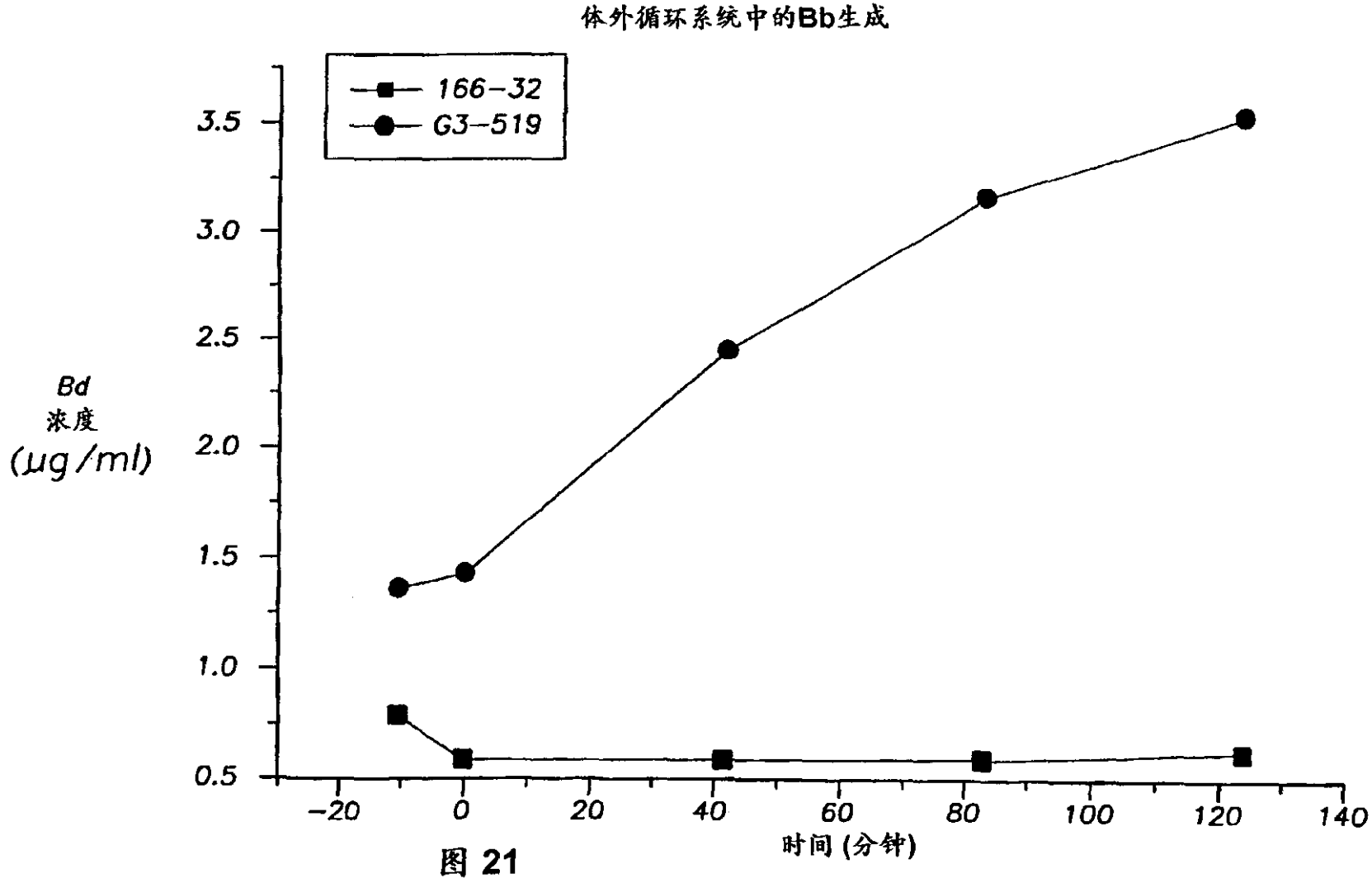

体外循环系统中的Bb生成
166-32
G3-519
Bd
浓度
(μg/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
-20
0
20
40
60
80
100
120
140
时间 (分钟)

