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(54) Title: NATURAL IGM ANTIBODIES AND INHIBITORS THEREOF

(57) Abstract: The invention provides natural IgM antibody inhibitors that may be used to treat various inflammatory diseases or disorders.

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Natural IgM Antibodies and Inhibitors Thereof

1. Government support

This invention was made with government support under grant No. GM52585, GM24891, and GM07560 from the National Institutes of Health. The government has certain rights in the invention.

2. Cross-reference to related applications

This application claims the benefit of U.S. Provisional Application No. 60/588,648, filed on July 16, 2004 and U.S. Provisional Application No. 60/549,123 filed on March 1, 2004; the content of each provisional application is specifically incorporated by reference herein.

3. Background of the Invention

Nucleated cells are highly sensitive to hypoxia and even short periods of ischemia in multi-cellular organisms can have dramatic effects on cellular morphology, gene transcription, and enzymatic processes. Mitochondria, as the major site of oxygen metabolism, are particularly sensitive to changes in oxygen levels and during hypoxia release reactive oxygen species that chemically modify intracellular constituents such as lipids and proteins. Clinically these effects manifest as an inflammatory response in the patient. Despite intensive investigations of cellular responses to hypoxia little is known regarding the initiation of acute inflammation.

Acute inflammatory responses can result from a wide range of diseases and naturally occurring events such as stroke and myocardial infarction. Common medical procedures can also lead to localized and systemic inflammation. Left untreated inflammation can result in significant tissue loss and may ultimately lead to multi-system failure and death. Interfering with the inflammatory response after injury may be one method to reduce tissue loss.

Inflammatory diseases and acute inflammatory responses resulting from tissue injury, however, cannot be explained by cellular events alone. Accumulating evidence supports a major role for the serum innate response or complement system in inflammation. Studies to date have looked at tissue injury resulting from ischemia and reperfusion as one type of inflammatory disorder that is complement dependent. For example, in the rat

myocardial model of reperfusion injury, pretreatment of the rats with the soluble form of the complement type 1 receptor dramatically reduced injury. Understanding how complement activation contributes to an inflammatory response is an area of active investigation.

Inflammatory diseases or disorders are potentially life-threatening, costly, and affect a large number of people every year. Thus, effective treatments of inflammatory diseases or disorders are needed.

4. Summary of the Invention

In one aspect, the invention features isolated natural immunoglobulins (IgMs). In one embodiment, the antibody is produced by ATCC Accession Number PTA-3507. In another embodiment, the antibody has a light chain variable region comprising the amino acid sequence shown as SEQ ID NO: 8. In yet another embodiment, the antibody has a heavy chain variable region comprising the amino acid sequence shown as SEQ ID NO: 2.

In another aspect, the invention features IgM inhibitors and pharmaceutical preparations thereof. In one embodiment, the IgM inhibitor is a peptide that specifically binds to a natural IgM and thereby blocks binding to the antigen and/or complement activation. In one embodiment, the peptide includes the following consensus sequence: xNNNxNNxNNNN (SEQ ID NO: 14). Certain inhibitory peptides are provided as SEQ ID NOs: 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, and 38. Inhibitory peptides may be modified, for example to increase *in vivo* half-life or bioavailability. Inhibitory peptides may also be labeled to facilitate detection.

In another aspect, the invention features nucleic acids encoding peptides that specifically bind to natural IgM antibodies, as well as vectors and host cells for expressing the peptides. Certain nucleic acids are provided as SEQ ID NOs: 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35 and 37.

In a further aspect, the invention features methods of treating an inflammatory disease in a subject by administering to the subject a pharmaceutical composition comprising an IgM inhibitor as disclosed herein.

In yet other aspects, the invention features method of detecting, diagnosing or monitoring inflammatory diseases in a subject using labeled inhibitory antibodies.

Other features and advantages of the invention will be apparent based on the following Detailed Description and Claims.

5. *Brief Description of the Drawings*

Figure 1 shows an IgM heavy chain sequence of B-1 hybridoma 22A5. **(A)** shows the IgM^{CM-22} (or 22A5 IgM) heavy chain nucleic acid sequence (SEQ ID NO: 1) and **(B)** shows the amino acid sequence corresponding to the heavy chain sequence of SEQ ID NO: 1 (SEQ ID NO: 2). Framework regions (FVWR) and complementarity-determining regions (CDR) are indicated above the nucleotides.

Figure 2 shows an IgM light chain sequence of B-1 hybridoma 22A5. **(A)** shows the IgM^{CM-22} (or 22A5 IgM) light chain nucleic acid sequence (SEQ ID NO: 7) and **(B)** shows the amino acid sequence corresponding to the light chain sequence of SEQ ID NO: 7 (SEQ ID NO: 8). Framework- regions (FVWR) and complementarity-determining regions (CDR) are indicated above the nucleotides.

Figure 3 is a bar graph depicting changes in intestinal permeability of inbred mice after intestinal ischemia and reperfusion or no injury (sham). WT represents parent strain for Cr2-/- mice. Cr2-/- was reconstituted with pooled IgG or IgM or saline control. Pooled IgM or IgG (0.5 mg) was administered intravenously approximately 1 hour before treatment. Values are means + standard error; n equals the number of mice in experimental groups.

Figure 4 demonstrates reconstitution of I/R injury in antibody deficient mice (RAG-1) by pooled IgM from a single B-1 cell hybridoma clone. IgM or saline was injected intravenously 30 minutes before initial laparotomy. At the end of reperfusion, blood is obtained and permeability index is calculated as the ratio of ^{125}I counts of dried intestine versus that of blood. Values represent means ± standard error; n equals the numbers of mice used in experimental groups. 1 = WT plus normal saline; 2 = RAG plus normal saline; 3 = RAG plus IgM hybridoma CM-22; 4 = WT sham control.

Figure 5 is a schematic diagram of the proposed role for complement and complement receptors in positive selection of peritoneal B-1 lymphocytes.

Figure 6A is a graph showing the ELISA screening of M-13 phage-display library for IgM^{CM-22}-specific peptides. Symbols: □-P1 clone; X-P2 clone, ○-P7 clone; ◊-P8 clone.

The plate was coated with a solution of IgM^{CM-22} before addition of varying concentrations of phage-clones. The results are representative of at least three independent experiments.

Figure 6B is a bar graph showing that the synthetic peptide P8 inhibits IgM^{CM-22} binding of phage clone P8. ELISA was performed with varying concentrations of the synthetic peptide P8 added to the IgM^{CM-22}-coated plate prior to the addition of $5x10^{11}$ PFU phage. The results are representative of at least three independent experiments.

Figure 6C is a bar graph showing specific binding of the P8 peptide to IgM^{CM-22}. The ELISA plates were coated with 50 μg/ml solution of P8 peptide, followed by addition of IgM^{CM-22} or IgM^{CM-75} at 1 or 10 μg/ml. IgM binding was detected with a biotinylated rat anti-mouse IgM followed by streptavidin-phosphatase and color reaction. The results are representative of at least three independent experiments.

Figure 7A is a series of photomicrographs showing that the P8 peptide blocked IgM^{CM-22} mediated injury *in vivo*. Two upper panels (i and ii) are representative sections (stained with Haematoxylin and Eosin) prepared following RI treatment in RAG-$1^{-/-}$ mice with IgM^{CM-22} alone or mixed with P8 peptide, respectively. Two lower panels (iii and iv) are representative sections prepared from wild type mice treated for intestinal reperfusion injury, which received either saline or peptide P8 5 minutes prior to reperfusion. Arrows indicate pathologic features of injury. Magnification 200x.

Figure 7B is a scatter plot indicating the mean pathology score of each group of treated animals. Each symbol represents the score from one animal. Control group is WT mice pretreated with a control peptide (ACGMPYVRIPTA; SEQ ID NO: 61) at a similar dose as the peptide P8. * indicates statistical significance determined by Student *t* test of the P8-treated versus untreated groups ($p<0.05$).

Figure 7C is a series of photomicrographs showing the absence of IgM and complement C3 or C4 within the microvilli of P8-treated animals. Representative cryosections of intestinal tissues were harvested following intestinal RI. Panels i-viii are IgM^{CM-22} reconstituted RAG-$1^{-/-}$ mice without pretreatment with P8 (panels i-iv) or with P8 (panels v-viii). Panels represent cryosections from the intestines of WT without P8 (panels ix - xii) or pretreated with P8 (panels xiii - xvi). Sections (i, iii, v, vii, ix, xiii, xv) were stained with anti-IgM-biotin followed by Streptavidin-Alexa-568 (red) and counterstained with DAPI (violet). Panels (ii, x, xiv) were stained with anti-C4-Alexa 488/FITC (green) and panels (iv, viii, xii, xvi) were stained with anti-C3-FITC (green). Magnification 400x.

Figure 8A is an immunoblot showing the immune precipitation of reperfusion injury (RI) specific antigens. Detection of a unique band (arrow) at approximately 250 kDa on a SDS-PAGE (10%). Size markers are indicated on the left. Intestinal lysates were prepared from RAG-$1^{-/-}$ mice reconstituted with IgM^{CM-22} and either sham control (no ischemia) or subjected to ischemia followed by reperfusion for 0 or 15 min.

Figure 8B is a series of graphs showing results of *in vitro* binding assays of IgM^{CM-22} to the isoforms of non-muscle myosin heavy chain-II (NMHC-II). ELISA plates were coated with monoclonal antibodies for 3 different isoforms of NMHC-II (upper left: isoform A, upper right: isoform B, lower left: isoform C and lower right: anti-pan myosin antibody). Bound myosin heavy chain from intestinal lysates was detected by IgM^{CM-22} or IgM^{CM-31}. The results represent mean ± standard error of OD 405 nm units and are representative of triplicate samples.

Figure 8C is a photomicrograph and a scatter plot showing the restoration of RI injury by anti-pan myosin antibody in RAG-$1^{-/-}$ mice. RAG-$1^{-/-}$ mice were reconstituted with affinity purified anti-pan myosin followed by RI surgery. The left panels represents morphologies of RAG-$1^{-/-}$ animals with saline control and with anti-pan myosin treatment. The right panel is the pathology scores of intestinal injury. The scatter plot (right panel) represents the pathology scores where each symbol represents a single animal.

Figure 9A is a graph showing the surface plasmon resonance for the self-peptide N2. Binding isotherms for samples of the self-peptide N2 with concentration from 10.5 μM to 120 μM injected over the IgM^{CM-22}-coupled surface.

Figure 9B is a graph showing the surface plasmon resonance for a control peptide. Binding isotherm for a same-length, random-sequence control peptide (AGCMPYVRIPTA; SEQ ID NO: 62), injected at a concentration of 117 μM over the IgM^{CM-22}-coupled surface.

Figure 9C is a graph showing the nonlinear curve fitting with a 1:1 Langmuir binding isotherm to the steady-state response levels for the injection showed in Figure 9A (χ^2=10).

Figure 9D is a graph showing the binding isotherm for the injection of the self-peptide N2 at 120 μM over a surface coupled with the control IgM^{CM-31}.

Figure 10A is a series of photomicrographs showing that the N2 self-peptide blocking RI in RAG-$1^{-/-}$ mice. Two upper panels show representative sections prepared

following RI treatment in RAG-$1^{-/-}$ mice with IgM^{CM-22} alone or mixed with N2 self-peptide. Two lower panels are representative sections prepared from WT mice treated for intestinal RI, which received either saline or N2 peptide 5 minutes prior to reperfusion.

Figure 10B is a scatter plot indicating the mean pathology score of each group of treated animals. Each symbol represents a single mouse. * indicates a statistical significance bases on a Student *t* test.

Figure 10C is a series of photomicrographs showing the prevention of the activation of classical pathway of complement in intestinal RI by the self-peptide N2. Representative cryosections of intestinal tissues were harvested following intestinal RI and treated with an antibody specific for the mouse IgM, C4 or C3 (400x magnification). IgM^{CM-22}-reconstituted RAG-$1^{-/-}$ mice without pretreatment with the self-peptide N2 are in panels i-iv or with the self-peptide N2 are in panels v-viii. Wild type mice without pretreatment with the self-peptide N2 are in panels ix-xii or with pretreatment with the self-peptide N2 are in panels xiii-xvi. The tissue in panels i, iii, v, vii, ix, xiii, xi, xv were stained with anti-IgM-biotin followed by Streptavidin-Alexa-568 (red) and counterstained with DAPI (violet). Panels ii, vi, x, and xiv were stained with anti-C4-FITC (green). Panels iv, viii, xii, xvi were stained with anti-C3-FITC (green).

Figure 11 shows the **(A)** nucleic acid sequence (SEQ ID NO: 47; Genbank Accession no. NM_022410) and **(B)** amino acid sequence (SEQ ID NO: 48; Genbank Accession no. NP_071855) of mouse non-muscle myosin heavy chain II-A (mNMHC-IIA).

Figure 12 shows the **(A)** nucleic acid sequence (SEQ ID NO: 49; Genbank Accession no. NM_002473) and **(B)** amino acid sequence (SEQ ID NO: 50; Genbank Accession no. NP_002464) of human non-muscle myosin heavy chain II-A (hNMHC-IIA).

Figure 13 shows the **(A)** nucleic acid sequence (SEQ ID NO: 51; Genbank Accession no. NM_175260) and **(B)** amino acid sequence (SEQ ID NO: 52; Genbank Accession no. NP_780469) of mouse non-muscle myosin heavy chain II-B (mNMHC-IIB).

Figure 14 shows the **(A)** nucleic acid sequence (SEQ ID NO: 53; Genbank Accession no. NM_005964) and **(B)** amino acid sequence (SEQ ID NO: 54; Genbank Accession no. NP_005955) of human non-muscle myosin heavy chain II-B (hNMHC-IIB).

Figure 15 shows the **(A)** nucleic acid sequence (SEQ ID NO: 55; Genbank Accession no. AY363100) and **(B)** amino acid sequence (SEQ ID NO: 56; Genbank

Accession no. AAQ24173) of mouse non-muscle myosin heavy chain II-C (mNMHC-IIC). **(C)** shows the nucleic acid sequence (SEQ ID No: 57; Genbank Accession no. NM_028021) and **(D)** shows the amino acid sequence (SEQ ID NO: 58; Genbank Accesion no. NP_079005) of another non-muscle myosin heavy chain II-C.

Figure 16 shows the **(A)** nucleic acid sequence (SEQ ID NO: 59; Genbank Accession no. NM_024729) and **(B)** amino acid sequence (SEQ ID NO: 60; Genbank Accession no. NP_079005) of human non-muscle myosin heavy chain II-C (hNMHC-IIC).

6. Detailed Description

6.1. Definitions:

For convenience, certain terms employed in the specification, examples, and appended claims are provided. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

"A" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"Amino acid" is used herein to refer to either natural or synthetic amino acids, including glycine and D or L optical isomers, and amino acid analogs and peptidomimetics.

"Antibody" is used herein to refer to binding molecules including immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen-binding site. Immunoglobulin molecules useful in the invention can be of any class (e.g., IgG, IgE, IgM, IgD, and IgA) or subclass. Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains. Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable domain at one end and a constant domain at its other end. Antibodies include, but are not limited to, polyclonal, monoclonal, bispecific, chimeric, partially or fully humanized antibodies, fully human antibodies (i.e., generated in a transgenic mouse expressing human immunoglobulin genes), camel antibodies, and anti-idiotypic antibodies. An antibody, or generally any molecule, "binds specifically" to an antigen (or other molecule) if the antibody binds preferentially to the antigen, and, e.g., has

less than about 30%, preferably 20%, 10%, or 1% cross-reactivity with another molecule. The terms "antibody" and "immunoglobulin" are used interchangeably.

"Antibody fragment" or "antibody portion" are used herein to refer to any derivative of an antibody which is less than full-length. In exemplary embodiments, the antibody fragment retains at least a significant portion of the full-length antibody's specific binding ability. Examples of antibody fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, scFv, Fv, dsFv diabody, minibody, Fd fragments, and single chain antibodies. The antibody fragment may be produced by any means. For instance, the antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody, it may be recombinantly produced from a gene encoding the partial antibody sequence, or it may be wholly or partially synthetically produced. The antibody fragment may optionally be a single chain antibody fragment. Alternatively, the fragment may comprise multiple chains which are linked together, for instance, by disulfide linkages. The fragment may also optionally be a multimolecular complex. A functional antibody fragment will typically comprise at least about 50 amino acids and more typically will comprise at least about 200 amino acids.

"Antigen-binding site" is used herein to refer to the variable domain of a heavy chain associated with the variable domain of a light chain.

"Bind" or "binding" are used herein to refer to detectable relationships or associations (e.g. biochemical interactions) between molecules.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

"Comprise" and "comprising" are used in the inclusive, open sense, meaning that additional elements may be included.

"Consensus sequence" is used herein to refer to the sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related sequences (See. e.g., Winnaker, From Genes to Clones, 1987). In a family of proteins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that

position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence. A "consensus framework" refers to the framework region in the consensus immunoglobulin sequence.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a natural immunoglobulin can be preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a natural immunoglobulin coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity.

"Detectable label" is used herein to refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorophores, chemiluminescent moieties, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, dyes, metal ions, ligands (e.g., biotin or haptens) and the like. "Fluorophore" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of labels which may be used under the invention include fluorescein, rhodamine, dansyl, umbelliferone, Texas red, luminol, NADPH, beta-galactosidase, and horseradish peroxidase.

"Inhibitor" or "IgM inhibitor" or "antagonist" as used herein refers to an agent that reduces or blocks (completely or partially) an interaction between a natural antibody and another molecule involved in an inflammatory cascade. An inhibitor may antagonize one or more of the following activities of a natural IgM: (i) inhibit or reduce an interaction (e.g., binding) between the IgM and an ischemia-specific antigen; (ii) inhibit or reduce an interaction (e.g., binding) between the natural IgM and a component of the complement pathway, e.g., Clq; (iii) neutralize the natural IgM by, e.g., sequestering the immunoglobulin and/or targeting its degradation; or (iv) inhibit or reduce production of the

natural IgM e.g., blocks synthesis, assembly, and/or posttranslational modifications of the IgM. The inhibitor can be a protein or a peptide, an antibody or fragment thereof (e.g., an anti-idiotypic antibody), a modified antibody, a carbohydrate, a glycoprotein, or a small organic molecule.

"Interaction" refers to a physical association between two or more molecules, e.g., binding. The interaction may be direct or indirect.

"Inflammatory disease" is used herein to refer to a disease or disorder that is caused or contributed to by a complicated set of functional and cellular adjustments involving acute or chronic changes in microcirculation, movement of fluids, and influx and activation of inflammatory cells (e.g., leukocytes) and complement, and included autoimmune diseases. Examples of such diseases and conditions include, but are not limited to: reperfusion injury, ischemia injury, stroke, autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, rheumatoid arthritis, celiac disease, hyper-IgM immunodeficiency, arteriosclerosis, coronary artery disease, sepsis, myocarditis, encephalitis, transplant rejection, hepatitis, thyroiditis (e.g. Hashimoto's thyroiditis, Graves disease), osteoporosis, polymyositis, dermatomyositis, Type I diabetes, gout, dermatitis, alopecia areata, systemic lupus erythematosus, lichen sclerosis, ulcerative colitis, diabetic retinopathy, pelvic inflammatory disease, periodontal disease, arthritis, juvenile chronic arthritis (e.g. chronic iridocyclitis), psoriasis, osteoporosis, nephropathy in diabetes mellitus, asthma, pelvic inflammatory disease, chronic inflammatory liver disease, chronic inflammatory lung disease, lung fibrosis, liver fibrosis, rheumatoid arthritis, chronic inflammatory liver disease, chronic inflammatory lung disease, lung fibrosis, liver fibrosis, Crohn's disease, ulcerative colitis, burns, and other acute and chronic inflammatory diseases of the Central Nervous System (CNS; e.g. multiple sclerosis), gastrointestinal system, the skin and associated structures, the immune system, the hepato-biliary system, or any site in the body where pathology can occur with an inflammatory component.

An "isolated" molecule, e.g., an isolated IgM, refers to a condition of being separate or purified from other molecules present in the natural environment.

"Natural IgM" is used herein to refer to an IgM antibody that is naturally produced in a mammal (e.g., a human). They have a pentameric ring structure wherein the individual monomers resemble IgGs thereby having two light (κ or λ) chains and two heavy (μ) chains. Further, the heavy chains contain an additional C_H4 domain. The monomers form a

pentamer by disulfide bonds between adjacent heavy chains. The pentameric ring is closed by the disulfide bonding between a J chain and two heavy chains. Because of its high number of antigen binding sites, a natural IgM antibody is an effective agglutinator of antigen. Production of natural IgM antibodies in a subject are important in the initial activation of B-cells, macrophages, and the complement system. IgM is the first immunoglobulin synthesized in an antibody response.

"Nucleic acid" is used herein to refer to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

"Operatively linked" is used herein to refer to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a coding sequence is "operably linked" to another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences ultimately process to produce the desired protein. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

"Patient", "subject" or "host" are used herein to refer to either a human or a non-human mammal.

"Peptide" is used herein to refer to a polymer of amino acids of relatively short length (e.g. less than 50 amino acids). The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The term also encompasses an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component.

"Promoter" is used herein to refer to a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the of a polynucleotide sequence. Both constitutive and inducible promoters, are included in the invention (see e.g., Bitter et al., *Methods in Enzymology* 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention. Tissue-specific regulatory elements may be used. Including, for example, regulatory elements from genes or viruses that are differentially expressed in different tissues.

"Specifically binds" is used herein to refer to the interaction between two molecules to form a complex that is relatively stable under physiologic conditions. The term is used herein in reference to various molecules, including, for example, the interaction of an antibody and an antigen (e.g. a peptide). Specific binding can be characterized by a dissociation constant of at least about $1x10^{-6}$ M, generally at least about $1x10^{-7}$ M, usually at least about $1x10^{-8}$ M, and particularly at least about $1x10^{-9}$ M or $1x10^{-10}$ M or greater. Methods for determining whether two molecules specifically bind are well known and include, for example, equilibrium dialysis, surface plasmon resonance, and the like.

"Stringency hybridization" or "hybridizes under low stringency, medium stringency, high stringency, or very high stringency conditions" is used herein to describes conditions for hybridization and washing. Guidance for performing hybridization reactions can be found in Current Protocols in *Molecular Biology,* John Wiley & Sons, N.Y. (1989), 6.3.1-6:3.6, which is incorporated by reference. Aqueous and non-aqueous methods are described in that reference and either can be used. Specific hybridization conditions referred to herein are as follows: 1) low stringency hybridization conditions in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by two washes in 0.2X SSC, 0.1% SDS at least at 50°C (the temperature of the washes can be increased to 55°C for low stringency conditions); 2) medium stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60°C; 3) high stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C; and preferably 4) very high stringency hybridization conditions are 0.5M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C. Very high stringency conditions (4) are the preferred conditions and the ones that should be used unless otherwise specified. Calculations of homology or sequence identity between sequences (the terms are used interchangeably herein) are performed as follows.

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position.

The percent identity between the two sequences is a function of the number of identical positions shared by the sequences and the percent homology between two sequences is a function of the number of conserved positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be

introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity and/or homology between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch ((1970) J. Mol. Biol. 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available on the world wide web with the extension gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available on the world wide web with the extension gcg.com), using a NWSgapdna CMP matrix and a gap weight of 40, 50, 60, 70; or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that should be used unless otherwise specified) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frame shift gap penalty of 5.

The percent identity and/or homology between two amino acid or nucleotide sequences can be determined using the algorithm of E. Meyers and W. Miller ((1989) CABIOS, 4:11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

"Treating" is used herein to refer to any treatment of, or prevention of, or inhibition of a disorder or disease in a subject and includes by way of example: (a) preventing the disease or disorder from occurring in a subject that may be predisposed to the disease or disorder, but has not yet been diagnosed as having it; (b) inhibiting the disease or disorder, i.e., arresting its progression; or (c) relieving or ameliorating the disease or disorder, i.e., causing regression. Thus, treating as used herein includes, for example, repair and regeneration of damaged or injured tissue or cells at the site of injury or prophylactic treatments to prevent damage, e.g., before surgery.

"Vector" as used herein refers to a nucleic acid molecule, which is capable of transporting another nucleic acid to which it has been operatively linked and can include a plasmid, cosmid, or viral vector. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors may be capable of directing the expression of genes to which they are operatively

linked. A vector may also be capable of integrating into the host DNA.. In the present specification, "plasmid" and "vector" are used interchangeably as a plasmid (a circular arrangement of double stranded DNA) is the most commonly used form of a vector. However, the invention is intended to include such other forms of vectors which serve equivalent functions and which become known in the art subsequently hereto. Viral vectors include, e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses.

6.2 Natural IgM antibodies

The present invention is based, at least in part, on the identification of natural immunoglobulins (Ig), in particular natural IgMs. Certain IgMs may be obtained from the hybridoma that has been deposited with the American Type Culture Collection and provided Accession Number PTA-3507.

The nucleotide sequence of the heavy chain variable region of the IgM produced from hybridoma PTA-3507, IgM^{CM-22} (also referred to as 22A5 IgM) is shown in Figure 1A (SEQ ID NO: 1), and the amino acid sequence is shown in Figure 1B (SEQ ID NO: 2). The CDR1 domain of the heavy chain variable region corresponds to amino acids 31 to 35 of SEQ ID NO: 2 (SEQ ID NO: 4), which is encoded by nucleotides 91-105 of SEQ ID NO: 1 (SEQ ID NO: 3), and the CDR2 domain of the heavy chain variable region corresponds to amino acids 50 to 66 of SEQ ID NO: 2 (SEQ ID NO: 6), which is encoded by nucleotides 148-198 of SEQ ID NO: 1 (SEQ ID NO: 5).

The nucleotide sequence of the light chain variable region of IgM^{CM-22} is shown in Figure 2A (SEQ ID NO: 7), and the amino acid sequence is shown in Figure 2B (SEQ ID NO: 8). The CDR1 domain of the light chain variable region corresponds to amino acids 23 to 37 of SEQ ID NO: 8 (SEQ ID NO: 10), which is encoded by nucleotides 67-111 of SEQ ID NO: 7 (SEQ ID NO: 9), and the CDR2 domain of the light chain variable region corresponds to amino acids 53 to 59 of SEQ ID NO: 8 (SEQ ID NO: 12), which is encoded by nucleotides 157 to 177 of SEQ ID NO: 7 (SEQ ID NO: 11). Due to the degeneracy of the genetic code, other nucleotide sequences can encode the amino acid sequences listed herein.

The nucleic acid compositions of the present invention, while often in a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures may be mutated, in accordance with standard techniques. For coding sequences, these mutations, may affect the amino acid sequence as desired. In particular, nucleotide sequences substantially identical to or derived from native V, D, J, constant, switches and other such sequences described herein are contemplated.

For example, an isolated nucleic acid can comprise an IgM^{CM-22} (or 22A5 IgM) heavy chain variable region nucleotide sequence having a nucleotide sequence as shown in Figure 1A (SEQ ID NO: 1), or a sequence, which is at least 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1. A nucleic acid molecule may comprise the heavy chain CDR1 nucleotide sequence of SEQ ID NO: 3, or a portion thereof. Further, the nucleic acid molecule may comprise the heavy chain CDR2 nucleotide sequence of SEQ ID NO: 5, or a portion thereof. In an exemplary embodiment, the nucleic acid molecule comprises a heavy chain CDR1 nucleotide sequence of SEQ ID NO: 3, or portion thereof, and a heavy chain CDR2 nucleotide sequence of SEQ ID NO: 5, or portion thereof. The nucleic acid molecules of the present invention may comprise heavy chain sequences, e.g. SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or combinations thereof, or encompass nucleotides having at least 80%, 90%, 95%, 96%, 97 %, 98%, and 99% sequence identity to SEQ ID NOs: 1, 3 or 5. Further, the nucleic acid molecules of the present invention may comprise heavy chain sequences, which hybridize under stringent conditions, e.g. low, medium, high or very high stringency conditions, to SEQ ID NOs: 1, 3 or 5.

In another embodiment, the invention features nucleic acid molecules having at least 80%, 90%, 95%, 96%, 97 %, 98%, and 99% sequence identity with a nucleic acid molecule encoding a heavy chain polypeptide, e.g., a heavy chain polypeptide of SEQ ID NOs: 2, 4 or 6. The invention also features nucleic acid molecules which hybridize to nucleic acid sequences encoding a heavy chain variable region of a natural antibody or portion thereof, e.g., a heavy chain variable region of SEQ ID NO: 2, 4 or 6.

In another embodiment, the isolated nucleic acid encodes a IgM^{CM-22} (22A5 IgM) light chain variable region nucleotide sequence having a nucleotide sequence as shown in Figure 2A (SEQ ID NO: 7), or a sequence at least 80%, 90%, 95%, 96%, 97%, 98%, 99% identical to SEQ ID NO: 7. The nucleic acid molecule may comprise the light chain CDR1 nucleotide sequence of SEQ ID NO: 9, or a portion thereof. In another preferred embodiment, the nucleic acid molecule may comprise the light chain CDR2 nucleotide

sequence of SEQ ID NO: 11, or a portion thereof. In an exemplary embodiment, the nucleic acid molecule comprises a light chain CDR1 nucleotide sequence of SEQ ID NO: 9, or portion thereof, and a light chain CDR2 nucleotide sequence of SEQ ID NO: 11, or portion thereof. The nucleic acid molecules of the present invention may comprise light chain sequences, e.g. SEQ ID NOs: 7, 9 or 11, or combinations thereof, or encompass nucleotides having at least 80%, 90%, 95%, 96%, 97 %, 98%, and 99% sequence identity to SEQ ID NOs: 7, 9 or 11. Further nucleic acid molecules may comprise light chain sequences, which hybridize under stringent conditions, e.g. low, medium, high or very high stringency conditions, to SEQ ID NOs: 7, 9 or 11.

Nucleic acid molecules can have at least 80%, 90%, 95%, 96%, 97 %, 98% or 99% sequence identity with a nucleic acid molecule encoding a light chain polypeptide, e.g., a light chain polypeptide of SEQ ID NOs: 8, 10, or 12. The invention also features nucleic acid molecules which hybridize to a nucleic acid sequence encoding a light chain variable region of a natural antibody or portion thereof, e.g., a light chain variable region of SEQ ID NOs: 8, 10 or 12.

In another embodiment, the invention provides an isolated nucleic acid encoding a heavy chain CDR1 domain comprising the amino acid sequence of SEQ ID NO: 4, or a fragment or modified form thereof. This nucleic acid can encode only the CDR1 region or can encode an entire antibody heavy chain variable region or a fragment thereof. For example, the nucleic acid can encode a heavy chain variable region having a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6. In yet another embodiment, the invention provides an isolated nucleic acid encoding a heavy chain CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6, or a fragment or modified form thereof. This nucleic acid can encode only the CDR2 region or can encode an entire antibody heavy chain variable region or a fragment thereof. For example, the nucleic acid can encode a light chain variable region having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 4.

In still another embodiment, the invention provides an isolated nucleic acid encoding a light chain CDR1 domain comprising the amino acid sequence of SEQ ID NO: 10, or a fragment or modified form thereof. This nucleic acid can encode only the CDR1 region or can encode an entire antibody light chain variable region. For example, the nucleic acid can encode a light chain variable region having a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 12. The isolated nucleic acid can

also encode a light chain CDR2 domain comprising the amino acid sequence of SEQ ID NO: 12, or a fragment or modified form thereof. This nucleic acid can encode only the CDR2 region or can encode an entire antibody light chain variable region. For example, the nucleic acid can encode a light chain variable region having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 10.

The nucleic acid encoding the heavy or light chain variable region can be of murine or human origin, or can comprise a combination of murine and human amino acid sequences. For example, the nucleic acid can encode a heavy chain variable region comprising the CDR1 of SEQ ID NO: 2 (SEQ ID NO: 4) and/or the CDR2 of SEQ ID NO: 2 (SEQ ID NO: 6), and a human framework sequence. In addition, the nucleic acid can encode a light chain variable region comprising the CDR1 of SEQ ID NO: 8 (SEQ ID NO: 10) and/or the CDR2 of SEQ ID NO: 8 (SEQ ID NO: 12), and a human framework sequence. The invention further encompasses vectors containing the above-described nucleic acids and host cells containing the expression vectors.

The invention also features polypeptides and fragments of the IgM^{CM-22} heavy chain variable regions and/or light chain variable regions. In exemplary embodiments, the isolated polypeptides comprise, for example, the amino acid sequences of SEQ ID NOs: 8, 10, or 12, or fragments or combinations thereof; or SEQ ID NO: 2, 4, or 6, or fragments or combinations thereof. The polypeptides of the present invention include polypeptides having at least, but not more than 20, 10, 5, 4, 3, 2, or 1 amino acid that differs from SEQ ID NOs: 8, 10, 12, 2, 4 or 6. Exemplary polypeptides are polypeptides that retain biological activity, e.g., the ability to bind an ischemia-specific antigen, and/or the ability to bind complement. In another embodiment, the polypeptides comprise polypeptides having at least 80%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity with a light chain variable region, or portion thereof, e.g. a light chain variable region polypeptide of SEQ ID NOs: 8, 10, or 12. In another embodiment, the polypeptides comprise polypeptides having at least 80%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity with a heavy chain variable region, or portion thereof, e.g. a heavy chain variable region polypeptide of SEQ ID NOs: 2, 4, or 6. In another embodiment, the invention features a polypeptide comprising the amino acid sequence of SEQ ID NO: 8 and SEQ ID NO: 2, further comprising an IRES sequence.

6.3 Inhibitors of Natural IgM Antibodies

6.3.1 Peptide Inhibitors of Natural IgM Antibodies

The invention further features IgM inhibitors. In one embodiment, the IgM inhibitor is a peptide that specifically binds to a natural IgM and thereby blocks binding to the antigen. Such peptides can include, but are not limited to, the asparagine-rich peptides described in Table 1 below.

Table 1: Amino acid sequences of natural IgM antibody-binding peptides

SEQ ID NO:	SEQUENCE	Name
14	xNNNxNNxNNNN	Asparagine-rich Consensus
16	YNNNNGNYTYRN	P1
18	ANTRNGATNNNM	P2
20	CDSSCDSVGNCN	P3
22	WNNNGRNACNAN	P4
24	HNSTSNGCNDNV	P5
26	NSNSRYNSNSNN	P6
28	KRNNHNNHNRSN	P7
30	NGNNVNGNRNNN	P8
32	NVANHNNSNHGN	P9
34	SYNNNNHVSNRN	P10

The peptides can also include certain "self-peptides" as described in Table 2 below.

Table 2: Amino acid sequences of self-peptides

SEQ ID NO:	SEQUENCE	Name
36	LMKNMDPLNDNI	Self-1
38	LMKNMDPLNDNV	Self-2 ("N-2")

As described in more detail in the Exemplification, self peptides bind to the natural IgM antibody IgM^{CM-22}.

In addition to the peptides described above, the present invention encompasses modified peptides whose activity may be identified and/or assayed using a variety of methods well known to the skilled artisan. For example, binding of the peptide to the IgM may be detected using biological assays, Western blotting, immunoprecipitation, or immonocytochemical techniques, such as those described below. In particular, the biological activity (e.g., the ability to a bind natural IgM antibody) of a modified peptide can be characterized relative to that of P8 (SEQ ID NO: 30) or N2 (SEQ ID NO: 38).

Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered "functional equivalents" of the peptides described in more detail herein. Such modified peptides may be produced, for instance, by amino acid substitution, deletion, or addition, which substitutions may consist in whole or part by conservative amino acid substitutions.

For instance, it is reasonable to expect that an isolated conservative amino acid substitution, such as replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine, will not have a major effect on the biological activity of the resulting molecule. Whether a change in the amino acid sequence of a peptide results in a functional homolog may be readily determined by assessing the ability of the variant peptide to produce a response similar to that of the wild-type peptide (e.g. ability to bind natural IgM antibodies). Peptides in which more than one replacement has taken place may readily be tested in the same manner.

Mutagenesis of the peptide may give rise to homologs, which have improved *in vivo* half-lives relative to the corresponding wild-type peptide. For example, the altered peptide may be rendered more stable to proteolytic degradation or other cellular processes which result in destruction or inactivation of the protein.

The amino acid sequences for a population of peptide homologs can be aligned, preferably to promote the highest homology possible. Such a population of variants may include, for example, homologs from one or more species, or homologs from the same species but which differ due to mutation. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In certain embodiments, the combinatorial library is produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential peptide sequences. For instance, a mixture of synthetic oligonucleotides may be

enzymatically ligated into gene sequences such that the degenerate set of potential nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display).

There are many ways by which the library of potential homologs may be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence may be carried out in an automatic DNA synthesizer, and the synthetic genes may then be ligated into an appropriate vector for expression. One purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential peptide sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al., (1981) Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp. 273-289; Itakura et al., (1984) Annu. Rev. Biochem. 53:323; Itakura et al., (1984) Science 198:1056; Ike et al., (1983) Nucleic Acid Res. 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) Science 249:386-390; Roberts et al., (1992) PNAS USA 89:2429-2433; Devlin et al., (1990) Science 249: 404-406; Cwirla et al., (1990) PNAS USA 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis may be utilized to generate a combinatorial library. For example, peptide homologs may be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) Biochemistry 33:1565-1572; Wang et al., (1994) J. Biol. Chem. 269:3095-3099; Balint et al., (1993) Gene 137:109-118; Grodberg et al., (1993) Eur. J. Biochem. 218:597-601; Nagashima et al., (1993) J. Biol. Chem. 268:2888-2892; Lowman et al., (1991) Biochemistry 30:10832-10838; and Cunningham et al., (1989) Science 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) Virology 193:653-660; Brown et al., (1992) Mol. Cell Biol. 12:2644-2652; McKnight et al., (1982) Science 232:316); by saturation mutagenesis (Meyers et al., (1986) Science 232:613); by PCR mutagenesis (Leung et al., (1989) Method Cell Mol. Biol. 1:11-19); or by random mutagenesis (Miller et al., (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) Strategies in Mol. Biol. 7:32-34).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and for screening cDNA libraries for gene products having a certain property (e.g., the ability to bind a natural IgM

antibody). Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of peptide homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In an illustrative embodiment of a screening assay, candidate combinatorial gene products are passed over a column containing beads having attached to it the binding protein, such as an IgM or portion thereof. Those candidate combinatorial gene products that are retained on the column may be further characterized for binding to IgMs in a manner that could be useful in blocking natural IgM antibody binding and treating inflammatory diseases.

In another example, the gene library may be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences may be expressed on the surface of infectious phage, thereby conferring two benefits. First, because these phage may be applied to affinity matrices at very high concentrations, a large number of phage may be screened at one time. Second, because each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage may be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins may be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al., PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al., (1992) J. Biol. Chem. 267:16007-16010; Griffiths et al., (1993) EMBO J. 12:725-734; Clackson et al., (1991) Nature 352:624-628; and Barbas et al., (1992) PNAS USA 89:4457-4461). Other phage coat proteins may be used as appropriate.

The invention also provides for mimetics (e.g., non-peptide agents) which are able to mimic binding of the authentic peptide to a natural IgM antibody. For example, the critical residues of a peptide which are involved in molecular recognition of a natural IgM

antibody may be determined and used to generate peptidomimetics that bind to a natural IgM antibody. The peptidomimetic may then be used as an inhibitor of the wild-type protein by binding to the natural IgM antibodies and covering up the critical residues needed for interaction with the wild-type protein, thereby preventing interaction of the protein and the natural IgM antibody. Peptidomimetic compounds may be generated which mimic those residues in binding to the natural IgM antibody. For instance, non-hydrolyzable peptide analogs of such residues may be generated using benzodiazepine (e.g., see Freidinger et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al., (1986) J. Med. Chem. 29:295; and Ewenson et al., in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al., (1985) Tetrahedron Lett 26:647; and Sato et al., (1986) J Chem Soc Perkin Trans 1:1231), and β-aminoalcohols (Gordon et al., (1985) Biochem Biophys Res Commun 126:419; and Dann et al., (1986) Biochem Biophys Res Commun 134:71).

6.3.2 Nucleic acids Encoding Peptide Inhibitors

The invention also features nucleic acids, which encode the peptides discussed above. Exemplary nucleic acids are provided in Table 3.

Table 3: Nucleic acids encoding natural IgM antibody-binding peptides

SEQ ID NO:	SEQUENCE	Name
13	NNN AAY AAY AAY NNN AAY AAY NNN AAY AAY AAY AAY	Aparagine-rich Consensus
15	TAY AAY AAY AAY AAY GGN AAY TAY ACN TAY MGN AAY	P1
17	GCN AAY ACN MGN AAY GGN GCN ACN AAY AAY AAY ATG	P2
19	TGY GAY WSN WSN TGY GAY WSN GTN GGN AAY TGY AAY	P3

SEQ ID NO:	SEQUENCE	Name
21	TGG AAY AAY AAY GGN MGN AAY GCN TGY AAY GCN AAY	P4
23	CAY AAY WSN ACN WSN AAY GGN TGY AAY GAY AAY GTN	P5
25	AAY WSN AAY WSN MGN TAN AAN WSN AAY WSN AAY AAY	P6
27	AAR MGN AAY AAY CAY AAY AAY CAY AAY MGN WSN AAY	P7
29	AAY GGN AAY AAY GTN AAY GGN AAY MGN AAY AAY AAY	P8
31	AAY GTN GCN AAY CAY AAY AAY WSN AAY CAY GGN AAY	P9
33	WSN TAY AAY AAY AAY AAY CAY GTN WSN AAY MGN AAY	P10
35	YTN ATG AAR AAY ATG GAY CCN YTN AAY GAY AAY ATH	Self-1
37	YTN ATG AAR AAY ATG GAY CCN YTN AAY GAY AAY GTN	Self-2

The isolated nucleic acids in Table 3 reflect degeneracy in the genetic code. In particular, an "R" corresponds to a base that may be a A or G; a "S" corresponds to a base that may be a G or C; a "V" corresponds to a base that may be an A, C or G; a "Y" corresponds to a base that may be a C or T; a "W" corresponds to a base that may be an A or T; a "D" corresponds to a base that may be an A, G or T; a "M" corresponds to a base that may be an A or C; a "H" corresponds to a base that may be an A, C or T; a "N" corresponds to a base that may be an A, C, G or T; a "K" corresponds to a base that may be a G or T and a "B" corresponds to a base that may be a C, G or T.

It is expected that DNA sequence polymorphisms that lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (from less than 1% up to about 3 or 5% or possibly more of the nucleotides) of the nucleic acids encoding a

particular peptide of the invention may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention. Preferred nucleic acids encode a peptide, which is at least about 60%, 70%, 80%, 85%, 90%, 95%, 98%, 99% homologous or more with an amino acid sequence of SEQ ID NO: 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38 or another peptide of the invention. Nucleic acids which encode peptides having an activity of a peptide of the invention and having at least about 60%, 70%, 80%, 85%, 90%, 95%, 98%, 99% homology or more with SEQ ID NO: 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, or another peptide of the invention are also within the scope of the invention.

Bias in codon choice within genes in a single species appears related to the level of expression of the protein encoded by that gene. Accordingly, the invention encompasses nucleic acid sequences which have been optimized for improved expression in a host cell by altering the frequency of codon usage in the nucleic acid sequence to approach the frequency of preferred codon usage of the host cell. Due to codon degeneracy, it is possible to optimize the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleotide sequence that encodes the peptides set forth in SEQ ID NO: 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38 or other peptides of the invention.

Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of such recombinant polypeptides.

A nucleic acid encoding a peptide of the invention may be obtained from mRNA or genomic DNA from any organism in accordance with protocols described herein, as well as those generally known to those skilled in the art. A cDNA encoding a peptide of the invention, for example, may be obtained by isolating total mRNA from an organism, e.g. a bacteria, virus, mammal, etc. Double stranded cDNAs may then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. A gene encoding a peptide of the invention may also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention.

In another aspect of the invention, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding a peptide of the invention and operably linked to at least one regulatory sequence. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

As will be apparent, the subject gene constructs may be used to cause expression of a peptide of the invention in cells propagated in culture, e.g., to produce proteins or polypeptides, including fusion proteins or polypeptides, for purification.

This invention also pertains to a host cell transfected with a recombinant gene in order to express a peptide of the invention. The host cell may be any prokaryotic or eukaryotic cell. For example, a polypeptide of the present invention may be expressed in bacterial cells, such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art. Additionally, the host cell may be supplemented with tRNA molecules not typically found in the host so as to optimize expression of the peptide. Other methods suitable for maximizing expression of the peptide will be known to those in the art.