图 21

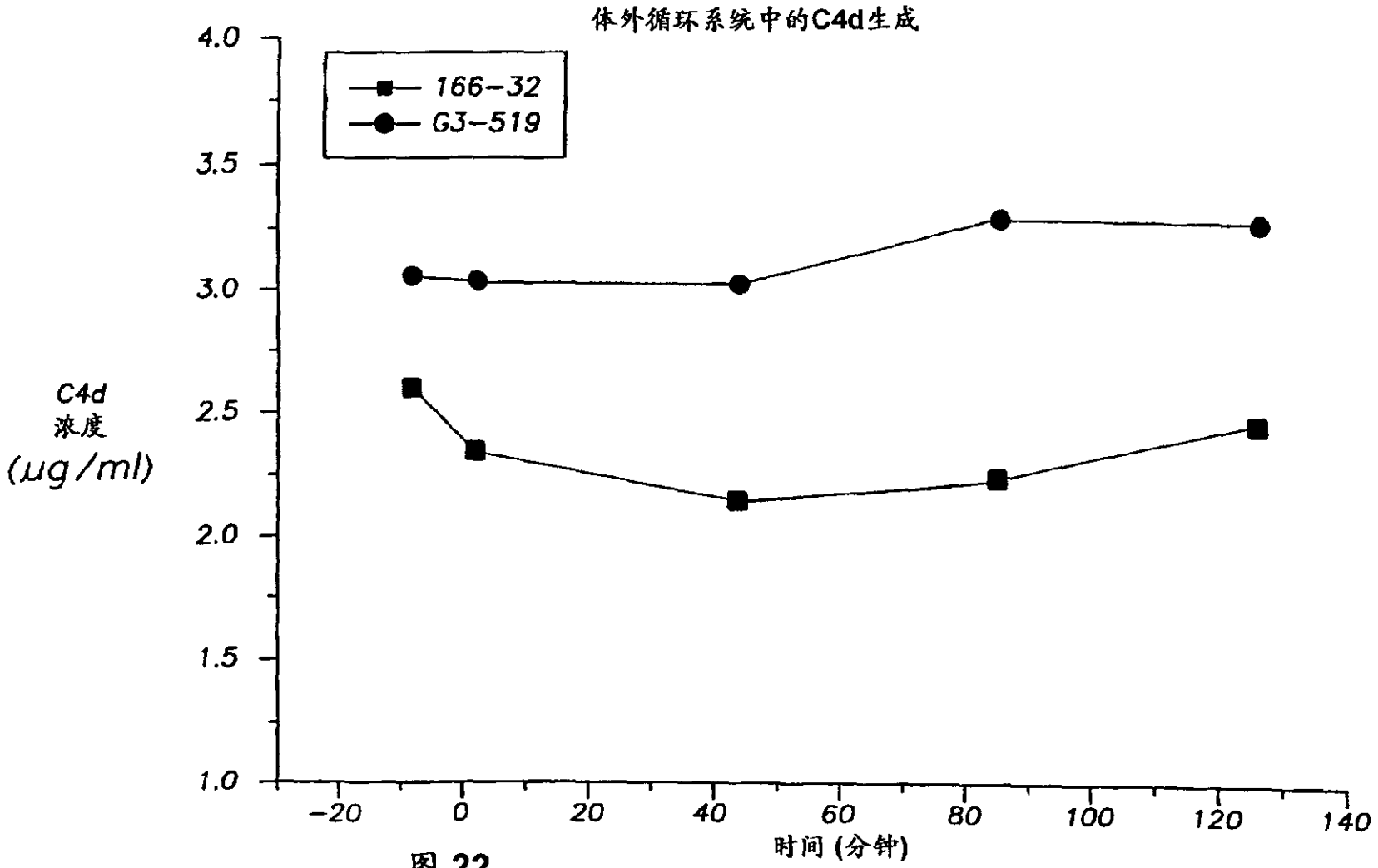

体外循环系统中的C4d生成
166-32
G3-519
C4d
浓度
(μg/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
-20
0
20
40
60
80
100
120
140
时间 (分钟)

图 22

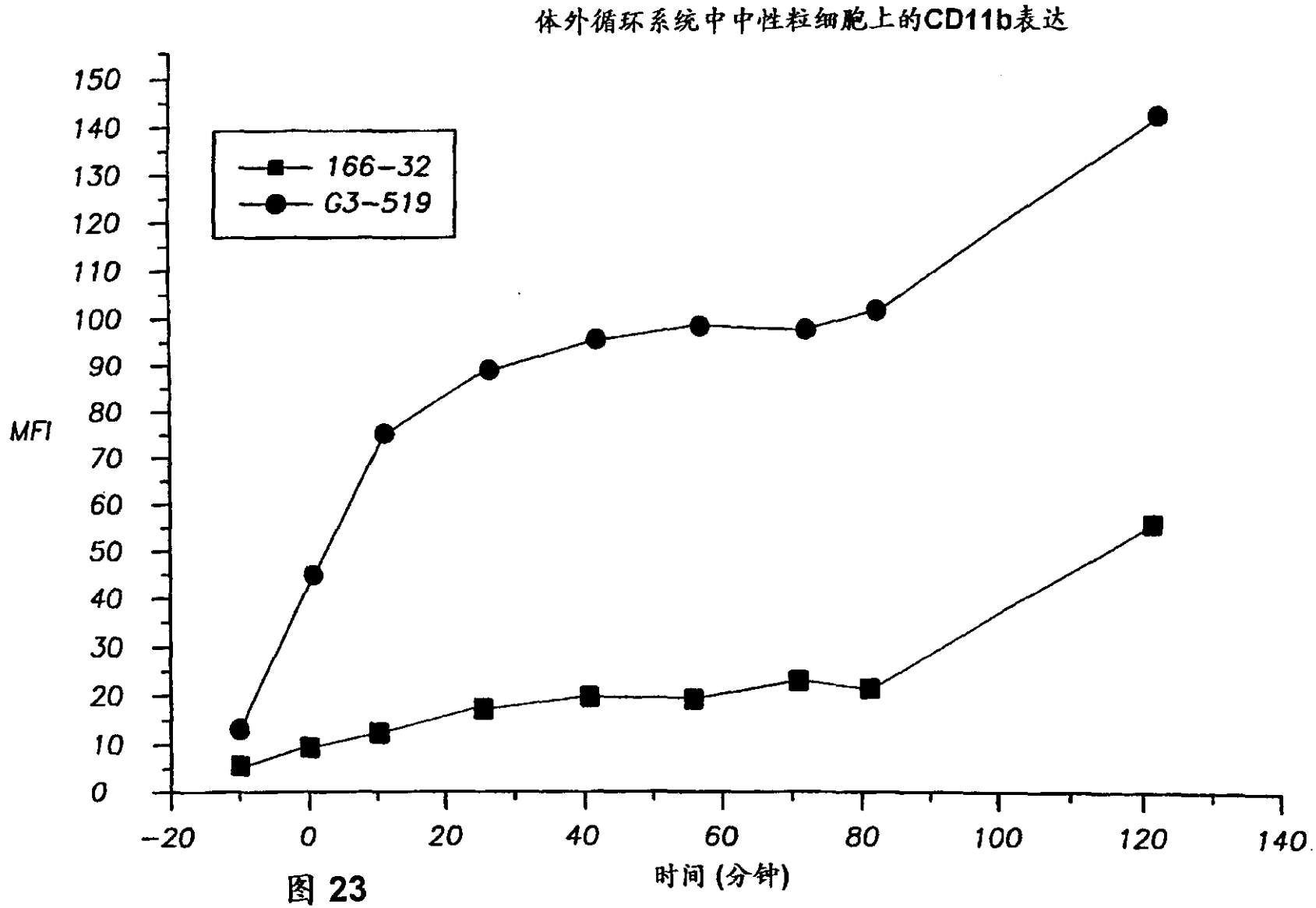

体外循环系统中中性粒细胞上的CD11b表达
166-32
G3-519
MFI
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-20
0
20
40
60
80
100
120
140
时间 (分钟)