6.3.3 Methods of Producing Peptide Inhibitors

Peptide inhibitors may be synthesized, for example, chemically, ribosomally in a cell free system, or ribosomally within a cell. Chemical synthesis of peptides of the invention may be carried out using a variety of art recognized methods, including stepwise solid phase synthesis, semi-synthesis through the conformationally-assisted re-ligation of peptide fragments, enzymatic ligation of cloned or synthetic peptide segments, and chemical ligation. Merrifield et al. in J. Am. Chem. Soc., Volume 85, page 2149 (1964), by Houghten et al. in Proc. Natl. Acad. Sci. USA, Volume 82, page 5132 (1985), and by Stewart and Young in Solid Phase Peptide Synthesis, Pierce Chem. Co, Rockford, Ill. (1984). Native chemical ligation employs a chemoselective reaction of two unprotected peptide segments to produce a transient thioester-linked intermediate. The transient thioester-linked intermediate then spontaneously undergoes a rearrangement to provide the full length ligation product having a native peptide bond at the ligation site. Full length ligation products are chemically identical to proteins produced by cell free synthesis. Full

length ligation products may be refolded and/or oxidized, as allowed, to form native disulfide-containing protein molecules. (see e.g., U.S. Patent Nos. 6,184,344 and 6,174,530; and T. W. Muir et al., Curr. Opin. Biotech. (1993): vol. 4, p 420; M. Miller, et al., Science (1989): vol. 246, p 1149; A. Wlodawer, et al., Science (1989): vol. 245, p 616; L. H. Huang, et al., Biochemistry (1991): vol. 30, p 7402; M. Schnolzer, et al., Int. J. Pept. Prot. Res. (1992): vol. 40, p 180-193; K. Rajarathnam, et al., Science (1994): vol. 264, p 90; R. E. Offord, "Chemical Approaches to Protein Engineering", in Protein Design and the Development of New therapeutics and Vaccines, J. B. Hook, G. Poste, Eds., (Plenum Press, New York, 1990) pp. 253-282; C. J. A. Wallace, et al., J. Biol. Chem. (1992): vol. 267, p 3852; L. Abrahmsen, et al., Biochemistry (1991): vol. 30, p 4151; T. K. Chang, et al., Proc. Natl. Acad. Sci. USA (1994) 91: 12544-12548; M. Schnlzer, et al., Science (1992): vol., 3256, p 221; and K. Akaji, et al., Chem. Pharm. Bull. (Tokyo) (1985) 33: 184).

In another variation, peptide production may be achieved using *in vitro* translation systems. An *in vitro* translation systems is, generally, a translation system which is a cell-free extract containing at least the minimum elements necessary for translation of an RNA molecule into a protein. An *in vitro* translation system typically comprises at least ribosomes, tRNAs, initiator methionyl-tRNAMet, proteins or complexes involved in translation, e.g., eIF2, eIF3, the cap-binding (CB) complex, comprising the cap-binding protein (CBP) and eukaryotic initiation factor 4F (eIF4F). A variety of *in vitro* translation systems are well known in the art and include commercially available kits. Examples of *in vitro* translation systems include eukaryotic lysates, such as rabbit reticulocyte lysates, rabbit oocyte lysates, human cell lysates, insect cell lysates and wheat germ extracts. Lysates are commercially available from manufacturers such as Promega Corp., Madison, Wis.; Stratagene, La Jolla, Calif.; Amersham, Arlington Heights, Ill.; and GIBCO/BRL, Grand Island, N.Y. *In vitro* translation systems typically comprise macromolecules, such as enzymes, translation, initiation and elongation factors, chemical reagents, and ribosomes. In addition, an *in vitro* transcription system may be used. Such systems typically comprise at least an RNA polymerase holoenzyme, ribonucleotides and any necessary transcription initiation, elongation and termination factors. *In vitro* transcription and translation may be carried out within in the same reaction to produce peptides from one or more isolated DNAs.

Nucleic acids encoding peptide inhibitors may be expressed *in vitro* by DNA transfer into a suitable host cell. Expression of peptides may be facilitated by inserting the

nucleic acids encoding the peptides into a vector, such as a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the natural antibody-binding peptide genetic sequences. Such vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg, et al., Gene, 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, J. Biol. Chem., 263:3521, 1988) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedrin promoters).

Nucleic acids encoding peptide inhibitors may be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect, and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors can incorporate DNA sequences of the invention. Methods which are well known to those skilled in the art can be used to construct vectors containing the natural antibody-binding peptide coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic techniques. (See, for example, the techniques described in Maniatis et al., 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.)

A variety of host-expression vector systems may be utilized. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors; yeast transformed with recombinant yeast expression vectors; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid); insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus); or animal cell systems infected with recombinant virus expression vectors (e.g., retroviruses,

adenovirus, vaccinia virus), or transformed animal cell systems engineered for stable expression.

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see e.g., Bitter et al., 1987, Methods in Enzymology 153:516-544). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage γ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 31987, Acad. Press, N.Y., Vol. 153, pp.516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II. A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used (Cloning in Yeast, Ch. 3, R. Rothstein In: DNA Cloning Vol. 11, A Practical Approach, Ed. D M Glover, 1986, IRL Press, Wash., D.C.). Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

Eukaryotic systems, and preferably mammalian expression systems, allow for proper post-translational modifications of expressed mammalian proteins to occur. Eukaryotic cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, phosphorylation, and advantageously, plasma membrane insertion of the gene product may be used as host cells.

Mammalian cell systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression

vectors, a natural antibody-binding peptide coding sequence may be ligated to an adenovirus transcription/-translation control complex, e.g., the late promoter and tripartite leader sequence. Alternatively, the vaccinia virus 7.5K promoter may be used. (e.g., see, Mackett et al., 1982, Proc. Natl. Acad. Sci. USA 79: 7415-7419; Mackett et al., 1984, J. Virol. 49: 857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. USA 79: 4927-4931). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver, et al., 1981, Mol. Cell. Biol. 1: 486). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as, for example, the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of a natural antibody-binding peptide gene in host cells (Cone & Mulligan, 1984, Proc. Natl. Acad. Sci. USA 81:6349-6353). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionine IIA promoter and heat shock promoters.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with a cDNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.) and a selectable marker. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11: 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48: 2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22: 817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77: 3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA

78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad Sci. USA 78: 2072; neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150: 1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30: 147) genes. Additional selectable genes include trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85: 8047); and ODC (ornithine decarboxylase) which confers resistance to the omithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-omithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

For stable recombinant cell lines, suitable cell types include but are not limited to cells of the following types: NIH 3T3 (Murine), C2C12, L6, and P19. C2C12 and L6 myoblasts will differentiate spontaneously in culture and form myotubes depending on the particular growth conditions (Yaffe and Saxel, 1977; Yaffe, 1968) P19 is an embryonic carcinoma cell line. Such cells are described, for example, in the Cell Line Catalog of the American Type Culture Collection (ATCC). These cells can be stably transformed by a method known to the skilled artisan. See, for example, Ausubel et al., Introduction of DNA Into Mammalian Cells, in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, sections 9.5.1-9.5.6 (John Wiley & Sons, Inc. 1995). "Stable" transformation in the context of the invention means that the cells are immortal to the extent of having gone through at least 50 divisions.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding natural antibody-binding peptides, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (see for example, Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

To interact with natural antibodies or for isolation and purification, natural antibody-binding proteins may need to be secreted from the host cell. Accordingly a signal sequence may be used to direct the peptide out of the host cell where it is synthesized.

Typically, the signal sequence is positioned in the coding region of nucleic acid sequence, or directly at the 5' end of the coding region. Many signal sequences have been identified, and any that are functional in the selected host cell may be used. Accordingly, the signal sequence may be homologous or heterologous to the polypeptide. Additionally, the signal sequence may be chemically synthesized using recombinant DNA techniques well known in the art.

The amount of peptide produced in the host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, HPLC separation, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

When natural antibody-binding peptides are secreted from the host cells, the majority of the peptide will likely be found in the cell culture medium. If, however, the peptide is not secreted, it will be present in the cytoplasm (for eukaryotic, Gram-positive bacteria, and insect host cells) or in the periplasm (for Gram-negative bacteria host cells).

If the natural antibody-binding peptide remains in the intracellular space, the host cells are typically first disrupted mechanically or osmotically to release the cytoplasmic contents into a buffered solution. The peptide is then isolated from this solution. Purification of the peptide from solution can thereafter be accomplished using a variety of techniques. If the peptide has been synthesized such that it contains a tag such as hexahistidine or other small peptides at either its carboxyl or amino terminus, it may be purified in a one-step process by passing the solution through an affinity column where the column matrix has a high affinity for the tag or for the peptide directly (i.e., a monoclonal antibody). For example, polyhistidine binds with great affinity and specificity to nickel, thus an affinity column of nickel (such as the Qiagen nickel columns) can be used for purification. (See, for example, Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1994).

Where, on the other hand, the peptide has no tag and it is not practical to use an antibody to purify the peptide, other well known procedures for purification can be used. Such procedures include, without limitation, ion exchange chromatography, molecular sieve chromatography, HPLC, native gel electrophoresis in combination with gel elution,

and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be combined to achieve increased purity.

If it is anticipated that the peptide will be found primarily in the periplasmic space of the bacteria or the cytoplasm of eukaryotic cells, the contents of the periplasm or cytoplasm, including inclusion bodies (e.g., Gram-negative bacteria) if the processed peptide has formed such complexes, can be extracted from the host cell using any standard technique known to the skilled artisan. For example, the host cells can be lysed to release the contents of the periplasm by the use of a French press, homogenization, and/or sonication. The homogenate can then be centrifuged.

6.3.4 Antibody Inhibitors of Natural IgM Antibodies

IgM inhibitors may also be antibodies that compete with natural IgMs in binding to antigen. Methods of producing antibodies are well known in the art. For example, a monoclonal antibody against a target (e.g., a pathogenic immunoglobulin or an ischemia specific antigen on a cell) can be produced by a variety of techniques, including conventional monoclonal antibody methodology e.g., the standard somatic cell hybridization technique of Kohler and Milstein, *Nature* 256: 495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed e.g., viral or oncogenic transformation of B lymphocytes. The preferred animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also known.

Human monoclonal antibodies can be generated using transgenic mice carrying the human immunoglobulin genes rather than mouse immunoglobulin genes. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, e.g., Wood et al. International Application WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. International Application WO 92/03918; Kay et al. International Application 92/03917; Lonberg, N. et al. 1994 Nature 368:856-859; Green, L.L. et al. 1994 Nature Genet. 7:13-21; Morrison, S.L. et al. 1994 Proc. Natl. Acad. Sci. USA 81:6851-6855; Bruggeman et al. 1993 Year Immuno. 17:33-40; Tuaillon et al. 1993 PNAS 90:3720-3724; Bruggeman et al. 1991 Eur. J. Immunol 21:1323-1326). In one

embodiment, hybridomas can be generated from human CD5+, B-1 cells. Alternatively, "humanized" murine hybridomas can be used that recognize cross-reactive "ischemic antigen".

Monoclonal antibodies can also be generated by other methods known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies (for descriptions of combinatorial antibody display see e.g., Sastry et al. 1989 PNAS 86:5,728; Huse et al. 1989 Science 246:1275; and Orlandi et al. 1989 PNAS 86:3833). After immunizing an animal with an immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al., 1991, *Biotechniques* 11:152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick et al., 1991, *Methods: Companion to Methods in Enzymology* 2:106-110).

In an illustrative embodiment, RNA is isolated from B lymphocytes, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. PNAS (1989) 86:3833-3837; Sastry et al., PNAS (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combination, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage; to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large variegated antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System,* catalog no. 27-9400-01; and the Stratagene SurfZAPTM phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating a variegated antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al. International Publication No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Human Antibody Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J. Mol. Biol.* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc. Acid Res.* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

In certain embodiments, the V region domains of heavy and light chains can be expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554, complete V_H and V_L domains of an antibody, joined by a flexible $(Gly_4\text{-}Ser)_3$ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFV antibodies immunoreactive with the antigen can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened with the target antigen, or peptide fragment thereof, to identify and isolate packages that express an antibody having specificity for the target antigen. Nucleic acid encoding the selected antibody can be recovered from the display package

(e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques.

Specific antibody molecules with high affinities for a surface protein can be made according to methods known to those in the art, e.g., methods involving screening of libraries (Ladner, R.C., *et al., U.S.* Patent 5,233,409; Ladner, R.C., *et al., U.S.* Patent 5,403,484). Further, these libraries can be used in screens to obtain binding determinants that are mimetics of the structural determinants of antibodies.

In particular, the Fv binding surface of a particular antibody molecule interacts with its target ligand according to principles of protein-protein interactions, hence sequence data for V_H and V_L (the latter of which may be of the κ or λ chain type) can be used in protein engineering techniques known to those with skill in the art. Details of the protein surface that comprises the binding determinants can be obtained from antibody sequence information, by a modeling procedure using previously determined three-dimensional structures from other antibodies obtained from NMR studies or crystallographic data. See for example Bajorath, J. and S. Sheriff, 1996, *Proteins: Struct., Funct., and Genet. 24 (2),* 152-157; Webster, D.M. and A. R. Rees, 1995, "Molecular modeling of antibody-combining sites," in S. Paul, Ed., *Methods in Molecular Biol. 51,* Antibody Engineering Protocols, Humana Press, Totowa, NJ, pp 17-49; and Johnson, G., Wu, T.T. and E.A. Kabat, 1995, "Seqhunt: A program to screen aligned nucleotide and amino acid sequences," in *Methods in Molecular Biol. 51, op. cit.,* pp 1-15.

In one embodiment, a variegated peptide library is expressed by a population of display packages to form a peptide display library. Ideally, the display package comprises a system that allows the sampling of very large variegated peptide display libraries, rapid sorting after each affinity separation round, and easy isolation of the peptide-encoding gene from purified display packages. Peptide display libraries can be in, e.g., prokaryotic organisms and viruses, which can be amplified quickly, are relatively easy to manipulate, and which allow the creation of large number of clones. Preferred display packages include, for example, vegetative bacterial cells, bacterial spores, and most preferably, bacterial viruses (especially DNA viruses). However, the present invention also contemplates the use of eukaryotic cells, including yeast and their spores, as potential display packages. Phage display libraries are described above.

Other techniques include affinity chromatography with an appropriate "receptor", e.g., a target antigen, followed by identification of the isolated binding agents or ligands by conventional techniques (e.g., mass spectrometry and NMR). Preferably, the soluble receptor is conjugated to a label (e.g., fluorophores, colorimetric enzymes, radioisotopes, or luminescent compounds) that can be detected to indicate ligand binding. Alternatively, immobilized compounds can be selectively released and allowed to diffuse through a membrane to interact with a receptor.

Combinatorial libraries of compounds can also be synthesized with "tags" to encode the identity of each member of the library (see, e.g., W.C. Still et al., International Application WO 94/08051). In general, this method features the use of inert but readily detectable tags that are attached to the solid support or to the compounds. When an active compound is detected, the identity of the compound is determined by identification of the unique accompanying tag. This tagging method permits the synthesis of large libraries of compounds which can be identified at very low levels among the total set of all compounds in the library.

An antibody of the present invention can be one in which the variable region, or a portion thereof, e.g., the complementarity determining regions (CDR or CDRs), are generated in a non-human organism, e.g., a rat or mouse. Chimeric, CDR-grafted, and humanized antibodies are within the invention. Antibodies generated in a non-human organism, e.g., a rat or mouse, and then modified, e.g., in the variable framework or constant region, to decrease antigenicity in a human are within the invention. Any modification is within the scope of the invention so long as the antibody has at least one antigen binding portion.

Chimeric antibodies (e.g. mouse-human monoclonal antibodies) can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the Fc constant region of a murine (or other species) monoclonal antibody molecule is digested with restriction enzymes to remove the region encoding the murine Fc, and the equivalent portion of a gene encoding a human Fc constant region is substituted. (see Robinson et al., International Patent Publication PCT/US86/02269; Akira, et al., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., International Application WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al., European Patent Application 125,023; Better et al. (1988 *Science* 240:1041-1043); Liu et al. (1987) PNAS

84:3439-3443; Liu et al., 1987, J. *Immunol.* 139:3521-3526; Sun et al. (1987) PNAS 84:214-218; Nishimura et al., 1987, *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al., 1988, J. *Natl. Cancer Inst.* 80:1553-1559).

A chimeric antibody can be further humanized by replacing sequences of the Fv variable region which are not directly involved in antigen binding with equivalent sequences from human Fv variable regions. General methods for generating humanized antibodies are provided by Morrison, S. L., 1985, *Science* 229:1202-1207 by Oi et al., 1986, *BioTechniques* 4:214, and by Queen et al. US 5,585,089, US 5,693,761 and US 5,693,762, the contents of all of which are hereby incorporated by reference. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from 7E3, an anti-$GPII_bIII_a$ antibody producing hybridoma. The recombinant DNA encoding the chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable humanized antibodies can alternatively be produced by CDR substitution. U.S. Patent 5,225,539; Jones et al. 1986 *Nature* 321:552-525; Verhoeyan et al. 1988 *Science* 239:1534; and Beidler et al. 1988 J *Immunol.* 141:4053-4060.

Humanized or CDR-grafted antibodies can be produced by CDR-grafting or CDR substitution, wherein one, two, or all CDRs of an immunoglobulin chain can be replaced. See e.g., U.S. Patent 5,225,539; Jones et al. 1986 *Nature* 321:552-525; Verhoeyan et al. 1988 *Science* 239:1534; Beidler et al. 1988 J. *Immunol.* 141:4053-4060; Winter US 5,225,539, the contents of all of which are hereby expressly incorporated by reference. Winter describes a CDR-grafting method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A, filed on . March 26, 1987; Winter US 5,225,539), the contents of which is expressly incorporated by reference.

A humanized or CDR-grafted antibody will have at least one or two but generally all recipient CDRs (of heavy and/or light immunoglobulin chains) replaced with a donor CDR. Preferably, the donor will be a rodent antibody, e.g., a rat or mouse antibody, and the recipient will be a human framework or a human consensus framework. Typically, the immunoglobulin providing the CDRs is called the "donor" and the immunoglobulin providing the framework is called the "acceptor." In one embodiment, the donor

immunoglobulin is a non-human (e.g., rodent). The acceptor framework can be a naturally-occurring (e.g., a human) framework or a consensus framework, or a sequence about 85% or higher, preferably 90%, 95%, 99% or higher identical thereto.

All of the CDRs of a particular antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding of the humanized antibody to the Fc receptor.

Also within the scope of the invention are chimeric and humanized antibodies in which specific amino acids have been substituted, deleted or added. In particular, preferred humanized antibodies have amino acid substitutions in the framework region, such as to improve binding to the antigen. For example, a humanized antibody will have framework residues identical to the donor framework residue or to another amino acid other than the recipient framework residue. As another example, in a humanized antibody having mouse CDRs, amino acids located in the human framework region can be replaced with the amino acids located at the corresponding positions in the mouse antibody. Such substitutions are known to improve binding of humanized antibodies to the antigen in some instances.

Antibody fragments of the invention are obtained using conventional procedures known to those with skill in the art. For example, digestion of an antibody with pepsin yields F(ab')2 fragments and multiple small fragments. Mercaptoethanol reduction of an antibody yields individual heavy and light chains. Digestion of an antibody with papain yields individual Fab fragments and the Fc fragment.

In another aspect, the invention also features a modified natural immunoglobulin, e.g., which functions as an agonist (mimetic) or as an antagonist. Preferably the modified natural immunoglobulin, e.g., modified pathogenic immunoglobulin, functions as an antagonist of complement activation. Variants of the pathogenic immunoglobulin can be generated by mutagenesis, e.g., discrete point mutation, the insertion or deletion of sequences or the truncation of a pathogenic immunoglobulin. An agonist of the natural immunoglobulin can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of the protein. An antagonist of a natural immunoglobulin can inhibit one or more of the activities of the naturally occurring form of the pathogenic immunoglobulin by, for example, being capable of binding to an ischemic specific antigen,

but incapable of activating a complement pathway. Thus, specific biological effects can be elicited by treatment with a variant of limited function.

In one embodiment, the site within the natural immunoglobulin (e.g., a pathogenic IgM) that binds C1q can be mutated such that it is no longer capable of binding Clq. For example, the CH2 domain of an IgG and the CH4 domain of an IgM, which are known to contain binding sites for Clq, can be mutated (see WO 94/29351). For example, the carboxyl terminal half of the CH2 domain of an IgG (residues 231 to 239, preferably within 234 to 239), which appear to mediate C1q binding and subsequent complement activation, can be mutated. As another example, Wright et al. have demonstrated that a single nucleotide change in the IgM constant region domain renders the antibody defective in initiating complement-dependent cytolysis. The single nucleotide change results in the encoding of a serine residue, rather than the normal proline residue, at amino acid position 436 in the third constant domain (Wright et al. 1988, *J. Biol. Chem.* 263: 11221). The amino acid substitutions that can be made to antibodies in order to alter complement binding or activity are well known in the art (see for example, Wright et al. 1988, J. *Biol. Chem.* 263: 11221; Shulman et al. (1986), *Proc. Natl. Acad. Sci. USA* 83: 7678-7682; Arya et al., (1994) J. *Immunol.* 253: 1206-1212; Poon et al., (1995) J. *Biol. Chem.* 270: 8571-8577, the contents of all of which are hereby incorporated by reference). Accordingly, in one embodiment, the antibodies of the present invention have a mutation that alters complement binding or activity. Antibodies in which amino acids have been added, deleted, or substituted are referred to herein as modified antibodies or altered antibodies. As will be appreciated by the skilled artisan, the methods used for causing such changes in nucleotide or amino acid sequence will vary depending upon the desired results.

Variants of a natural immunoglobulin can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants, of a natural immunoglobulin for agonist or antagonist activity.

Libraries of fragments e.g., N terminal, C terminal, or internal fragments, of a natural immunoglobulin coding sequence can be used to generate a variegated population of fragments for screening and subsequent selection of variants of this protein. Variants in which a cysteine residue is added or deleted or in which a residue that is glycosylated is added or deleted are particularly preferred.

Methods for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants (Arkin and Yourvan (1992) *Proc. Natl. Acad. Sci.USA* 89:7811-7815; Delgrave et al. (1993) *Protein Engineering* 6(3):327-331).

Cell based assays can be exploited to analyze a variegated library. For example, a library of expression vectors can be transfected into a cell line, e.g., a cell line, which ordinarily responds to the protein in a substrate-dependent manner. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of signaling by the pathogenic immunoglobulin-substrate, and the individual clones further characterized.

The invention also features a method of making a natural immunoglobulin, e.g., a pathogenic immunoglobulin having a non-wild type activity, e.g., an antagonist, agonist, or super agonist of a naturally occurring pathogenic immunoglobulin. The method includes: altering the sequence of a natural immunoglobulin, e.g., by substitution or deletion of one or more residues of a non-conserved region, a domain or residue disclosed herein, and testing the altered polypeptide for the desired activity.

Further, the invention features a method of making a fragment or analog of a natural immunoglobulin, e.g., a pathogenic immunoglobulin having an altered biological activity of a naturally occurring pathogenic immunoglobulin. The method includes: altering the sequence, e.g., by substitution or deletion of one or more residues, of a pathogenic immunoglobulin, e.g., altering the sequence of a non-conserved region, or a domain or residue described herein, and testing the altered polypeptide for the desired activity. In an exemplary embodiment, the modified natural immunoglobulin may have a reduced ability to activate complement. For example, one or more of the amino acid residues involved in complement binding and/or activation are mutated.

In certain embodiment, the modified natural antibody may comprise at least the CDR1 region of SEQ ID NO: 8 (SEQ ID NO: 10), or antigen binding portions thereof, and/or at least the CDR2 region of SEQ ID NO: 8 (SEQ ID NO: 12), or antigen binding portions thereof. In another embodiment, the modified antibody may comprise at least the CDR1 region of SEQ ID NO: 2 (SEQ ID NO: 4), or antigen binding portions thereof,

and/or at least the CDR2 region of SEQ ID NO: 2 (SEQ ID NO: 6), or antigen binding portions thereof. In an exemplary embodiment, the modified antibody comprises the CDR1 region of SEQ ID NO: 8 (SEQ ID NO: 10) and the CDR2 region of SEQ ID NO: 8 (SEQ ID NO: 12) or antigen binding portions thereof. In another exemplary embodiment, the modified antibody comprises the CDR1 region of SEQ ID NO: 2 (SEQ ID NO: 4) and the CDR2 region of SEQ ID NO: 2 (SEQ ID NO: 6) or antigen binding portions thereof. The modified antibody may also comprise the CDR1 region of SEQ ID NO: 8 (SEQ ID NO: 10) and the CDR2 region of SEQ ID NO: 8 (SEQ ID NO: 12) and the modified antibody comprises the CDR1 region of SEQ ID NO: 2 (SEQ ID NO: 4) and the CDR2 region of SEQ ID NO: 2 (SEQ ID NO: 6) or antigen binding portions thereof.

The modified natural antibody can be a human antibody having a binding affinity to the ischemic-specific antigen, similar, e.g., greater than, less than, or equal to, the binding affinity of the antibody produced by the hybridoma deposited with the ATCC, having the accession number PTA-3507. In another embodiment, the natural antibody can be a non-human antibody, e.g., a cow, goat, mouse, rat, sheep, pig, or rabbit. In an exemplary embodiment, the non-human antibody is a murine antibody. The natural antibody may also be a recombinant antibody. In an exemplary embodiment, the natural antibody is a humanized antibody. The modified natural antibody may be an IgG or IgM antibody. In another embodiment, the isolated natural immunoglobulin possess the same antigenic specificity as the immunoglobulin produced by the hybridoma deposited with the ATCC, having accession number PTA-3507.

6.4 Screening Assay to Identify Additional Inhibitors

Other inhibitors of an interaction between a natural IgM antibody and an antigen or a component of the complement pathway may be identified from one or more (e.g., a plurality of) test compounds, comprising (i) providing a reaction mixture which includes the natural IgM antibody and the antigen or the component of the complement pathway under conditions that allow binding of the natural IgM antibody and the antigen or the component of the complement pathway to occur; (ii) contacting the natural IgM antibody and the antigen or the component of the complement pathway with one or more test compounds (e.g., members of a combinatorial library); and (iii) detecting any changes in binding of the natural IgM antibody and the antigen or the component of the complement in

the presence of a given test compound relative to that detected in the absence of the test compound. A change (e.g., decrease) in the level of binding between the natural IgM antibody and the antigen or the component of the complement pathway in the presence of the test compound relative to that detected in the absence of the test compound indicates that the test compound is an inhibitor of the interaction between the natural IgM antibody and the antigen or the component of the complement pathway.

The method can further include pre-treating the natural IgM antibodies with one or more test compounds. The pre-treated natural IgM antibodies can then be injected into mice deficient in natural immunoglobulins.

In certain embodiments, the methods is performed *in vitro*. In an exemplary embodiment, the contacting step is effected *in vivo*. In an exemplary embodiment, the antigen is myosin. In other embodiments, the antigen is an endothelial tissue or lysate obtained from a subject e.g., a human patient with reperfusion or ischemic injury. In another exemplary embodiment, the component of the complement pathway is a component of the classical pathway of complement. In a further exemplary embodiment, the component of the complement pathway is a Cl molecule or a subunit thereof (e.g., Clq).

In exemplary embodiments, either the natural IgM antibody or the antigen (or both) is labeled with a detectable signal, e.g., fluorophores, colorimetric enzymes, radioisotopes, luminescent compounds, and the like. The method can further include repeating at least one step, e.g., the contacting step with a second or subsequent member or members of the library.

In an exemplary embodiment, a plurality of test compounds, e.g., library members, is tested. The plurality of test compounds, e.g., library members, can include at least 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 compounds. In a preferred embodiment, the plurality of test compounds, e.g., library members, share a structural or functional characteristic. The test compound can be a peptide or a small organic molecule.

In one embodiment, the inhibitor is a small organic molecule that may be identified in a combinatorial library. In one embodiment, the invention provides libraries of inhibitors. The synthesis of combinatorial libraries is well known in the art and has been reviewed (see, e.g., E.M. Gordon *et al., J. Med. Chem.* (1994) 37:1385-1401 ; DeWitt, S. H.; Czarnik, A. W. Acc. *Chem. Res.* (1996) 29:114; Armstrong, R. W.; Combs, A. P.; Tempest, P. A.; Brown, S. D.; Keating, T. A. *Acc. Chem. Res.* (1996) 29:123; Ellman, J. A.

Acc. Chem. Res. (1996) 29:132; Gordon, E. M.; Gallop, M. A.; Patel, D. V. *Acc. Chem.* Res. (1996) 29:144; Lowe, G. *Chem. Soc. Rev.* (1995) 309, Blondelle et al. *Trends Anal. Chem.* (1995) 14:83; Chen et al. J.. *Am. Chem.* Soc. (1994) 116:2661; U.S. Patents 5,359,115, 5,362,899, and 5,288,514; PCT Publication Nos. W092/10092, W093/09668, W091/07087, W093/20242, W094/08051).

Libraries of compounds of the invention can be prepared according to a variety of methods, some of which are known in the art. For example, a "split-pool" strategy can be implemented in the following way: beads of a functionalized polymeric support are placed in a plurality of reaction vessels; a variety of polymeric supports suitable for solid-phase peptide synthesis are known, and some are commercially available (for examples, see, e.g., M. Bodansky "Principles of Peptide Synthesis", 2nd edition, Springer-Verlag, Berlin (1993)). To each aliquot of beads is added a solution of a different activated amino acid, and the reactions are allowed to proceed to yield a plurality of immobilized amino acids, one in each reaction vessel. The aliquots of derivatized beads are then washed, "pooled" (i.e., recombined), and the pool of beads is again divided, with each aliquot being placed in a separate reaction vessel. Another activated amino acid is then added to each aliquot of beads. The cycle of synthesis is repeated until a desired peptide length is obtained. The amino acid residues added at each synthesis cycle can be randomly selected; alternatively, amino acids can be selected to provide a "biased" library, e.g., a library in which certain portions of the inhibitor are selected non-randomly, e.g., to provide an inhibitor having known structural similarity or homology to a known peptide capable of interacting with an antibody, e.g., the an anti-idiotypic antibody antigen binding site. It will be appreciated that a wide variety of peptidic, peptidomimetic, or non-peptidic compounds can be readily generated in this way.

The "split-pool" strategy results in a library of peptides, e.g., inhibitors, which can be used to prepare a library of test compounds of the invention. In another illustrative synthesis, a "diversomer library" is created by the method of Hobbs DeWitt *et al. (Proc. Natl. Acad. Sci. USA 90:*6909 (1993)). Other synthesis methods, including the "tea-bag" technique of Houghten (see, e.g., Houghten *et al., Nature* 354:84-86 (1991)) can also be used to synthesize libraries of compounds according to the subject invention. Libraries of compounds can be screened to determine whether any members of the library have a desired activity, and, if so, to identify the active species. Methods of screening combinatorial libraries have been described (see, e.g., Gordon *et al., J Med. Chem.,*

supra). Soluble compound libraries can be screened by affinity chromatography with an appropriate receptor to isolate ligands for the receptor, followed by identification of the isolated ligands by conventional techniques (e.g., mass spectrometry, NMR, and the. like). Immobilized compounds can be screened by contacting the compounds with a soluble receptor; preferably, the soluble receptor is conjugated to a label (e.g., fluorophores, colorimetric enzymes, radioisotopes, luminescent compounds, and the like) that can be detected to indicate ligand binding. Alternatively, immobilized compounds can be selectively released and allowed to diffuse through a membrane to interact with a receptor. Exemplary assays useful for screening the libraries of the invention are described below.

In one embodiment, compounds of the invention can be screened for the ability to interact with a natural immunoglobulin by assaying the activity of each compound to bind directly to the immunoglobulin or to inhibit an interaction between the immunoglobulin and an ischemic antigen, e.g., by incubating the test compound with an immunoglobulin and a lysate, e.g., an endothelial cell lysate, e.g., in one well of a multiwell plate, such as a standard 96-well microtiter plate. In this embodiment, the activity of each individual compound can be determined. A well or wells having no test compound can be used as a control. After incubation, the activity of each test compound can be determined by assaying each well. Thus, the activities of a plurality of test compounds can be determined in parallel.

6.5 Modified Inhibitors and Pharmaceutical and Diagnostic Preparations

IgM inhibitors may be modified, for example to increase solubility and/or facilitate purification, identification, detection, and/or structural characterization. Exemplary modifications, include, for example, addition of: glutathione S-transferase (GST), protein A, protein G, calmodulin-binding peptide, thioredoxin, maltose binding protein, HA, myc, poly-arginine, poly-His, poly-His-Asp or FLAG fusion proteins and tags. In various embodiments, an IgM inhibitors may comprise one or more heterologous fusions. For example, peptides may contain multiple copies of the same fusion domain or may contain fusions to two or more different domains. The fusions may occur at the N-terminus of the peptide, at the C-terminus of the peptide, or at both the N- and C-terminus of the peptide. It is also within the scope of the invention to include linker sequences between a peptide of

the invention and the fusion domain in order to facilitate construction of the fusion protein or to optimize protein expression or structural constraints of the fusion protein. In another embodiment, the peptide may be constructed so as to contain protease cleavage sites between the fusion peptide and peptide of the invention in order to remove the tag after protein expression or thereafter. Examples of suitable endoproteases, include, for example, Factor Xa and TEV proteases.

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene may be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments may be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments, which may subsequently be annealed to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al., John Wiley & Sons: 1992).

IgM inhibitors may be chemically modified based on linkage to a polymer. The polymer is typically water soluble so that the inhibitor to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer may have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so that the degree of polymerization may be controlled. A preferred reactive aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see U.S. Pat. No. 5,252,714). The polymer may be branched or unbranched. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. The water soluble polymer, or mixture thereof if desired, may be selected from the group consisting of, for example, polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol.

IgM inhibitors may be labeled, for example with an isotopic label to facilitate its detection using nuclear magnetic resonance or another applicable technique. Exemplary

isotopic labels include radioisotopic labels such as, for example, potassium-40 (^{40}K), carbon-14 (^{14C}), tritium (^{3}H), sulphur-35 (^{35}S), phosphorus-32 (^{32}P), technetium-99m (^{99m}Tc), thallium-201 (^{201}Tl), gallium-67 (^{67}Ga), indium-111 (^{111}In), iodine-123 (^{123}I), iodine-131 (^{131}I), yttrium-90 (^{90}Y), samarium-153 (^{153}Sm), rhenium-186 (^{186}Re), rhenium-188 (^{188}Re), dysprosium-165 (^{165}Dy) and holmium-166 (^{166}Ho). The isotopic label may also be an atom with non zero nuclear spin, including, for example, hydrogen-1 (^{1}H), hydrogen-2 (^{2}H), hydrogen-3 (^{3}H), phosphorous-31 (^{31}P), sodium-23 (^{23}Na), nitrogen-14 (^{14}N), nitrogen-15 (^{15}N), carbon-13 (^{13}C) and fluorine-19 (^{19}F). In certain embodiments, the inhibitor is uniformly labeled with an isotopic label, for example, wherein at least 50%, 70%, 80%, 90%, 95%, or 98% of the inhibitor is labeled. In other embodiments, the isotopic label is located in one or more specific locations within the inhibitor, for example, the label may be specifically incorporated into one or more of the leucine residues of a peptide. A single inhibitor may comprise two or more different isotopic labels, for example, a peptide may comprise both ^{15}N and ^{13}C labeling.

Inhibitors may be labeled with a fluorescent label. In an exemplary embodiment, an inhibitor is fused to a heterologous polypeptide sequence which produces a detectable fluorescent signal, including, for example, green fluorescent protein (GFP), enhanced green fluorescent protein (EGFP), *Renilla reniformis* green fluorescent protein, GFPmut2, GFPuv4, enhanced yellow fluorescent protein (EYFP), enhanced cyan fluorescent protein (ECFP), enhanced blue fluorescent protein (EBFP), citrine and red fluorescent protein from discosoma (dsRED).

Toxicity and therapeutic efficacy of natural antibody inhibitors including natural IgM antibody-binding peptides or modified natural IgM antibodies can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Natural antibody inhibitors which exhibit large therapeutic effects are preferred. While natural antibody inhibitors or natural antibody-binding peptides that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such peptides or modified antibodies to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of a natural antibody inhibitor or a natural antibody-binding peptides lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any inhibitor or peptide used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In another embodiment, a single bolus of a natural antibody inhibitor including a natural IgM antibody-binding peptide and modified natural IgM antibodies is administered prior to, contemporaneously with, or subsequent to a tissue injury. Typically a single dose injection will be a few hours, a few days or a few weeks after tissue injury. The present invention is based in part upon the discovery that a natural IgM antibody inhibitor preventsreperfusion injury. A single unit dosage delivery can be immediately adjacent to the site of injury or can be, for example, to a vessel that drains or flows to the site of injury.

A natural IgM antibody inhibitor such as natural IgM antibody-binding peptide or modified natural IgM antibody is administered initially at a point in time prior to the time of damage of the target organ or tissue. This may be a useful approach in subjects who are determined to be at risk for reperfusion injury, such as those with a history of reperfusion injury or those about to undergo surgery.

In yet another embodiment, a single bolus of a natural IgM antibody inhibitor can be followed by subsequence administrations of a natural IgM antibody inhibitor as continuous infusions or additional single bolus deliveries. The inhibitor may be administer in sequential exposures over a period of hours, days, weeks, months or years. In addition, it is contemplated that additional therapeutic agents can be combined with, administered prior to or subsequent to administration of a natural antibody-binding peptide or another natural antibody inhibitor. Other therapeutic agents that may be administered with an natural IgM antibody inhibitor include, but are not limited to, anti-coagulation agents and complement inhibitors.

The subject inhibitors may be provided in pharmaceutically acceptable carriers or formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remmington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. In certain embodiments, the inhibitor is provided for transmucosal or transdermal delivery. For such administration, penetrants appropriate to the barrier to be permeated are used in the formulation with the polypeptide. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the inhibitors of the invention are formulated into ointments, salves, gels, or creams as generally known in the art.

The pharmaceutical compositions according to the invention are prepared by bringing a natural IgM antibody inhibitors into a form suitable for administration to a subject using carriers, excipients and additives or auxiliaries. Frequently used carriers or auxiliaries include magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talc, milk protein, gelatin, starch, vitamins, cellulose and its derivatives, animal and vegetable oils, polyethylene glycols and solvents, such as sterile water, alcohols, glycerol and polyhydric alcohols. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described, for instance, in Remington's Pharmaceutical Sciences, 15th ed. Easton: Mack Publishing Co., 1405-1412, 1461-1487 (1975) and The National Formulary XIV., 14th ed. Washington: American Pharmaceutical Association (1975), the contents of which are hereby incorporated by reference. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art. See Goodman and Gilman's The Pharmacological Basis for Therapeutics (7th ed.).

The pharmaceutical compositions are preferably prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories and including, for example, alginate based pH dependent release gel caps. For treatment of a subject, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the subject, different daily doses are necessary. Under certain

circumstances, however, higher or lower daily doses may be appropriate. The administration of the daily dose can be carried out both by single administration in the form of an individual dose unit or by several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

The pharmaceutical compositions according to the invention may be administered locally or systemically in a therapeutically effective dose. Amounts effective for this use will, of course, depend on the severity of the disease and the weight and general state of the subject. As discussed above, dosages used *in vitro* may provide useful guidance in the amounts useful for in situ administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, e.g., in Langer, Science, 249: 1527, (1990); Gilman et al. (eds.) (1990), each of which is herein incorporated by reference.

In one embodiment, the invention provides a pharmaceutical composition useful for administering a natural antibody-binding peptide to a subject in need of such treatment. "Administering" the pharmaceutical composition of the invention may be accomplished by any means known to the skilled artisan. Preferably a "subject" refers to a mammal, most preferably a human.

The natural IgM antibody inhibitor can be administered parenterally, enterically, by injection, rapid infusion, nasopharyngeal absorption, dermal absorption, rectally and orally. Pharmaceutically acceptable carrier preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers for occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners and elixirs containing inert diluents commonly used in the art, such as purified water. Where the disease or disorder is a gastrointestinal disorder oral formulations or suppository formulations are preferred.

Sterile injectable solutions can be prepared by incorporating a natural antibody-binding peptide in the required amount (e.g., about 10 μg to about 10 mg/kg) in an appropriate solvent and then sterilizing, such as by sterile filtration. Further, powders can be prepared by standard techniques such as freeze drying or vacuum drying.

In another embodiment, a natural IgM antibody inhibitor is prepared with a biodegradable carrier for sustained release characteristics for either sustained release in the GI tract or for target organ implantation with long term active agent release characteristics to the intended site of activity. Biodegradable polymers include, for example, ethylene vinyl acetate, polyanhydrides, polyglycolic acids, polylactic acids, collagen, polyorthoesters, and poly acetic acid. Liposomal formulation can also be used.

Another means of delivering natural IgM antibody inhibitor (e.g., a natural IgM antibody-binding peptide) is by delivering host cells that express natural antibody-binding peptides to a site or tissue in need of repair. Alternatively, the cells may be delivered in conjunction with various delivery vehicles, including biocompatible biodegradable or non-biodegradable sponges (e.g., collagen, or other extracellular matrix materials), cotton, polyglycolic acid, cat gut sutures, cellulose, gelatin, dextran, polyamide, a polyester, a polystyrene, a polypropylene, a polyacrylate, a polyvinyl, a polycarbonate, a polytetrafluorethylene, or a nitrocellulose compound formed into a three-dimensional structure (see, for example, U.S. Pat. No. 5,858,721 to Naughton et al., the disclosure of which is incorporated herein by reference).

Any route of administration compatible with the active principle can be used. The preferred is parenteral administration, such as subcutaneous, intramuscular or intravenous injection. The dose of the active ingredient to be administered depends on the basis of the medical prescriptions according to age, weight and the individual response of the patient.

The daily non-weighted dosage for the patient can be between about 2.5-5.0 mg/Kg, e.g., about 2.5-3.0 mg/Kg, about 3.0-3.5 mg/Kg, about 3.5-4.0 mg/Kg, about 4.0-4.5 mg/Kg, and about 4.5-5.0 mg/Kg.

The pharmaceutical composition for parenteral administration can be prepared in an injectable form comprising the active principle and a suitable vehicle. Vehicles for the parenteral administration are well known in the art and comprise, for example, water, saline solution, Ringer solution and/or dextrose.

The vehicle can contain small amounts of excipients in order to maintain the stability and isotonicity of the pharmaceutical preparation.

The preparation of the cited solutions can be carried out according to the ordinary modalities.

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims. The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

6.6 Diseases and conditions that can be treated with natural IgM antibody inhibitors

IgM inhibitors, such as natural IgM antibody-binding peptides or modified natural IgM antibodies, may be used for treating a number of inflammatory diseases and conditions that are triggered by binding of natural IgM antibodies. For instance, the inhibitors may be used to treat inflammatory diseases or conditions such as reperfusion injury, ischemia injury, stroke, autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, rheumatoid arthritis, celiac disease, hyper-IgM immunodeficiency, arteriosclerosis, coronary artery disease, sepsis, myocarditis, encephalitis, transplant rejection, hepatitis, thyroiditis (e.g., Hashimoto's thyroiditis, Graves disease), osteoporosis, polymyositis, dermatomyositis, Type I diabetes, gout, dermatitis, alopecia areata, systemic lupus erythematosus, lichen sclerosis, ulcerative colitis, diabetic retinopathy, pelvic inflammatory disease, periodontal disease, arthritis, juvenile chronic arthritis (e.g., chronic iridocyclitis), psoriasis, osteoporosis, nephropathy in diabetes mellitus, asthma, pelvic inflammatory disease, chronic inflammatory liver disease, chronic inflammatory lung disease, lung fibrosis, liver fibrosis, rheumatoid arthritis, chronic inflammatory liver disease, chronic inflammatory lung disease, lung fibrosis, liver fibrosis, Crohn's disease, ulcerative colitis, burn injury (or thermal injury), and other acute and chronic inflammatory diseases of the Central Nervous System (CNS; e.g., multiple sclerosis), gastrointestinal system, the skin

and associated structures, the immune system, the hepato-biliary system, or any site in the body where pathology can occur with an inflammatory component.

An inflammatory condition such as reperfusion or ischemic injury may result following a naturally occurring episode, e.g., as a stroke or myocardial infarction. Reperfusion or ischemic injury may also occur during and/or following a surgical procedure. Exemplary surgical procedures that cause can cause injury include a vessel-corrective technique selected from the group consisting of angioplasty, stenting procedure, atherectomy, and bypass surgery. In an exemplary embodiment, reperfusion or ischemic injury occurs in a cardiovascular tissue, such as the heart.

In addition, diseases or conditions that are triggered by binding of natural IgM antibodies may be treated or prevented in a subject by removing from the subject or inactivating a natural or pathogenic IgM and/or B cells producing the pathogenic immunoglobulin (e.g., B-1 cells as described herein), thereby reducing the amount of the pathogenic immunoglobulin and/or B cells present in the subject.

The methods described herein may comprise removing from the subject or inactivating a pathogenic immunoglobulin, e.g., a pathogenic IgM as described herein, and/or B-cells producing the pathogenic IgM (e.g., B-1 cells as described herein), thereby reducing the amount of the pathogenic immunoglobulin and/or B cells present in the subject.