图 23

体外循环系统中血小板上的CD62P表达

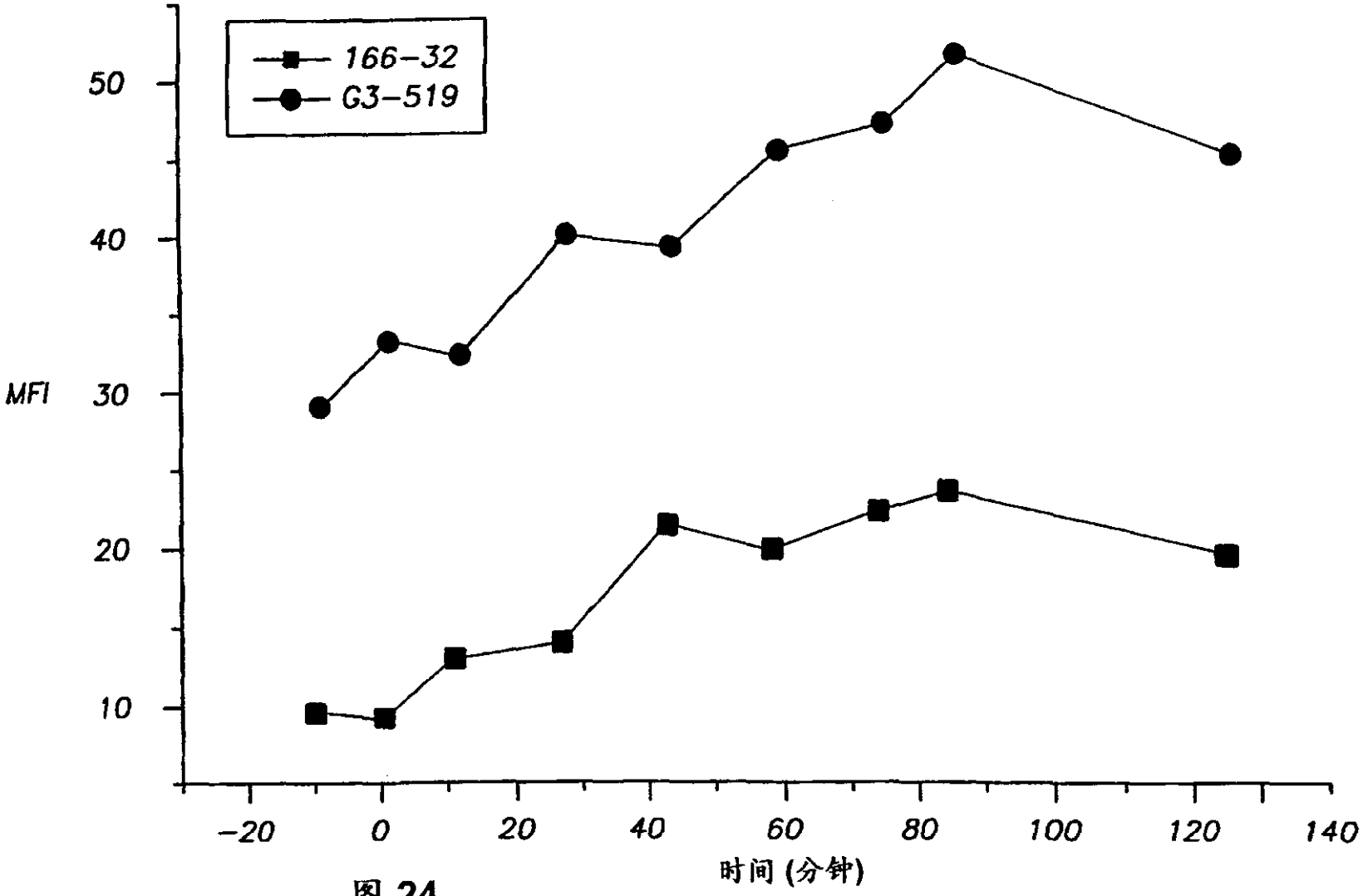


图 24

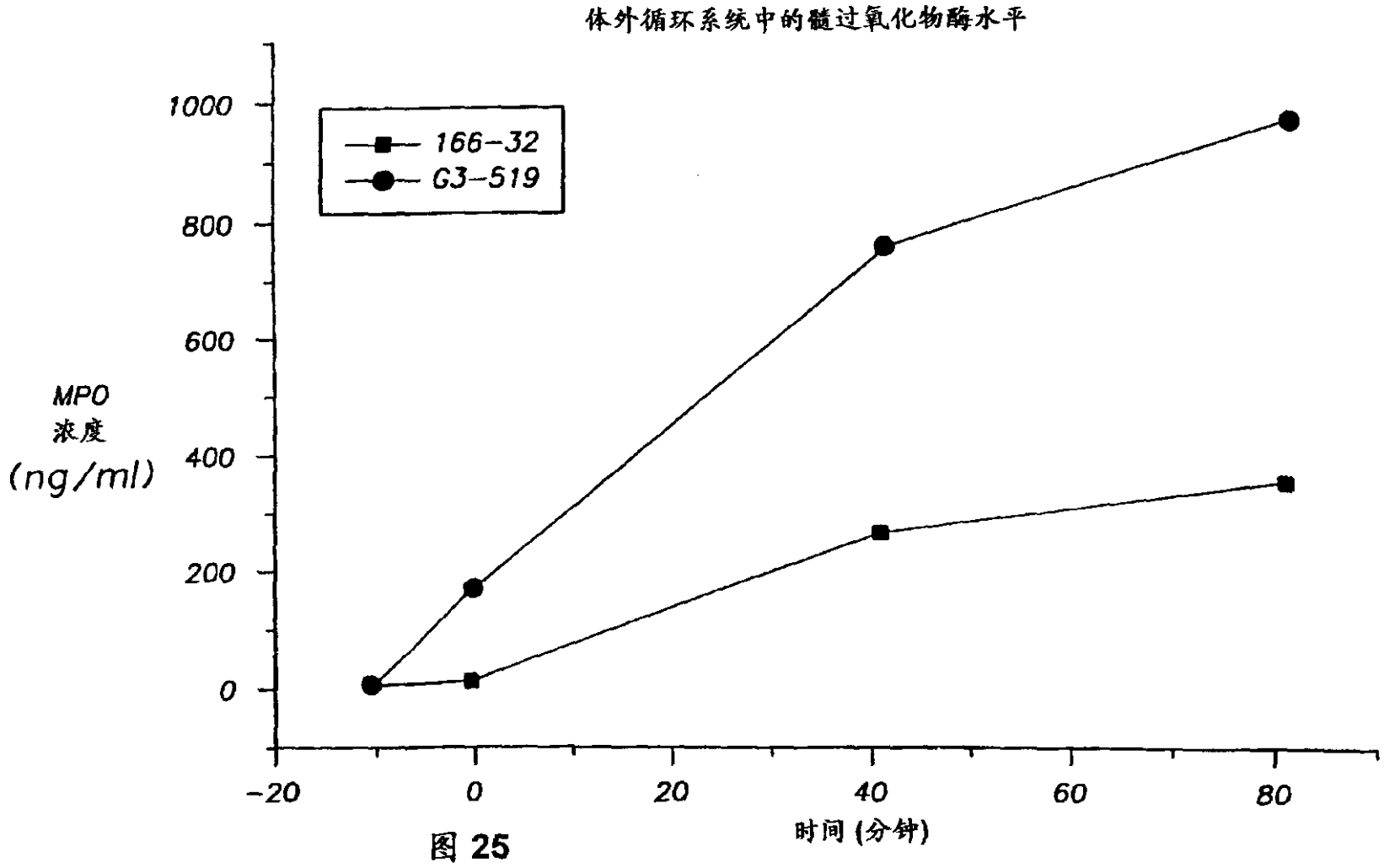

体外循环系统中的髓过氧化物酶水平
166-32
G3-519
MPO
浓度
(ng/ml)
1000
800
600
400
200
0
-20
0
20
40
60
80
时间 (分钟)

图 25