In one embodiment, the removing or inactivating step is performed *ex vivo*. The pathogenic immunoglobulins or B cells can be removed by hemoperfusion. Alternatively, the B cells can be removed using a B cell-specific antibody (e.g., an anti-B-1 antibody or an anti-CD5 antibody or anti-CD 11 G/CD 18). The pathogenic immunoglobulin, e.g., an IgM, can be removed by contacting blood from a subject with an immobilized antigen (e.g., an ischemia-specific antigen) or an immobilized anti-idiotypic antibody. The removing or inactivating step of the pathogenic immunoglobulin may be performed by administering an anti-idiotypic antibody to the subject. In another embodiment, the removing or inactivating step of the B cell is performed by administering to the subject a B cell targeting moiety (e.g., an antibody or an antigen binding fragment thereof, or an antigen) coupled to a toxin, e.g., ricin or diphteria toxin. The subject is a mammal, e.g., a rodent (e.g., a mouse) or a primate (e.g., a human). In a exemplary embodiment, the subject has sustained a reperfusion or ischemic injury following a naturally occurring episode, e.g., as a stroke, and

the removing step is carried out within minutes, one to five hours, five to ten hours, ten to twenty hours, one to five days, following the naturally occurring episode. In another exemplary embodiment, the reperfusion or ischemic injury occurs in a cardiovascular tissue, e.g., the heart, and the reperfusion or ischemic injury is prevented and/or decreased by, removing from the subject, the pathogenic immunoglobulin, and/or the B cells, prior to, during, and/or following the surgical procedure. For example, the removing step can be carried out at least one to five hours, five to ten hours, ten to twenty hours, or one, two or three days prior to the surgical procedure. The removing step can also be continued for appropriate time intervals during and after the surgical procedure.

6.7 Diagnostic Assays

The invention further provides a method for detecting the presence of a natural IgM antibody in a biological sample. Detection of a natural IgM antibody in a subject, particularly a mammal, and especially a human, will provide a diagnostic method for diagnosis of an inflammatory disease or condition in the subject. In general, the method involves contacting the biological sample with a compound or an agent capable of detecting natural IgM antibody of the invention or a nucleic acid of the invention in the sample. The term "biological sample" when used in reference to a diagnostic assay is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject.

The detection method of the invention may be used to detect the presence of a natural IgM antibody or a nucleic acid of the invention in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of a nucleic acid of the invention include Northern hybridizations and in situ hybridizations. *In vitro* techniques for detection of polypeptides of the invention include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations, immunofluorescence, radioimmunoassays and competitive binding assays.

Nucleic acids for diagnosis may be obtained from an infected individual's cells and tissues, such as bone, blood, muscle, cartilage, and skin. Nucleic acids, e.g., DNA and RNA, may be used directly for detection or may be amplified, e.g., enzymatically by using PCR or other amplification technique, prior to analysis. Using amplification, characterization of the species and strain of prokaryote present in an individual, may be

made by an analysis of the genotype of the prokaryote gene. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the genotype of a reference sequence. Point mutations can be identified by hybridizing a nucleic acid, e.g., amplified DNA, to a nucleic acid of the invention, which nucleic acid may be labeled. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence differences may also be detected by alterations in the electrophoretic mobility of the DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing. See, e.g. Myers et al., Science, 230: 1242 (1985). Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and S1 protection or a chemical cleavage method. See, e.g., Cotton et al., Proc. Natl. Acad. Sci., USA, 85: 4397-4401 (1985).

Agents for detecting a nucleic acid of the invention, e.g., comprising the sequence set forth in a subject nucleic acid sequence, include labeled nucleic acid probes capable of hybridizing to a nucleic acid of the invention. The nucleic acid probe can comprise, for example, the full length sequence of a nucleic acid of the invention, or an equivalent thereof, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to a subject nucleic acid sequence, or the complement thereof. Agents for detecting a polypeptide of the invention, e.g., comprising an amino acid sequence of a subject amino acid sequence, include labeled anti-antibodies capable of binding to a natural IgM antibody of the invention. Anti-idiotypic antibodies may be polyclonal, or alternatively, monoclonal. An intact anti-idiotypic antibody, or a fragment thereof can be used. Labeling the probe or antibody also encompasses direct labeling of the probe or antibody by coupling (e.g., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin.

In certain embodiments, detection of a nucleic acid of the invention in a biological sample involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) Science 241:1077-1080; and Nakazawa et al. (1994) PNAS 91:360-364), the latter of which can be

particularly useful for distinguishing between orthologs of polynucleotides of the invention (see Abravaya et al. (1995) Nucleic Acids Res. 23:675-682). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a nucleic acid of the invention under conditions such that hybridization and amplification of the polynucleotide (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample.

In one aspect, the present invention contemplates a method for detecting the presence of a natural IgM antibody in a sample, the method comprising: (a) providing a sample to be tested for the presence of a natural IgM antibody; (b) contacting the sample with an anti-idiotypic antibody reactive against about eight consecutive amino acid residues of a subject amino acid sequence from such species under conditions which permit association between the anti-idiotypic antibody and its ligand; and (c) detecting interaction of the anti-idiotypic antibody with its ligand, thereby detecting the presence of a natural IgM antibody in the sample.

In another aspect, the present invention contemplates a method for detecting the presence of a natural IgM antibody in a sample, the method comprising: (a) providing a sample to be tested for the presence of a natural IgM antibody; (b) contacting the sample with an anti-idiotypic antibody that binds specifically to a polypeptide of the invention from such species under conditions which permit association between the anti-idiotypic antibody and its ligand; and (c) detecting interaction of the anti-idiotypic antibody with its ligand, thereby detecting the presence of such species in the sample.

In yet another example, the present invention contemplates a method for diagnosing a patient suffering from an inflammatory disease or condition related to the presence of a natural IgM antibody, comprising: (a) obtaining a biological sample from a patient; (b) detecting the presence or absence of a polypeptide of the invention, e.g., a natural IgM antibody, or a nucleic acid encoding a polypeptide of the invention, in the sample; and (c) diagnosing a patient suffering from such an inflammatory disease or condition based on the presence of a polypeptide of the invention, or a nucleic acid encoding a polypeptide of the invention, in the patient sample.

The diagnostic assays of the invention may also be used to monitor the effectiveness of a treatment in an individual suffering from an inflammatory disease or condition related to a natural IgM antibody. For example, the presence and/or amount of a nucleic acid of the invention or a polypeptide of the invention can be detected in an individual suffering from an inflammatory disease or condition related to a natural IgM antibody before and after treatment with a natural IgM antibody therapeutic agent. Any change in the level of a polynucleotide or polypeptide of the invention after treatment of the individual with the therapeutic agent can provide information about the effectiveness of the treatment course. In particular, no change, or a decrease, in the level of a polynucleotide or polypeptide of the invention present in the biological sample will indicate that the therapeutic is successfully combating such disease or disorder.

Alternatively, polypeptides of the invention, e.g., natural IgM antibodies, can be detected *in vivo* in a subject by introducing into the subject a labeled antibody specific for a polypeptide of the invention, e.g., an anti-idiotypic antibody to detect natural IgM antibodies. For example, the anti-idiotypic antibody can be labeled with a radionuclide marker whose presence and location in a subject can be detected by standard imaging techniques.

A "radionuclide" refers to molecule that is capable of generating a detectable image that can be detected either by the naked eye or using an appropriate instrument, e.g. positron emission tomography (PET), and single photon emission tomography (SPECT). Radionuclides useful within the present disclosure include penetrating photon emitters including gamma emitters and X-ray emitters. These rays accompany nuclear transformation such as electron capture, beta emission and isomeric transition. Radionuclides useful include those with photons between 80 and 400 keV and positron producers, 511 keV annihilation photons and acceptable radiation doses due to absorbed photons, particles and half life. Radionuclides include radioactive isotopes of an element. Examples of radionuclides include ^{123}I, ^{125}I, ^{99m}Tc, ^{18}F, ^{68}Ga, ^{62}Cu, ^{111}In, ^{131}I, ^{186}Re, ^{188}Re, ^{90}Y, ^{212}Bi, ^{211}At, ^{89}Sr, ^{166}Ho, ^{153}Sm, ^{67}Cu, ^{64}Cu, ^{100}Pd, ^{212}Pb, ^{109}Pd, ^{67}Ga, ^{94}Tc, ^{105}Rh, ^{95}Ru, ^{177}Lu, ^{170}Lu ^{11}C, and ^{76}Br.

In one embodiment, an anti-idiotypic antibody that recognizes a natural IgM antibody of the present invention may be labeled with ^{99m}Tc. ^{99m}Tc, a commonly used radionuclide in Nuclear Medicine, combines desirable physical properties with a 6 hr half-

life and a 140-KeV gamma energy (85% as gamma photons) and widespread availability, since it can readily be eluted from molybdenum generators.

The imaging agents of the disclosure may be used in the following manner. An effective amount of the imaging agent (from 1 to 50 mCi) may be combined with a pharmaceutically acceptable carrier for use in imaging studies. In accordance with the disclosure, "an effective amount" of the imaging agent of the disclosure is defined as an amount sufficient to yield an acceptable image using equipment which is available for clinical use. An effective amount of the imaging agent of the disclosure may be administered in more than one injection. Effective amounts of the imaging agent of the disclosure will vary according to factors such as the degree of susceptibility of the individual, the age, sex, and weight of the individual, idiosyncratic responses of the individual and dosimetry. Effective amounts of the imaging agent of the disclosure will also vary according to instrument and film-related factors. Optimization of such factors is well within the level of skill of a person skilled in the art.

The amount of imaging agent used for diagnostic purposes and the duration of the imaging study will depend upon the nature and severity of the condition being treated, on the nature of therapeutic treatments which the patient has undergone, and on the idiosyncratic responses of the patient. Ultimately, the attending physician will decide the amount of imaging agent to administer to each individual patient and the duration of the imaging study.

The pharmaceutically acceptable carrier for an imaging agent of the disclosure may include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic agents, absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. The imaging agent of the disclosure may further be administered to an individual in an appropriate diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as human serum albumin or liposomes. Supplementary active compounds can also be incorporated into the imaging agent of the disclosure. Pharmaceutically acceptable diluents; include saline and aqueous buffer solutions. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and nhexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diethylpyrocarbonate, and trasylol. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.* (1984) *J. Neuroimmunol.* 7, 27).

In one embodiment, the imaging agent of the disclosure is administered parenterally as injections (intravenous, intramuscular or subcutaneous). The imaging agent may be formulated as a sterile, pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. Certain pharmaceutical compositions of this disclosure suitable for parenteral administration comprise one or more imaging agents in combination with one or more pharmaceutically acceptable sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. A formulation for injection should contain, in addition to the cardiovascular imaging agent, an isotonic vehicle such as sodium chloride solution, Ringer's solution, dextrose solution , dextrose and sodium chloride solution, lactated Ringer's solution, dextran solution, sorbitol solution, a solution containing polyvinyl alcohol, or an osmotically balanced solution comprising a surfactant and a viscosity-enhancing agent, or other vehicle as known in the art. The formulation used in the present disclosure may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The invention also encompasses kits for detecting the presence of a natural IgM antibody in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting a polynucleotide or polypeptide of the invention in a biological sample; means for determining the amount of a natural IgM antibody in the sample; and means for comparing the amount of a natural IgM antibody in the sample with a standard. An unlabeled compound may also be provided with instructions for labeling the compound. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect a polynucleotide or polypeptide of the invention.

Exemplification

The invention, having been generally described, may be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention in any way.

Example 1: Mechanism of Ischemia-Reperfusion Injury

This Example shows that mice deficient in the complement system were resistant to ischemia-reperfusion injury.

To examine the mechanism of ischemia-reperfusion injury, mice deficient in complement C3 were treated in the hindlimb model. The C3-/- mice were partially protected from injury based on an approximate 50% reduction in permeability index (see Weiser et al. (1996) J. *Exp. Med.* 1857-1864). Thus, complement C3 is essential for induction of full injury in this murine model.

The experiments in Weiser et al. did not identify how complement was activated. The serum complement system can be activated by at least three distinct pathways, classical, lectin or alternative. Knowing which pathway is involved, is important as it suggests a mechanism for injury. For example, the classical pathways is activated very efficiently by IgM and IgG isotypes of immunoglobulin or by the serum recognition protein C-reactive protein. Whereas, the lectin pathway is activated following recognition of specific carbohydrates such as mannan by mannan binding lectin (MBL) (Epstein et al., *(1996) Immunol 8,* 29-35). In both pathways, complement C4 is required in forming an enzyme complex with C2 that catalyzes cleavage of the central component C3. By contrast, the alternative pathway activates spontaneously leading to conversion of C3 to its active form (C3b) and attachment to foreign-or self-tissues. The pathway is tightly regulated as all host cells express inhibitors of amplification of the complement pathway by inactivating, or displacing the C3 convertase (Muller-Eberhard, H.J., (1988) *Ann. Rev. Biochem.* 57, 321-347). One approach for determining the pathway involved is use of mice deficient in C4, i.e., cannot form C3 convertase via classical or lectin pathways. Comparison of mice deficient in either C3 or C4 with wild type (WT) controls in the hindlimb model, revealed that C4 was also required for induction of full injury (Weiser et al. *supra).* This finding was important as it suggested that antibody or MBL might be involved.

Example 2: Natural IgM Mediates Ischemia Reperfusion (I/R) Injury

This Example shows that mice deficient in immunoglobulin were resistant to ischemia-reperfusion injury.

To determine if antibody was involved in mediating I/R injury, mice totally deficient in immunoglobulin, RAG2-/- (recombinase activating gene-2 deficient) were characterized along with the complement deficient animals in the intestinal model. Significantly, the RAG-2-/- mice were protected to a similar level as observed in the

complement deficient animals (Weiser et al. *supra).* Since the RAG2-/- animals are also missing mature lymphocytes, it was important to determine that the pathogenic effect was antibody dependent (Shinkai et al. (1992) Cell 68, 855-867). To confirm that injury was mediated by serum antibody, the deficient animals were reconstituted with either normal mouse sera (Weiser et al. supra) or purified IgM (Williams et al. (1999) J. Appl. Physiol 86; 938-42). In both cases, the reconstituted RAG-2-/- mice were no longer protected and injury was restored. In the latter experiments, a model of intestinal injury was used as in this model, injury is thought to be mediated primarily by complement.

The interpretation of these results is that during the period of ischemia, neoantigens are either expressed or exposed on the endothelial cell surface. Circulating IgMs appear to recognize the new determinant, bind and activate classical pathway of complement. While the nature of the antigen is not known, IgM rather than IgG seems to be primarily responsible for activation of complement as reconstitution of deficient mice with pooled IgG did not significantly restore injury in the mice. An alternative hypothesis is that there is another initial event such as the MBL pathway that recognizes the altered endothelial surface, induces low level complement activation which in turn exposes new antigenic sites and the pathway is amplified by binding of IgM.

Example 3: Pathogenic IgM is a Product of B-1 cells

Since a major fraction of circulating IgM is thought to represent natural antibody, i.e. product of rearranged germline genes, it is possible that mice bearing deficiencies in the B-1 fraction of lymphocytes might also be protected. B-1 cells have a distinct phenotype from more conventional B-2 cells in that they express low levels of IgD and CD23 and a major fraction express the cell surface protein CD5 (Hardy et al., (1994) *Immunol. Rev.:* 137, 91; Kantor et al. (1993) *Annu. Rev. Immunol. 11,* 501-538, 1993. B-1 cells are also distinguished by reduced circulation in mice, limited frequency in the peripheral lymph nodes and spleen and are primarily localized within the peritoneal cavity. To examine a role for B-1 cells as a source of pathogenic IgM, antibody-deficient mice (RAG-2-/-) were reconstituted with 5×10^5 peritoneal B-1 cells and rested approximately 30 days before treatment. Circulating IgM levels reach a near normal range within a month following adoptive transfer. Characterization of the B-1 cell reconstituted mice in the intestinal ischemia model confirmed that B-1 cells were a major source of pathogenic IgM

(see Williams et al. (1999) supra). This was an important observation because the repertoire of B-1 cell natural antibody is considerably more limited than would be expected for conventional B-2 cells. Therefore, it is possible that the pathogenic antibody represents a product of the germline.

Example 4: Cr2-/- Mice are protected from Ischemia Reperfusion Injury

The initial characterization of Cr2-/- knockout mice revealed an approximate 50% reduction in the frequency of B-1a or CD5 + B-1 cells (Ahearn et al. (1996) *Immunity* 4: 251-262). Although characterization of another strain of Cr2-deficient mice did not identify a similar reduction (Molina et al. (1996) *Proc. Natl. Acad. Sci.* USA 93, 3357-3361). Whether the difference in frequency of CD5 + cells was due to variation in strain background or environmental differences is not known. Despite the reduced frequency of B-1 a cells in the Cr2-/- mice, circulating levels of IgM were within the normal range. These findings suggested that the repertoire of IgM might be different in the Cr2-deficient animals. To test this hypothesis, mice in the intestinal I/R model were characterized. Surprisingly, the Cr2-/- mice were equally protected as the complete-antibody deficient mice (Figure 3). Comparison of survival over a five-day period following treatment in the intestinal model demonstrated a significant increase in mortality of the WT compared to Cr2-deficient animals. Consistent with an increased mortality, a dramatic reduction in injury was observed in tissue sections harvested from treated WT or Cr2-/- deficient mice.

Extensive injury to the mucosal layer of the intestine was observed in WT mice or Cr2-/- mice reconstituted with pooled IgM or B-1 cells. By contrast, tissue sections isolated from treated Cr2-/- mice were similar to that of sham controls. Thus, despite normal circulating levels of IgM, the Cr2-deficient mice were protected from injury. These results not only confirm the importance of B-1 cells as a source of pathogenic antibody but suggest that the complement system is somehow involved in formation or maintenance of the repertoire of natural antibody. For example, complement may be involved in positive selection of B-1 cells.

Example 5: Identification of Pathogenic IgMs

This Example describes the generation of a specific hybridoma clone from normal B-1 cells and the identification of one clone that produces a pathogenic IgM. The pathogenic IgM was shown to restore injury in vivo to antibody deficient mice.

Studies in mice bearing a deficiency in complement receptors CD21/CD35, revealed that the mice were missing the pathogenic antibody. This finding was unexpected because they have a normal level of IgM in their blood. These findings led to the hypothesis that a special population of B cells termed B-1 cells are responsible for secreting the pathogenic IgM. For example, engraftment of the receptor deficient mice (Cr2-/-) with B-1 cells from normal mice restored injury, confirming the importance of B-I cells. To identify the specific antibody or antibodies responsible for injury, a panel of hybridoma clones were constructed from an enriched pool of peritoneal B-1 cells harvested from normal mice. The general approach for preparing hybridomas from enriched fraction of peritoneal cells includes harvesting peritoneal cells from mice treated 7 days earlier with IL-10 and subsequently enriched for CD23 negative B cells by negative selection with magnetic beads. Enriched B cells are analyzed by FACS following staining with IgM, Mac-1 and CD23 specific Mab. The enriched population is further activated by culturing with LPS for 24 hours. Activated cells are hybridized with fusion partner myeloma cells in the presence of PEG and grown in HAT-selective medium. Hybridomas are screened for IgM secreting clones by ELISA , and positive wells are expanded for purification of IgM.

Twenty-two IgM-secreting hybridoma clones were analyzed by pooling an equal amount of IgM product from each of the clones. Treatment of antibody-deficient mice with the pooled IgM restored injury similar to that seen with pooled IgM from serum. This finding confirmed that the pathogenic IgM was among the twenty-two hybridomas produced. By dividing the pools into two fractions, i.e., 1-11 and 12-22, and treatment mice with the two fractions, the pathogenic antibody was found to fractionate with the pool that included clone # 22. Finally, mice were reconstituted with either clone 17 or 22. Clone 22 restored injury whereas the other clones did not (see Figure 4).

Example 6: Complement involvement in B-1 cell selection

Two different models have been proposed to explain the development of B-1 cells. The lineage hypothesis proposes that B-1 cells develop in early fetal life as a distinct population (Kantor et al. (1993) *supra).* Alternatively, B-1 cells develop from the same progenitors as conventional B cells but depending on their environment, i.e., encounter with antigen, they develop into B-1 or retain the B-2 cell phenotype (Wortis, H.H. (1992) Int. Rev. Immunol. 8, 235; Clarke, J. (1998) Exp. Med. 187, 1325-1334). Irrespective of their origin, it is known that B-1 cells are not replenished from adult bone marrow at the same

frequency as B-2 cells and that their phenotype is more similar to that of early fetal liver B cells or neonatal bone marrow (BM) cells. Consistent with an early origin, their repertoire tends to be biased towards expression of more proximal V_H genes and N-nucleotide addition is limited (Gu et al. (1990) *EMBO J 9,* 2133; Feeney, J. (1990) *Exp. Med. 172,* 1377). It seems reasonable that given the reduced replenishment by adult BM stem cells, B-1 cells are self-renewed and that antigen stimulation might be important in their renewal, expansion or even initial selection (Hayakawa et al., (1986) *Eur. J. Immunol.* 16, 1313). Indeed inherent to the conventional model, B-1 cells must be antigen selected.

Evidence in support of a B-cell receptor (BCR) signaling requirement for positive selection of B-1 cells comes from mice bearing mutations that alter BCR signaling. For example, impairment of BCR signaling through CD 19, vav, or Btk dramatically affects development of B-1 cells. By contrast, loss of negative selection such as in CD22- or SHIP-1 deficient mice can lead to an increase in B-1 cell frequency (O'Keefe et al. (1996) *Science 274,* 798-801; Shultz et al. (1993) *Cell* 73, 1445). Recent, elegant studies with mice bearing two distinct Ig transgenes, V_H12 (B-1 cell phenotype) or V_HB1-8 (B-2 cell phenotype) support the view that B-1 cells are positively selected by self-antigens. For example, B cells expressing V_H12 either alone or together with B1-8 developed a B-1 cell phenotype. Whereas, few if any B cells were identified that expressed the B1-8 transgene only. Thus, these results suggested that encounter of transgenic B cells with self-PtC resulted in expansion of those expressing V_H 12. Selection of B-1 cells was recently reported by Hardy et al. (1994) *Immunol. Rev. 137,* 91). In their model, B cells expressing an immunoglobulin transgene specific for Thy 1.1 were selected and expanded in mice expressing the cognate antigen. By contrast, transgene + B-1 cells were not found in mice that expressed the alternative allotype Thy 1.2.

Where does complement fit into B-1 cell development? The overall reduction in B-1a cell frequency and the more specific loss of B-1 cells expressing IgM involved in I/R injury suggests a role for CD21/CD35 in either positive selection or maintenance of B-1a cells. One possible role for complement is that it enhances BCR signaling on encounter with cognate antigen. Biochemical studies and analysis of CD21/CD35 deficient mice demonstrate the importance of co-receptor signaling in activation and survival of conventional B cells (Carroll, M.C., (1998) *Ann. Rev. Immunol.* 16, 545-568; Fearon et al. (1995) *Annu. Rev. Immunol.* 13, 127-149). It is very likely that B-1 cells likewise utilize co-receptor signaling to enhance the BCR signal. For example, bacteria express typical B-1

cell antigens such as phosphoryl choline and it is not unreasonable that coating of bacteria with complement ligand C3d would enhance crosslinking of the co-receptor with the BCR and enhance overall signaling. Thus, antigens expressed at lower concentrations might require complement enhancement in order for the cognate B-cell to recognize it and expand or be positively selected. Another role for complement receptors is in localizing antigen on follicular dendritic cells (FDC) within the lymphoid compartment. However, since the major population of B-1 cells occupy the peritoneal tissues it is not clear if they would encounter FDC within lymphoid structures. The actual site or sites in which B-1 cells undergo positive selection are not known. It is possible that they must encounter cognate antigen in early fetal development or in neonatal BM. If this is the case, it might be expected that complement receptors on stromal cells within these compartments bind antigen for presentation to B cells. It is possible that complement receptors could participate in both stages of development. First, they might enhance antigens signaling in initial positive selection. Secondly, as selected B-1 cells are replenished at peripheral sites, complement receptors might again be involved in enhancement of BCR signaling.

Figure 5 is a schematic diagram of the proposed role for complement and complement receptors in positive selection of peritoneal B-1 lymphocytes. The interaction of complement-ligand coated antigens (self- and non-self) results in co-ligation of the CD21/CD19 co-receptor and BCR on the cell surface leading to enhanced signaling and positive selection.

Example 7: Materials and Methods for Examples 8-11

Phage display peptide library and peptide synthesis

A 12-mer M-13 phage display library (New England Biolab, MA) was screened by 4 rounds with MBL-beads coated with IgM^{CM-22} and 2 rounds with IgM^{CM-75} according to the manufacturer's recommendation. Phage clones were selected from the enriched pool and the nucleotide sequence of the relevant phage gene determined for at least ten clones. Selected peptides were synthesized with purity>95% in Harvard Proteomic Core or New England Peptide, Inc. (Gardner, MA).

Binding assays

ELISA was performed as described earlier (Zhang et al. (2004) *PNAS USA* 101:3886-91). Briefly, IgM binding to phage or phage-specific peptides was determined by

coating a 96-well plate with saturating amounts of antigen. Subsequent to blocking, IgM was added (1 or 10 μg/ml) for 2 hr at 37°C. Plates were washed and then developed with alkaline phosphatase-labeled goat anti-mouse IgM (Sigma, MO). Binding of IgM to NMHC-II was determined by culturing 96-well plates previously coated with specific rabbit antibody (NMHC-II A & B; Covance Research Products; NMHC-II C a gift from Dr. Adelstein, NHLBI, NIH, Bethesda, MD) or pan-myosin Hc (Sigma, MO) with intestinal lysates prepared from IgM^{CM-22} reconstituted RAG-$1^{-/-}$ mice either sham treated or treated for ischemia as described (Zhang et al. (2004) *PNAS USA* 101:3886-91). Lysates were prepared as described for immune precipitation (see below). Alkaline-phosphatase labeled goat anti-mouse IgM (Sigma, MO) was then used to detect bound IgM.

Intestinal RI model

Surgical protocol for RI was performed as previously described (Zhang et al. (2004) *PNAS USA* 101:3886-91). Briefly, a laparotomy is performed, and a microclip (125g pressure, Roboz, MD) was applied to the superior mesenteric artery and bilateral circulation limited with silk sutures flanking a 20 cm segment of the jejunum. After 40 minutes of ischemia, the microclip was removed, and reperfusion of the mesenteric vasculature was confirmed by the return of pulsation to the vascular arcade and a change to pink color. The incision was closed, and all animals kept warm for 3 hours. Reconstituted RAG-$1^{-/-}$ animals received either IgM mixed with peptide or saline in 0.2 ml volume intravenously 30 min before the initial laparotomy. WT animals were treated with saline or peptide i.v. 5 minutes prior to reperfusion. At the end of reperfusion, the ischemic segment of the jejunum was harvested and the central 4 cm was cut for pathological analysis.

Histopathology and immuno-histochemistry analysis

Cryostat sections of intestinal tissues were stained by hematoxylin and eosin (H&E) and examined by light microscopy for mucosal damage. Pathology score was assessed based on procedure by Chiu (Chiu et al, *Arch Surg* 101: 484-488, 1970; Chiu, et al, *Arch Surg* 101: 478-483, 1970) that included direct inspection of all microvilli over a 4 cm stretch of jejuneum as described. Zhang et al. (2004) *PNAS USA* 101:3886-91. For immuno-fluorescence, cryosections fixed with 4% (w/v) paraformaldehyde were incubated for varying periods with either biotin-labeled anti-mouse IgM (Becton Dickinson, CA) followed by 1 hour with streptavidin-Alexa-568 (1:500 dilution, Molecular Probes, OR). C4 deposition was detected by staining with FITC-labeled rabbit anti-huC4c (DAKO, CO),

followed by anti-rabbit-Alexa 488 (Molecular Probes, OR). The specificity of anti-C4c staining was confirmed by staining serial sections with biotin-labeled anti-mouse C4 for 1 hour followed by streptavidin-FITC (Becton Dickinson, CA). C3 deposition was detected by treating with FITC-labeled anti-C3 (DAKO, CO). Sections were mounted in Anti-fade Mounting Medium with DAPI (Molecular Probes, OR).

SPR analysis of peptide binding to antibody

An IgM (IgM^{CM-22} or IgM^{CM-31}) antibody was immobilized by amine coupling in a BiaCore SPR CM5™ chip flowcell at a density of 33,400 response units (RU) ~33 ng/mm^2 as described. Vorup-Jensen et al, *PNAS USA* 100: 1873-1878, 2003. Briefly, a reference flow cell was prepared by coupling of ethanolamine-HCl. Peptides, diluted in PBS running buffer, were flowed separately over the IgM-coupled surface and the reference at a rate of 10 µl/min at 25 °C and with the data collection rate at 10 Hz. The injection phase had a duration of 240 s (end of injection phases are marked by arrow heads in Figures 9A, B and D). Binding isotherms were derived by subtracting the response in the reference cell from the response of the IgM-coupled surface. Following each run, the surface was regenerated by injecting 40 µl 0.05% (v/v) polyoxyethylenesorbitan monolaureate/PBS.

Immune precipitation

Frozen tissues were homogenized in a lysis buffer containing detergent and a cocktail of enzyme inhibitors. A sample of lysate is analyzed for total protein content (Bio-Rad kit) to insure similar levels of protein for analysis. Lysates are mixed with sepharose beads coated with rat anti-mouse IgM for 1 hr at 4°C. Subsequently, beads were pelleted gently, washed in lysis buffer and then boiled in SDS-sample buffer under reducing conditions to elute bound complexes. Samples were fractionated on 6% (w/v) polyacrylamide SDS gels and subsequently fixed and then stained with either coomassie blue or silver stain to identify protein bands.

Protein identification by tandem mass spectrometry

Individual Coomassie Blue-stained bands were excised from SDS-gels, destained, and subjected to enzyme digestion as described previously. Borodovsky et al, *Chem Biol* 9: 1149-1159, 2002. The peptides were separated using a nanoflow liquid coupled chromatography system (Waters Cap LC) and amino acid sequences determined by tandem mass spectrometer (Q-TOF micro, Waters, MA). MS/MS data were processed and

subjected to database searches using Mascot (Matrixscience) against Swissprot, TREMBL/New or the NCBY non-redundant database.

Example 8: Identification of asparagine-rich peptides that bind natural IgM antibody

We previously identified a hybridoma clone of a natural IgM antibody (IgM^{CM-22}) that binds ischemic tissue in the intestinal RI model, which support our hypothesis that ischemic tissue was altered relative to normal tissue and that neo-epitopes expressed during ischemia were targets for an innate response to self. To characterize the ligand bound by pathogenic IgM^{CM-22}, a M-13 phage-display library of random 12-mer amino acid sequences was screened using beads coated with the specific IgM.

After four rounds of specific screening and two rounds with a control IgM (clone IgM^{CM-75}), ten phage clones were isolated and the nucleotide sequence of the relevant M-13 gene sequenced. Notably, all ten clones contain sequences rich in asparagine. Five of the clones were selected for a relative binding assay with IgM^{CM-22} and one of these clones, P8, which bound with the highest efficiency was selected for further study (Table 4 and Figure 6A).

Table 4: Phage displayed peptides bind to IgM^{CM-22}

Phage Clone	Sequence	SEQ ID NO:
P1	YNNNNGNYTYRN	16
P2	ANTRNGATNNNM	18
P3	CDSSCDSVGNCN	20
P4	WNNNGRNACNAN	22
P5	HNSTSNGCNDNV	24
P6	NSNSRYNSNSNN	26
P7	KRNNHNNHNRSN	28
P8	NGNNVNGNRNNN	30
P9	NVANHNNSNHGN	32
P10	SYNNNNHVSNRN	34
Asparagine-rich Consensus	xNNNxNNxNNNN	14

A 12- amino acid peptide (P8) was synthesized based on the phage sequence and assayed for inhibition of phage P8 binding to IgM^{CM-22} (Figure 6B). Titration of increasing amounts of P8 peptide yielded 50% inhibition at an estimated concentration of 10 μmole. This assay indicates a reasonable overall avidity of binding based on multiple binding sites expressed on the phage surface. This result suggested that IgM^{CM-22} binding to phage P8 was specific for the peptide region and that the synthetic peptide could be used as a mimotope for the actual antigen. To further characterize binding of P8 peptide to IgM^{CM-22}, ELISA plates were coated with the peptide and tested with IgM^{CM-22} or control IgM^{CM-75} for binding (Figure 6C). At the lower concentration of 1 μg/ml, neither IgM bound above background. However, at 10 μg/ml, significantly more IgM^{CM-22} bound than IgM^{CM-75}. Together, the three results suggest that peptide P8 binds specifically to IgM^{CM-22} and can be used for identification of the actual antigen.

Example 9: Asparagine-rich peptide P8 blocks intestinal RI

Previous studies had demonstrated that intestinal RI in RAG-$1^{-/-}$ mice was IgM-dependent and that IgM^{CM-22} alone was sufficient to restore injury. As expected, reconstitution of RAG-$1^{-/-}$ mice with IgM^{CM-22} but not saline prior to reperfusion resulted in RI (Figure 7A(i) and Figure 7B). By contrast, mixing of IgM^{CM-22} with P8 prior to injection in ischemic mice significantly blocked apparent injury (mean pathology score 6±3 versus 31±13; $p<0.001$) (Figure 7Aii and Figure 7B). Previous titration of peptide with IgM^{CM-22} suggested an optimal concentration of 10 μM of P8 was sufficient to block 50-100 μg of IgM^{CM-22} (0.1-0.2 μM).

Immunohistological analyses of serial sections of reperfused intestinal tissue (jejuneum) following RI identified co-localization of IgM and complement C4 and C3 within the microvilli in RAG-$1^{-/-}$ mice reconstituted with IgM^{CM-22} (Figure 7Ci-iv). By contrast, sections prepared from mice receiving P8 showed no evidence of IgM or complement binding (Figure 7Cv-viii). No binding of IgM or complement was observed in IgM^{CM-22} reconstituted sham controls, nor RAG-$1^{-/-}$ mice reconstituted with control IgM^{CM-31} or RAG-$1^{-/-}$ mice reconstituted with saline only (Zhang et al. (2004) *PNAS USA* 101:3886-91). Thus, P8 blocks the binding of IgM^{CM-22} and the induction of injury *in vivo*.

The identification of a single natural IgM antibody that could initiate RI in RAG-$1^{-/-}$ mice led to the general question of the number of possible neo-epitopes expressed on

ischemic tissues and the corresponding number of pathogenic clones of IgM in the repertoire of wild type (WT) mice. It might be predicted that the number of antibodies is limited based on the current understanding that the repertoire of natural IgMs is relatively small. Herzenberg et al, *Immunol Today* 14: 79-83, discussion 88-90, 1993; Arnold et al, *J Exp Med* 179: 1585-1595, 1994.. Moreover, ligands of natural IgM antibodies are considered highly conserved structures and also are probably limited in number. To test if P8 represented a mimotope for a major self-antigen, WT mice were pretreated with P8 (approximately 10 μM) five minutes prior to reperfusion in the intestinal model. Analysis of jejuneum tissues of mice treated with saline or a control peptide prior to reperfusion identified significant injury to the microvilli as expected (Figure 7Aiii). By contrast, pretreatment of WT mice with P8 five minutes prior to reperfusion blocked apparent injury (mean pathology score 5±3 versus 24±16 and 23±19; $p<0.005$ and 0.027, respectively) (Figure 7A(iv) and Figure 7B). As expected, IgM, C4 and C3 co-localized within microvilli of RI treated WT mice (Figure 7Cix-xii). By contrast, no apparent deposits of IgM or complement were observed in reperfused tissues of mice administered P8 (Figure 7Cxiii-xvi). These results suggest that the number of key epitopes required to initiate RI is limited as a single peptide blocks injury and deposition of IgM and complement.

Example 10: Immunoprecipitation of self-peptides with IgM^{CM-22}

Using the amino acid sequence of P8, a homology search of the genomic database revealed no exact matches. Therefore, an immune-precipitation approach was used to identify the ischemia antigen/antigens in RAG-$1^{-/-}$ mice reconstituted with IgM^{CM-22}.

RAG-$1^{-/-}$ mice were reconstituted with an optimal amount of IgM^{CM-22}, treated for intestinal ischemia and reperfused for varying lengths of time, i.e., 0 minutes or 15 minutes before harvesting of tissues. Immune complexes of IgM-antigen were isolated from lysates of jejuneum at the varying time points and fractionated by SDS-PAGE under reducing conditions. Analysis of the stained gels indicated common bands at lower molecular weight for all time points (Figure 8A). However, at 15 minutes, a band at high molecular weight (> 200 kD) was identified (Figure 8A).

Protein bands were excised from stained gels, enzymatically digested and peptides analyzed by Tandem Mass Spec as described. Kocks et al, *Mol Cell Proteomics* 2: 1188-1197, 2003. Analysis of eluted peptides indicated that the common bands at approximately

25, 50 and 75 kDa represented immunoglobulin light chain (Lc), and IgG heavy chain (Hc) and IgM Hc, respectively. Analysis of the high molecular weight band yielded peptide sequences homologous to non-muscle myosin heavy chain (NMHC) type II isoforms A and C (Table 5).

Table 5: Mass Spectrometry Results

Matched proteins	Mass Spectroscopy sequenced peptides
Mouse non muscle myosin heavy chain II-A (gi/20137006; GenBank™ Accession NO: NP_071855) *total score=130; peptides matched=6*	VVFQEFR (MS-1; SEQ ID NO: 39) CNGVLEGIR (MS-2; SEQ ID NO: 40) KFDQLLAEEK (MS-3; SEQ ID NO: 41) KFDQLLAEEK EQADFAIEALAK (MS-4; SEQ ID NO: 42) QLLQANPILEAFGNAK (MS-5; SEQ ID NO: 43)
Mouse non muscle myosin heavy chain II-C (gi/33638127; GenBank™ Accession NO: AAQ24173) *total score=133; peptides matched=7*	CNGVLEGIR VKPLLQVTR (MS-6; SEQ ID NO: 44) KFDQLLAEEK KFDQLLAEEK EQADFALEALAK LAQAEEQLEQESR (MS-7; SEQ ID NO: 45) QLLQANPILEAFGNAK (MS-8; SEQ ID NO: 46)

*Score is -10XLog (P), where P is the probability that the observed match is a random event. Individual ion scores >53 indicate identity or extensive homology ($p<0.05$).

In similar experiments using lysates prepared from WT mice treated for 3 hours in intestinal RI, a similar size band at 200 kD was also observed and sequence analysis identified NMHC-A and C peptides.

Three forms of type II NMHC have been identified (A, B and C) in the mouse and human genome. Golomb et al, J Biol Chem 279: 2800-2808, 2004; Kelley et al, J Cell Biol 134: 675-687, 1996. All eukaryotic cells express type II NMHC but the distribution of the three isoforms varies. NMHC-II A and B are approximately 85% homologous; whereas NMHC-II C is approximately 65% similar. Golomb et al, J Biol Chem 279: 2800-2808, 2004. The three isotypes are highly conserved among mice and humans.

To confirm the binding of IgM^{CM-22} to type II NMHC, an ELISA approach was used. Plates were coated with antibody specific for each of the three forms of NMHC or with a pan-myosin antibody to capture the relevant antigen from lysates prepared from jejuneum of RAG-$1^{-/-}$ mice. Subsequently, IgM^{CM-22} or IgM^{CM-31} were added and then developed with a labeled anti-mouse IgM antibody. Above background binding of IgM^{CM-22} but not IgM^{CM-31} to all three of the isoforms of NMHC-II was observed (Figure 8B). The combined sequence analysis and ELISA results show that IgM^{CM-22} recognizes a conserved region of the type II NMHC.

To determine whether myosin is exposed to circulating antibody following ischemia, RAG-$1^{-/-}$ mice were reconstitute with a purified IgG fraction of rabbit anti-pan myosin heavy chain. Analysis of tissues of sham treated RAG-$1^{-/-}$ following reconstitution with the rabbit IgG mice showed no evidence of injury or deposition of IgG. By contrast, ischemic RAG-$1^{-/-}$ mice reconstituted with the pan-myosin IgG prior to reperfusion developed significant RI compared to saline controls (33±11 versus 11±8, $p<0.028$) (Figure 8C). Accordingly, myosin is exposed to antibody in circulation following ischemia.

Comparison of the sequences of the three NMHC-II isoforms with the P8 peptide sequence identified one region of apparent homology (Table 6). All three isoforms include a motif of NxxxxNxNx that has similarity with the P8 sequence. A 12- amino acid self-peptide (N2) sequence (NMHC-II C isoform) was prepared for further study.

Table 6: Conserved homologous sequence in NMHC-II A-C

Phage Clone	Sequence
P8	NGNNVNGNRNNN (SEQ ID NO:30)
Consensus	xNNNx(N/D)NxN(N/D)N(N/V) (SEQ ID NO:14)

NMHC-II	Sequence
Mouse-IIA (542-556)	LMKNMDPLNDI (SEQ ID NO:36)
Human-IIA (585-596)	LMKNMDPLNDI
Mouse-IIB (592-603)	LMKNMDPLNDNV (N2; SEQ ID NO:38)
Human-IIB (592-603)	LMKNMDPLNDNV

Mouse-IIC (607-619)	LMKNMDPLNDNV (N2; SEQ ID NO:38)
Human-IIC (611-622)	LMKNMDPLNDNV

To test that this region bound IgM^{CM-22} surface, plasmon resonance analysis was used (Figure 9). N2 peptide was injected over a surface coupled with IgM^{CM-22} (Figure 9A) and generated a robust response, which corresponded to a K_D of 123±61 μM (mean±SD, n=2) as calculated from the steady-state response levels (Figure 9C). In contrast, no binding was observed when a control peptide was injected over the specific IgM-coupled surface (Figure 9B) or when the N2 peptide was injected over a surface coupled with the IgM^{CM-31} control (Figure 9D).

Example 11: Self-peptide N2 blocks intestinal RI

To test the functional binding of N2 with pathogenic IgM, approximately 100 nmoles of the peptide (or saline control) was mixed with IgM^{CM-22} prior to reconstitution of RAG-$1^{-/-}$ mice and treatment in the RI model. Analysis of histology of tissue sections prepared from the reperfused jejuneum of IgM^{CM-22}- and saline-treated mice identified injury and deposition of IgM and complement as expected (Figure 5Ai and 5B). By contrast, mixing the N2 peptide with IgM^{CM-22} prior to reperfusion was protective from injury (mean pathology score 13±8 versus 31±10; $p<0.049$) (Figure 10Aii and 10B). In addition, no deposition of IgM and complement was observed in reperfused jejuneum when IgM^{CM-22} was mixed with the N2 peptide prior to injection in RAG-$1_{-/-}$ mice (Figure 10Ci-viii). Thus, as observed with the synthetic peptide P8, the self-peptide N2 blocked functional binding of IgM^{CM-22} *in vivo*.

To test if self-peptide N2 represents the major self-epitope in intestinal RI, WT mice were treated with approximately 40 μM of the synthetic peptide P8 prior to reperfusion in the intestinal model. Histological analysis of tissue sections of saline treated WT mice identified injury and deposition of IgM and complement as expected (Figure 10Aiii and 10Cix-xii). By contrast, treatment of WT mice with self-peptide N2 blocked both injury (mean pathology score 8±5 versus 22±17) and deposition of IgM and complement (Figure 10Aiv; Figure 10B; Figure 10Cxiii-xvi). These results suggest that a conserved region within type II NMHC proteins represents the major epitope for binding of natural IgM following ischemia in the intestinal model.

Incorporation by Reference

All publications, patents, and patent applications mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Also incorporated by reference in their entirety are any polynucleotide and polypeptide sequence which reference an accession number correlating to an entry in a public database, such as those maintained by The Institute for Genomic Research (TIGR) on the world wide web with the extension tigr.org and or the National Center for Biotechnology Information (NCBI) on the world wide web with the extension ncbi.nlm.nih.gov.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims:

1. An isolated nucleic acid encoding a peptide comprising SEQ ID NO: 14.

2. The isolated nucleic acid of claim 1, wherein the nucleic acid is SEQ ID NO: 13.

3. An isolated nucleic acid encoding a peptide comprising SEQ ID NOs: 16, 18, 20, 22, 24, 26, 28, 30, or 32.

4. The isolated nucleic acid of claim 3, wherein the nucleic acid is SEQ ID NOs: 15, 17, 19, 21, 23, 25, 27, 29, or 31.

5. The isolated nucleic acid of claim of claim 3, wherein the peptide comprises SEQ ID NO: 30.

6. The isolated nucleic acid of claim 4, wherein the nucleic acid is SEQ ID NO: 29.

7. An isolated nucleic acid encoding a peptide comprising a natural IgM antibody-binding portion of SEQ ID NOs: 48, 50, 52, 54, 56, 58 or 60.

8. An isolated nucleic acid of encoding a peptide comprising SEQ ID NO: 36 or 38.

9. The isolated nucleic acid of claim 8, wherein the peptide comprises SEQ ID NO: 38.

10. The isolated nucleic acid of claims 1, 3, 7, or 8 operably linked to a promoter.

11. A vector comprising the nucleic acid of claim 10.

12. A host cell comprising the vector of claim 11.

13. A composition comprising a peptide having the amino acid sequence of SEQ ID NO: 14.

14. A composition comprising a peptide having the amino acid sequence of SEQ ID NOs: 16, 18, 20, 22, 24, 26, 28, 30, or 32.

15. The composition of claim 14, wherein the peptide comprises SEQ ID NO: 30.

16. A composition comprising a peptide having the amino acid sequence of SEQ ID NOs: 36 or 38.

17. The composition of claim 16, wherein the peptide comprises SEQ ID NO: 38.

18. The composition of claims 13, 14, or 16, wherein the peptide is pegylated.

19. The composition of claims 13, 14, or 16, wherein the peptide is labeled with a detectable label.

20. A method of treating an inflammatory disease or disorder comprising administering to a subject the composition of claims 13, 14, or 16.

21. The method of claim 20, wherein the inflammatory disease or disorder is reperfusion injury.

22. The method of claim 20, wherein the subject is a mammal.

23. The method of claim 20, wherein the mammal is a human.

24. An isolated nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule comprising a nucleotide sequence which is at least 96% identical to the nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 5;

b) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 5; or

c) a nucleic acid molecule which hybridizes to the nucleotide sequence of SEQ ID NO: 1 under stringent conditions.

25. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO: 6.

26. A vector comprising the nucleic acid of claim 24.

27. A cell comprising the nucleic acid of claim 26.

28. An isolated nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule comprising a nucleotide sequence which is at least 96% identical to the nucleotide sequence of SEQ ID NO: 7, SEQ ID NO: 9, or SEQ ID NO: 11;

b) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 7, SEQ ID NO: 9, or SEQ ID NO: 11; or

c) a nucleic acid molecule which hybridizes to the nucleotide sequence of SEQ ID NO: 7 under stringent conditions.

29. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 8, SEQ ID NO: 10, or SEQ ID NO: 12.

30. A vector comprising the nucleic acid of claim 28.

31. A cell comprising the nucleic acid of claim 30.

32. An isolated polypeptide comprising the amino acid sequence SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO: 6.

33. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 8, SEQ ID NO: 10, or SEQ ID NO: 12.

34. An isolated natural immunoglobulin, or antigen binding portion thereof, having one or more of the following properties: (i) is capable of interacting with an ischemia-specific antigen; (ii) is capable of fixing complement; or (iii) is produced by a subpopulation of B cells.

35. The isolated natural immunoglobulin of claim 34, which is an IgM.

36. The isolated natural immunoglobulin of claim 34, wherein the immunoglobulin is produced by B-1 cells.

37. The isolated natural immunoglobulin of claim 34, which is produced by a cell having ATCC deposit number PTA-3507.

38. The isolated natural immunoglobulin of claim 34, wherein the immunoglobulin is a recombinant antibody.

39. An antibody comprising the nucleic acid sequence of claim 24, which binds an ischemia-specific antigen.

40. An antibody comprising the nucleic acid sequence of claim 28, which binds an ischemia-specific antigen.

41. An antibody of claim 39, further comprising the nucleic acid sequence of claim 28.

42. The antibody of claim 41, comprising a nucleic acid sequence which encodes the polypeptide of SEQ ID NO: 2 and a nucleic acid sequence which encodes the polypeptide of SEQ ID NO: 8.

43. A modified natural immunoglobulin having a mutation that alters complement binding or activity.

44. The modified natural immunoglobulin of claim 43, which is capable of interacting with an ischemia-specific antigen.

45. The modified natural immunoglobulin of claim 43, which is an IgM.

46. The modified natural immunoglobulin of claim 43, which is an IgG.

47. A composition comprising a therapeutically effective amount of an antibody of claim 43 and a pharmaceutically acceptable carrier.

FIGURE 1A

```
<----------------------------- FWR1 ------------------------------
CAG GTT CAG CTG CAG CAG TCT GGG GCT GAG CTG GTG AAG CCT GGG GCC TCA GTG AAG ATT TCC

-------------------------------->  _____CDR1_____  <----------------------
TGC AAA GCT TCT GGC TAC GCA TTC AGT AGC TAC TGG ATG AAC TGG GTG AAG CAG AGG CCT GGA

---FWR2----------------------->  _____CDR2_____
AAG GGT CTT GAG TGG ATT GGA CAG ATT TAT CCT GGA GAT GGT GAT ACT AAC TAC AAC GGA

_____  <-------------------------------- FWR3 ----------------------------
AAG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC

-------------------------------------------------->  ____D____  _____
AGC AGC CTG ACC TCT GAG GAC TCT GCG GTC TAT TTC TGT GCA AGA GAA GAT TAC TAC GGT AGT

<---------------------- J ----------------------->
 GAC TGG TAC TTC GAT GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC TCC TCA GGT AAG CTG GCT

                    *
TTT TTC TTT CTG CAC ATT CCA TTC TGA (SEQ ID NO:1)
```

FIGURE 1B

```
----------------FWR1-----------> <CDR1> <----FWR2---->
QVQLQQSGAELVKPGASVKISCKASGYAFS  SYWMN  WVKQRPGKGLEWIG

<----CDR2------->   <-------------FWR3------------->
QIYPGDGDTNYNGKFKG   KATLTADKSSSTAYMQLSSLTSEDSAVYFCAR

<-D--> <-------J------->
EDYYGS DWYFDVWGTGTTVTVSSGKLAFFFLHIPF*(SEQ ID NO:2)
```

FIGURE 2A

```
<----------------------------- FWR1-------------------------------->
ATT GTG ATG ACC CAG TCT GCT GCT TCC TTA GCT GTA TCT CTG GGG CAG AGG GCC ACC ATC TCA TAC

                  CDR1                                  <-------------------------
AGG GCC AGC AAA AGT GTC AGT ACA TCT GGC TAT AGT TAT ATG CAC TGG AAC CAA CAG AAA CCA GGA

FWR2------------------------->      CDR2                <-------------------------
CAG CCA CCC AGA CTC CTC ATC TAT CTT GTA TCC AAC CTA GAA TCT GGG GTC CCT GCC AGG TTC AGT

------------------------------ FWR3-------------------------------------------
GGC AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG GAG GAG GAT GCT GCA ACC

---------->                J                  *
TAT TAC TGT CAG CAC ATT AGG GAG CTT ACA CGT TCG GAG GGG GGA CCA AGC TGG AAA TAA (SEQ ID NO:7)
```

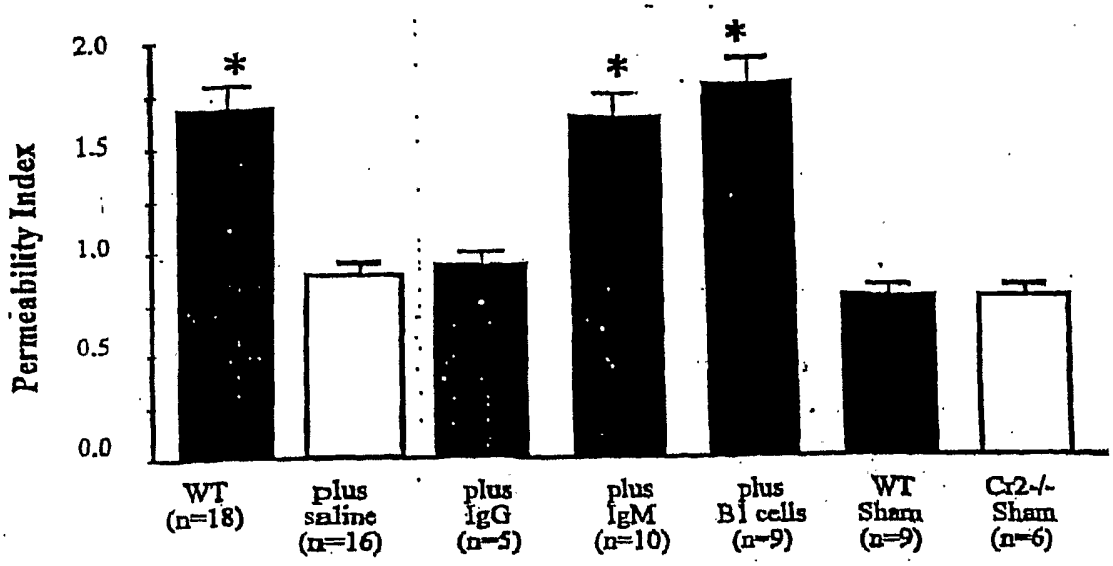
FIGURE 2B

```
--------FWR1--------->   <----CDR1----->   <-----FWR2----->
IVMTQSAASLAVSLGQRATISY   RASKSVSTSGYSYMH   WNQQKPGQPPRLLIY

<-CDR2->   <-----------------FWR3---------->
LVSNLES    GVPARFSGSGSGTDFTLNIHPVEEEDAATYYC QHIRE

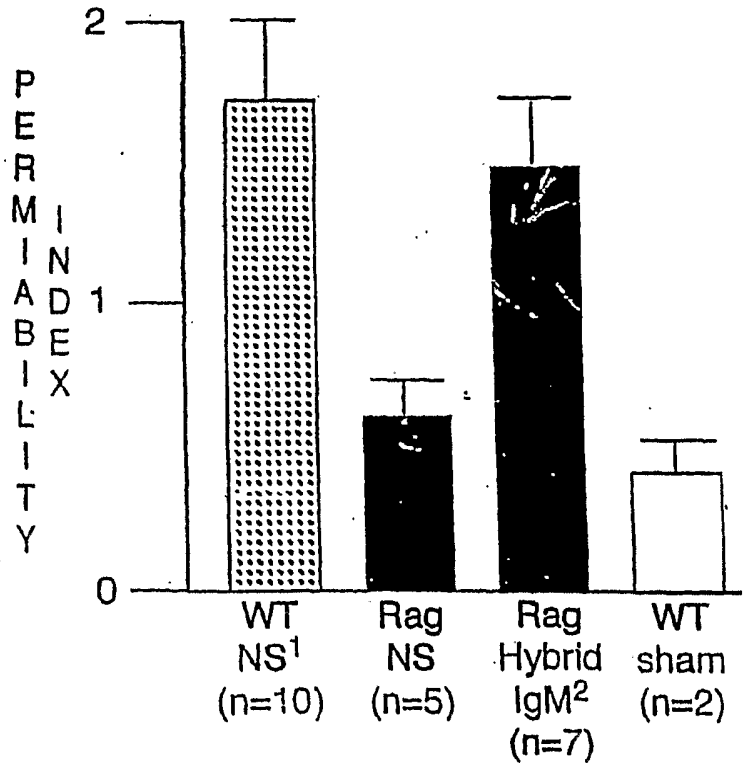
 <----J--->
LTRSEGGPSWK*  (SEQ ID NO:8)
```

FIGURE 3



* $p < 0.00001$

FIGURE 4


PERMIABILITY INDEX
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1
0
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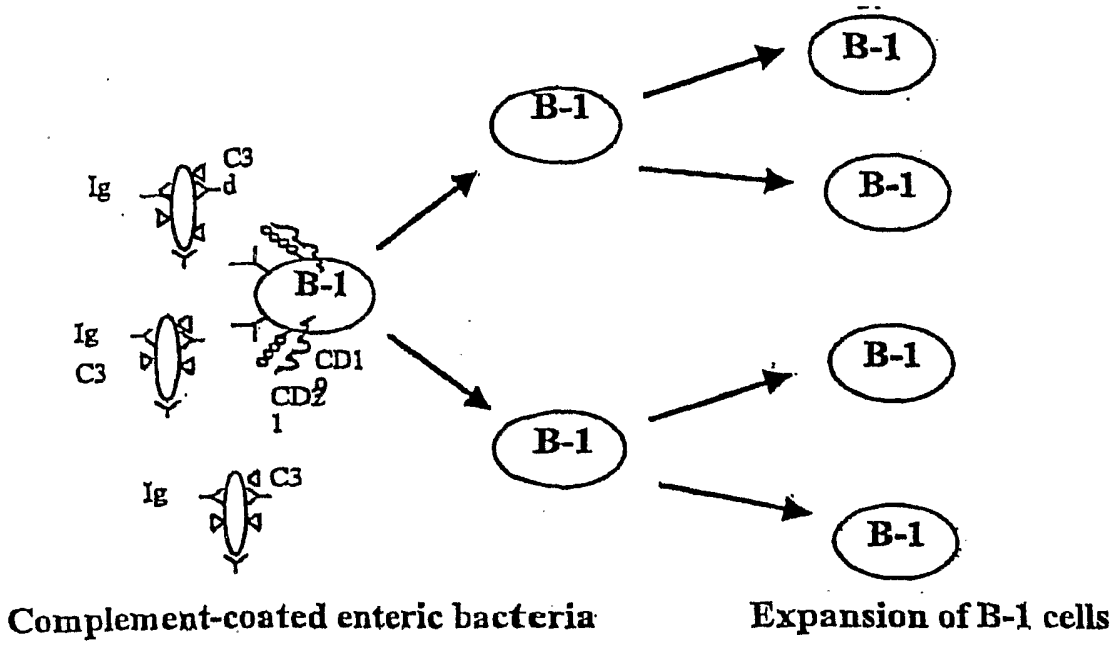
Rag NS (n=5)
Rag Hybrid IgM[2] (n=7)
WT sham (n=2)

FIGURE 5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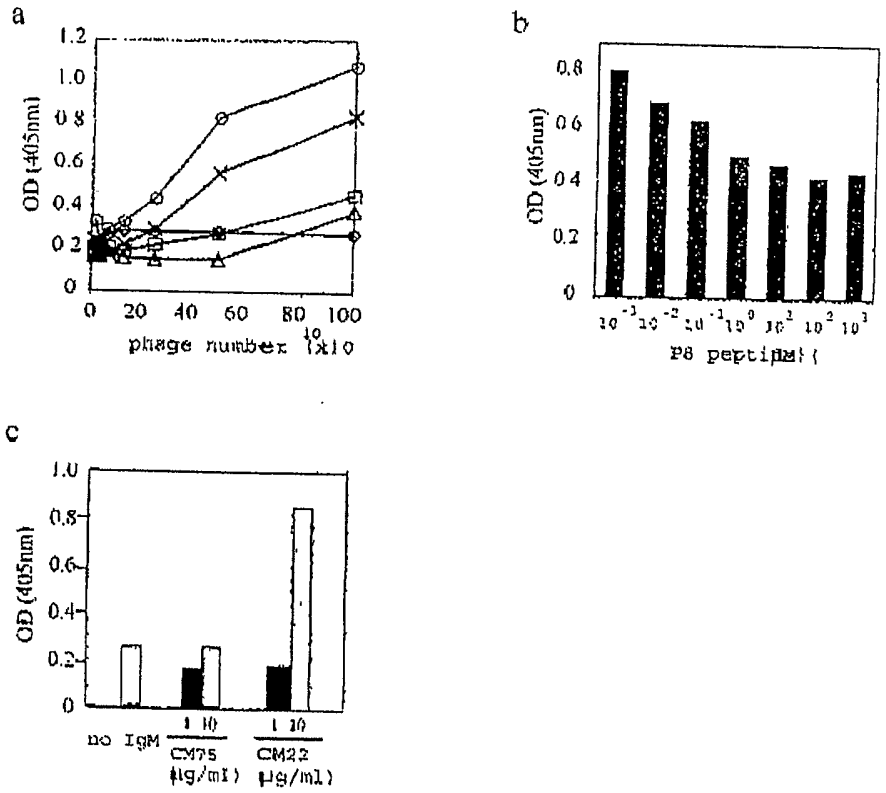
B-1
B-1
B-1
B-1
B-1
Ig
C3
d
Ig
C3
CD1
CD2
1
Ig
C3
Complement-coated enteric bacteria
Expansion of B-1 cells

FIGURE 6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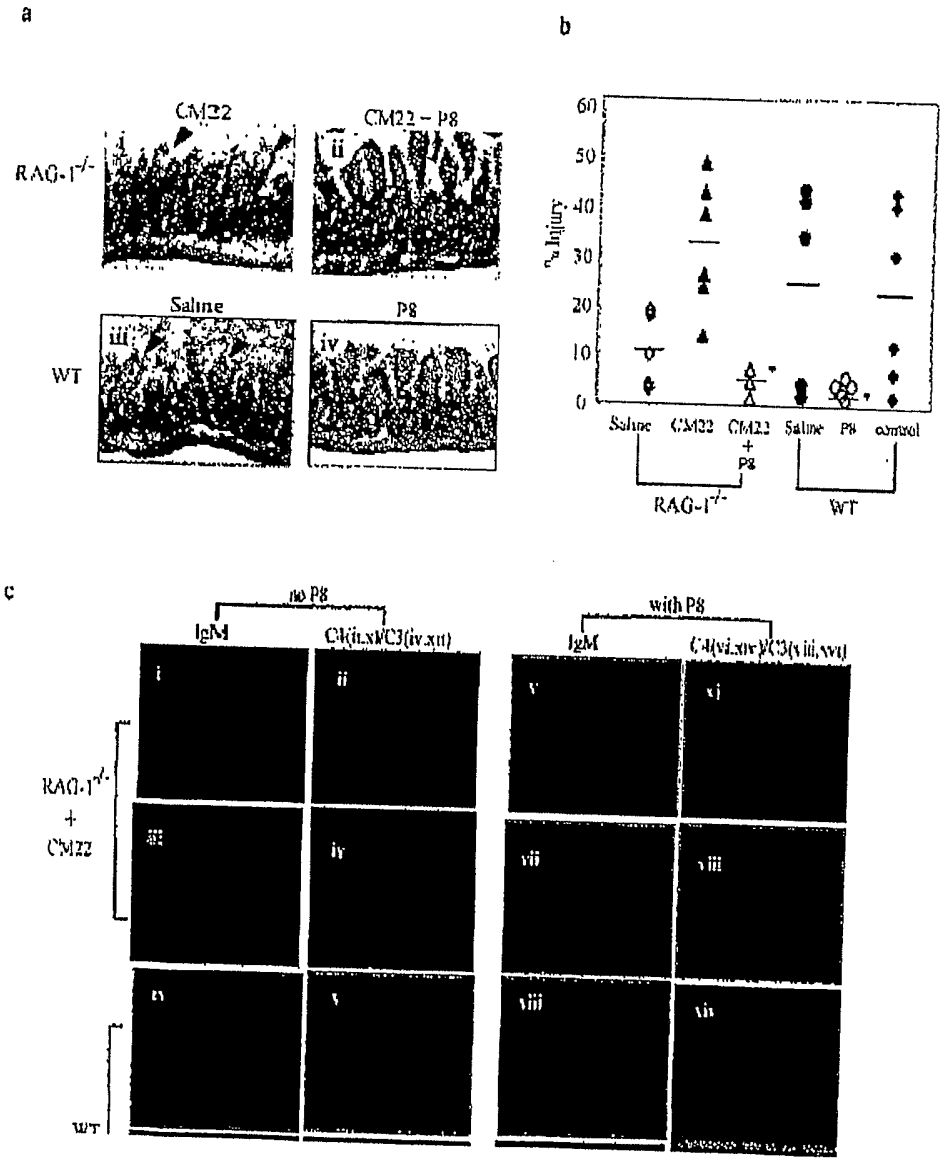
OD (405nm)
phage number (x10^10)
b
OD (405nm)
P8 peptide
c
OD (405nm)
no IgM
CM75 (μg/ml)
CM22 (μg/ml)

FIGURE 7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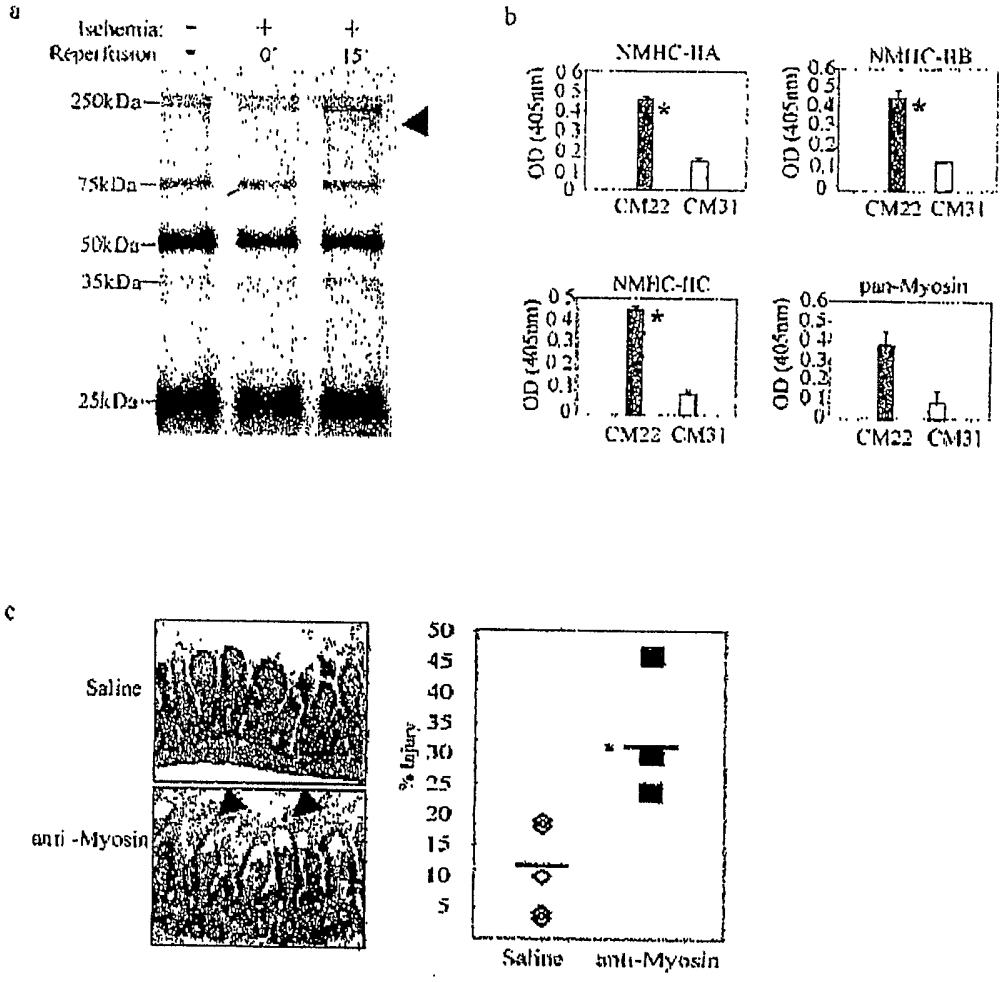
CM22
CM22 – P8
RAG-1-/-
Saline
P8
WT
b
% Injury
60
50
40
30
20
10
0
Saline
CM22
CM22 + P8
Saline
P8
control
RAG-1-/-
WT
c
no P8
with P8
IgM
C4(i,xi)C3(iv,xii)
C4(vi,xiv)C3(viii,xvi)
RAG-1-/- + CM22
WT

FIGURE 8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Ischemia: - + +
Reperfusion - 0' 15'
250kDa
75kDa
50kDa
35kDa
25kDa
b
NMHC-IIA
NMHC-IIB
NMHC-IIC
pan-Myosin
OD (405nm)
CM22 CM31
c
Saline
anti -Myosin
% Injury
Saline
anti-Myosin

FIGURE 9

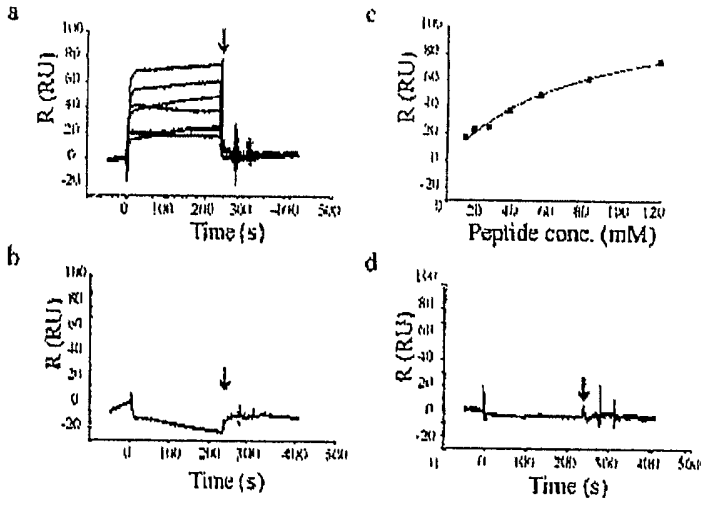

FIGURE 10

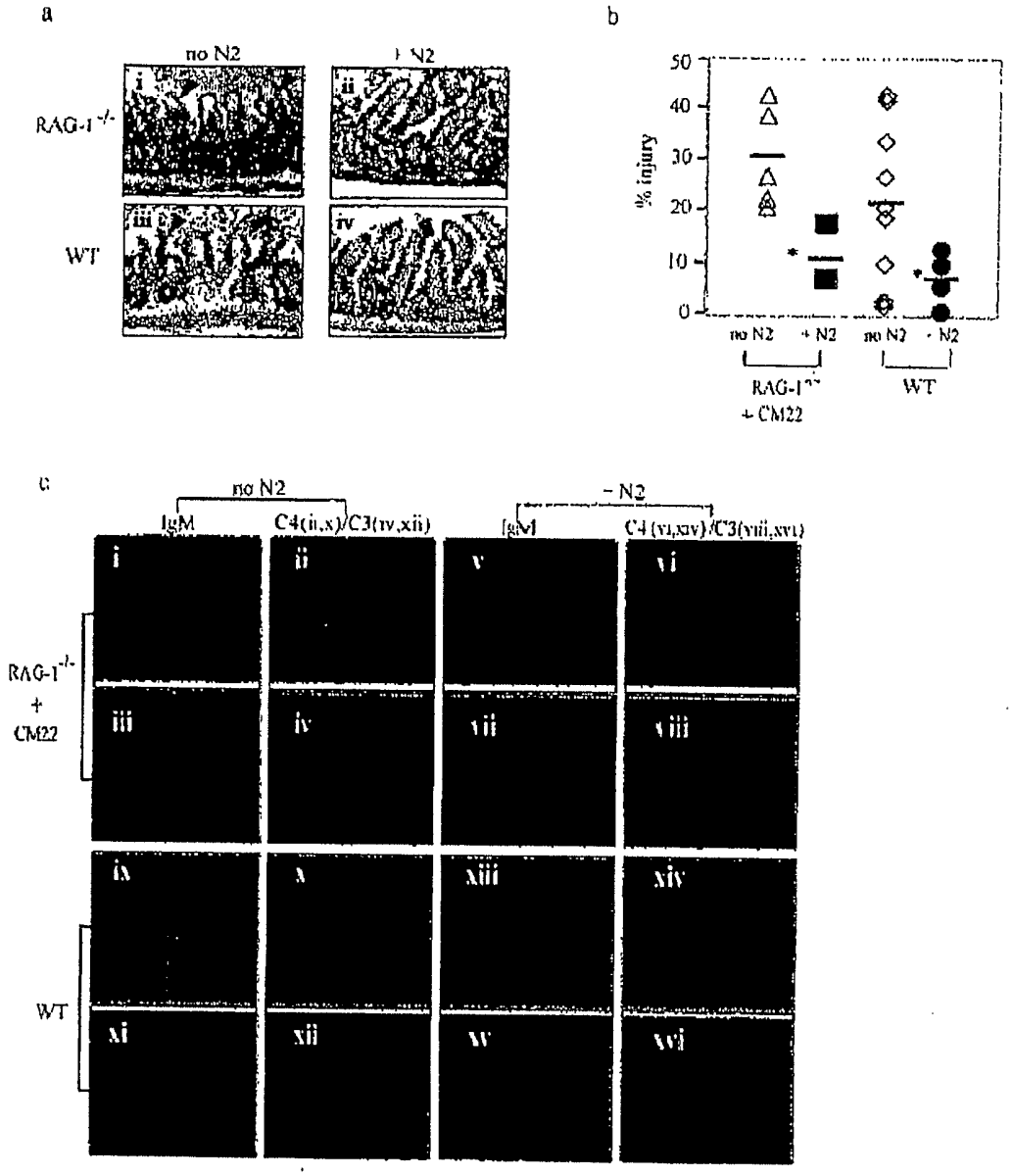
a
no N2
+ N2
RAG-1 -/-
WT
i
ii
iii
iv
b
% injury
50
40
30
20
10
0
no N2
+ N2
no N2
- N2
RAG-1 -/-
+ CM22
WT
c
no N2
- N2
IgM
C4(ii,x)/C3(iv,xii)
IgM
C4 (vi,xiv)/C3(viii,xvi)
RAG-1 -/-
+
CM22
WT
i
ii
v
vi
iii
iv
vii
viii
ix
x
xiii
xiv
xi
xii
xv
xvi

Figure 11

(A)

```
   1 tgggcagggc acggaaggct caagaacctg acctgctgca gcttccagtc tcgcgttcgc
  61 cccaccccgc cgcgccgccc gagcgctcga gaaagtccac tcggaagaac cagcgcctgt
 121 tccccgggca gacccaggtt caggtcctgg ccgcaagtca ccatggctca gcaggctgca
 181 gacaagtacc tctatgtgga taaaaacttc atcaataacc cgctggccca agctgactgg
 241 gctgccaaga agttggtatg ggtgccttcc agcaagaatg gctttgaacc agctagcctc
 301 aaggaggagg tgggagaaga ggccattgta gagctggtag agaatgggaa gaaggtgaag
 361 gtgaacaagg acgacatcca gaagatgaac ccacccaagt tctccaaggt ggaggacatg
 421 gcagagctca cgtgcctcaa cgaagcttcg gtgctgcaca acctcaagga gcgatactac
 481 tcagggctta tctacaccta ttcaggcctg ttctgtgtgg tcatcaaccc ttataagaac
 541 ctgcccatct actcagagga gatcgtggag atgtacaagg gcaagaagag gcacgagatg
 601 ccaccccaca tctacgccat cacagatact gcctaccgga gcatgatgca ggaccgggaa
 661 gatcagtcca tcctgtgcac gggggagtct ggagcaggga agacagagaa caccaagaaa
 721 gtcatccagt acctggcaca tgtggcctcc tcacacaaga gcaagaagga ccagggggag
 781 ttggagcggc agctgctaca ggccaaccct atcctagagg cctttggaaa cgccaagacg
 841 gtgaagaatg acaactcctc tcgattcggt aaattcattc gtatcaactt tgatgtcaat
 901 ggctacattg ttggtgccaa cattgagact tatcttctgg agaaatctcg tgctatccgc
 961 caagccaaag aggagcggac cttccacatc ttctactacc tgctgtctgg ggccggagaa
1021 cacctgaaga ctgatctcct gttggagcca tacaacaaat accgcttcct gtccaacggg
1081 cacgtcacca tccctgggca gcaggacaag gacatgttcc aggagacaat ggaggccatg
1141 agaattatgg gtatcccaga ggatgagcag atgggcttgc tgcgggtcat ctctggggtc
1201 cttcagcttg gcaacattgc cttcaagaag gagcggaaca ctgaccaggc gtccatgccg
1261 gacaacacag ctgctcaaaa ggtgtcccac ctcctgggga tcaatgtgac cgacttcacc
1321 agaggcatcc tcaccccacg catcaaggtg ggcagagact atgtgcagaa ggcgcagact
1381 aaagagcagg ctgactttgc cattgaggcc ttggccaagg ctacctatga gcggatgttc
1441 cgctggctgg tgcttcgcat caacaaagct ctggacaaga ccaagaggca gggcgcctca
1501 tttatcggga tcctggacat cgctggcttt gagatctttg atctgaactc cttcgagcag
1561 ctgtgcatca actacaccaa cgagaagctg cagcagctgt tcaaccacac catgttcatc
1621 ctggagcagg aggagtacca gcgagagggc atcgagtgga acttcatcga cttcggcctg
1681 gacctgcagc cctgcatcga cctcattgag aagccggcgg gtcccccagg catcctggcc
1741 ctgctagatg aggagtgctg gtttcctaag gccactgaca agagcttcgt ggagaaggtg
1801 gtgcaggagc agggcaccca ccccaagttc cagaagccca agcaactgaa ggacaaggct
1861 gatttctgca ttatccacta tgccggcaag gtggactata aagctgacga gtggctgatg
1921 aagaacatgg acccccttgaa cgacaacatc gccacgctgc ttcaccagtc ctcagacaag
1981 tttgtctctg agctgtggaa ggatgtggat cggatcattg gcttggacca agtggctgga
2041 atgtccgaga cagcactacc tggtgccttc aagacccgga agggcatgtt ccgtactgtc
2101 ggacagctgt acaaggagca gctggccaag ctcatggcca cgttgaggaa taccaacccc
2161 aacttcgtgc gctgcatcat tcccaaccat gagaagaagg ccggcaaact ggacccgcac
2221 ttggtgctgg accagctgcg ctgcaatggc gtccttgagg gcatccggat ctgccgccag
2281 ggctttccca acagggtggt cttccaggag ttccggcaga ggtatgagat cctcaccccc
2341 aactccatcc cgaagggctt catggatggc aagcaagcgt gtgtgctcat gatcaaagcc
2401 ttggagcttg acagcaacct gtaccgcatc ggccagagca aagtgttctt ccgggcagga
2461 gtgctagccc acctggagga agagcgggac ctgaagatca ccgatgtcat cattggcttc
2521 caggcctgct gcaggggcta cctggccagg aaggcctttg ccaagaggca gcaacagctg
2581 accgccatga aggtcctaca gaggaactgt gctgcgtacc tcaggctgcg caactggcag
2641 tggtggaggc tcttcaccaa ggtcaagccc ctgttgaact caataagaca tgaggatgag
2701 ctgttagcca aggaggcgga actgacaaag gttcgagaga aacatctggc tgcagagaac
2761 aggctgacag agatggagac gatgcagtct cagctcatgg cagagaagct gcagcttcag
2821 gagcagctgc aggcggagac agagctgtgt gccgaggctg aggagctccg ggcccgtctg
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2941 gaggaggagc gctgccagta cctgcaggcc gagaagaaga agatgcagca gaacatccag
3001 gaacttgagg agcagttgga ggaggaggag agcgcccggc agaagctgca gcttgagaag
3061 gtgaccaccg aggccaagct gaagaaactg gaggaggacc agatcatcat ggaggaccag
3121 aactgcaaac tggccaagga gaagaaactg ctggaagaca gagtagctga attcactacc
```

3181 aacctcatgg aagaggagga gaagtccaag agcctggcca agctcaagaa caagcacgag
3241 gcaatgatca ccgacctgga agagcgcctc cgtagggagg agaagcagag gcaggagttg
3301 gagaagaccc gtcgcaagct ggagggagac tccacagacc tcagtgacca gattgctgag
3361 ctccaggcgc agatagcaga gctcaagatg cagctggcca agaaggagga ggagttgcag
3421 gctgccttgg ccagagtgga agaagaagct gctcagaaga atatggccct gaagaagatc
3481 cgagaactgg aaactcagat ctctgagctc caggaggacc tggagtcgga gcgagcctcc
3541 aggaataaag ccgagaagca gaaacgggat ctgggagagg agctggaggc gctgaagaca
3601 gagctggagg acacgctgga ctccacggct gcccagcagg agctgaggtc gaagcgtgag
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3721 atccaggaga tgaggcagaa gcactcacag gctgtggagg agctggcaga tcagttggag
3781 cagacgaagc gggtaaaagc taccccttgag aaggcgaagc agaccctgga gaatgagcgg
3841 ggagagctgg ccaatgaggt gaaggccctg ctgcaaggca agggcgactc agagcacaag
3901 cgcaagaagg tggaggcgca gctgcaagaa ctgcaggtca agttcagcga gggagagcgc
3961 gtgcgaaccg aactggccga caaggtcacc aagctgcagg ttgaactgga cagtgtgacc
4021 ggtctcctta gccagtctga cagcaagtcc agcaagctta cgaaggactt ctctgcgctg
4081 gagtcccagc ttcaggacac acaggagttg ctccaggagg agaaccggca gaagctgagc
4141 ctgagcacca agctcaagca gatggaggat gagaaaaact ccttcaggga gcagctggag
4201 gaggaggagg aggccaagcg caacttggag aagcagatcg ccacgctcca tgcccaggtg
4261 accgacatga agaagaagat ggaggacggt gtagggtgcc tggagactgc agaggaggcg
4321 aagcggaggc ttcagaagga cttggaaggc ctgagccagc ggcttgagga gaaggtggct
4381 gcctacgata agctggagaa gaccaagaca cggctgcagc aggagctgga cgacctgctg
4441 gttgacctgg accaccagcg gcagagcgtc tccaacctgg aaaagaagca gaagaagttc
4501 gaccagctcc tagccgagga gaagaccatc tcggccaagt atgcagagga gcgtgaccga
4561 gctgaggctg aggcccgtga gaaggagaca aaggcgctat cactggcccg ggcgcttgag
4621 gaggccatgg agcagaaggc agagctggag cggctcaaca agcagttccg cacggagatg
4681 gaggacctca tgagctccaa ggatgacgtg ggcaagagtg tccacgagct ggagaagtcc
4741 aagcgggcct tggagcagca ggtggaggag atgaagaccc agctggagga gctggaggat
4801 gagctgcagg ccacggagga tgccaagctc cgcctggagg tgaacctgca ggccatgaag
4861 gcccagtttg agcgggatct gcagggccgg gatgaacaga gcgaggagaa gaagaagcag
4921 ctggtcagac aggtgcggga gatggaggcg gagctggagg atgagaggaa gcagcgctcc
4981 atggccatgg ccgcacgcaa gaaactggag atggatctga aggacctgga ggcacacatt
5041 gacacagcca ataagaaccg ggaagaggcc atcaaacagc tgcggaagct tcaggcccag
5101 atgaaggact gcatgcggga gctggacgac acgcgcgcct cccgggagga gatcctggcg
5161 caggccaagg agaatgagaa gaagctgaag agcatggagg ccgagatgat tcagctgcag
5221 gaggaactgg cagctgctga gcgtgctaag cgtcaggccc aacaggaacg ggacgagctg
5281 gctgatgaga tcgccaacag cagtggcaaa ggggccctag cattagagga gaagcggcga
5341 ctggaggccc gcattgccct gctggaggag gagctggagg aggaacaggg caacacggag
5401 ctgatcaacg atcggctgaa gaaggccaac ctgcagatcg accaaataaa caccgacctg
5461 aacctggaac gcagccacgc acagaagaat gagaatgcgc gacagcagct ggaacgccag
5521 aacaaggagc tcaaggccaa gctgcaggaa atggagagtg ctgtcaagtc caaatacaag
5581 gcctccatcg cggccttgga ggccaaaatt gcacagctgg aggaacagct ggacaacgag
5641 accaaggagc gccaggcagc ctccaagcag gtgcgccgga cggagaagaa gctgaaggac
5701 gtgctgctgc aggtggagga cgagcggagg aacgcggaac agttcaagga ccaggctgac
5761 aaggcgtcca cccgcctgaa gcagcttaaa cggcagctag aggaggctga agaggaggcc
5821 cagcgggcca atgcctcacg ccggaagctg cagcgtgagc tggaagatgc cacagagacc
5881 gctgatgcta tgaaccgcga ggtcagctcc ctgaagaaca aactgaggcg tggggacctg
5941 ccatttgtcg tgactcgccg aattgttcgg aaaggcactg gcgactgctc agacgaggag
6001 gtcgacggta aagcagatgg ggccgatgcc aaggcagctg aataggagct tctcctgcag
6061 cccaggcgga tggacaaacg gctctgcctc cctcccccaa ccctccacac ccctgccttg
6121 agactgctct gaccatgtcc ccctcctccc aaggccttcc cgagggcatt ggcttcctct
6181 gctgcagccc ttccagtcct ccataccctt tgagaatctg ataccaaaga gtccaggctg
6241 gctcaggccg gatgacccac agggtcttgt cctccttgcc tgaaagcacg ggtggtgggc
6301 aagaagggcg gccattggag taggcacaag agttttctat gaatctattt tgtcttcaga
6361 taaagatttt gatagctcag gcctctagta gtgttaccct ccccgacctc ggctgtcccc
6421 gtcccccgtc cccctgctg ttggcaatca cacacggtaa cctcatacct gccctatggc
6481 ccccttccct gggccctatt ggtccagaag gagcctctgt ctgggtgcag aacatggggc
6541 actctgggaa tccccccact cccttctggg cagcactggt gcctctgctc ctccgactgt

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6601 aaaccgtctc aagtgcaatg cccctcccct cccttgccaa ggacagaccg tcctggcacc
6661 ggggcaaacc agacagggca tcagggccac tctagaaagg ccaacagcct tccggtggct
6721 tctcccagca ctctagggga ccaaatatat ttaatggtta agggacttgc agggcctggc
6781 agccagaata tccaagggct ggagcccact gtgcgctctg gtgcctctcc taggactggg
6841 gccaagggtg gtcgagctgt gccacccact ctatagcttc aagtctgcct tccacaagga
6901 tgcttttgaa agaaaaaaaa aggttttatt tttcccttct tgtagtaagt gctctagttc
6961 tgggtgtctt cactgccttg ccctggaact gtgtttagaa gagagtagct tgccctacaa
7021 tgtctacact ggtcgctgag ttcccctgcgc actgcacctc actgtttgta aatgctgtga
7081 ttaggttccc ttatggcagg aaggcttttt ttttcttttt ttttttcttt tctttttttt
7141 ttttttaaag gaaaaccagt caaatcatga agccacatac gctagagaag ctgaatccag
7201 gtcccaaagg cgctgtcata aaggagcaag tgggacccgc acccctttt ttatataata
7261 caagtgcctt agcatgtgtc gcagctgtca ccactacagt aagctggttt acagatgttt
7321 ccactgagcg tcacaataaa gagtaccatg tccta  (SEQ ID NO: 47)
```

(B)

```
   1 maqqaadkyl yvdknfinnp laqadwaakk lvwvpsskng fepaslkeev geeaivelve
  61 ngkkvkvnkd diqkmnppkf skvedmaelt clneasvlhn lkeryysgli ytysglfcvv
 121 inpyknlpiy seeivemykg kkrhempphi yaitdtayrs mmqdredqsi lctgesgagk
 181 tentkkviqy lahvasshks kkdqgelerq llqanpilea fgnaktvknd nssrfgkfir
 241 infdvngyiv ganietylle ksrairqake ertfhifyyl lsgagehlkt dlllepynky
 301 rflsnghvti pgqqdkdmfq etmeamrimg ipedeqmgll rvisgvlqlg niafkkernt
 361 dqasmpdnta aqkvshllgi nvtdftrgil tprikvgrdy vqkaqtkeqa dfaiealaka
 421 tyermfrwlv lrinkaldkt krqgasfigi ldiagfeifd lnsfeqlcin ytneklqqlf
 481 nhtmfileqe eyqregiewn fidfgldlqp cidliekpag ppgilallde ecwfpkatdk
 541 sfvekvvqeq gthpkfqkpk qlkdkadfci ihyagkvdyk adewlmknmd plndniatll
 601 hqssdkfvse lwkdvdriig ldqvagmset alpgafktrk gmfrtvgqly keqlaklmat
 661 lrntnpnfvr ciipnhekka gkldphlvld qlrcngvleg iricrqgfpn rvvfqefrqr
 721 yeiltpnsip kgfmdgkqac vlmikaleld snlyrigqsk vffragvlah leeerdlkit
 781 dviigfqacc rgylarkafa krqqqltamk vlqrncaayl rlrnwqwwrl ftkvkpllns
 841 irhedellak eaeltkvrek hlaaenrlte metmqsqlma eklqlqeqlq aetelcaeae
 901 elrarltakk qeleeichdl earveeeeer cqylqaekkk mqqniqelee qleeeesarq
 961 klqlekvtte aklkkleedq iimedqnckl akekklledr vaefttnlme eeekskslak
1021 lknkheamit dleerlrree kqrqelektr rklegdstdl sdqiaelqaq iaelkmqlak
1081 keeelqaala rveeeaaqkn malkkirele tqiselqedl eserasrnka ekqkrdlgee
1141 lealkteled tldstaaqqe lrskreqevs ilkktledea ktheaqiqem rqkhsqavee
1201 ladqleqtkr vkatlekakq tlenergela nevkallqgk gdsehkrkkv eaqlqelqvk
1261 fsegervrte ladkvtklqv eldsvtglls qsdsksskslt kdfsalesql qdtqellqee
1321 nrqklslstk lkqmedekns freqleeeee akrnlekqia tlhaqvtdmk kkmedgvgcl
1381 etaeeakrrl qkdleglsqr leekvaaydk lektktrlqq elddllvdld hqrqsvsnle
1441 kkqkkfdqll aeektisaky aeerdraeae areketkals laraleeame qkaelerlnk
1501 qfrtemedlm sskddvgksv helekskral eqqveemktq leeledelqa tedaklrlev
1561 nlqamkaqfe rdlqgrdeqs eekkkqlvrq vremeaeled erkqrsmama arkklemdlk
1621 dleahidtan knreeaikql rklqaqmkdc mrelddtras reeilaqake nekklksmea
1681 emiqlqeela aaerakrqaq qerdeladei anssgkgala leekrrlear ialleeelee
1741 eqgntelind rlkkanlqid qintdlnler shaqknenar qqlerqnkel kaklqemesa
1801 vkskykasia aleakiaqle eqldnetker qaaskqvrrt ekklkdvllq vederrnaeq
1861 fkdqadkast rlkqlkrqle eaeeeaqran asrrklqrel edatetadam nrevsslknk
1921 lrrgdlpfvv trrivrkgtg dcsdeevdgk adgadakaae (SEQ ID NO: 48)
```

Figure 12

(A)

```
   1 atacgactca ctatagggcg atcaggtgct ggaaagaagg ctaagcaagg ctgacctgct
  61 gcagctcccg cctcgtgcgc tcgccccacc cggccgccgc ccgagcgctc gagaaagtcc
 121 tctcgggaga agcagcgcct gttcccgggg cagatccagg ttcaggtcct ggctataagt
 181 caccatggca cagcaagctg ccgataagta tctctatgtg gataaaaact tcatcaacaa
 241 tccgctggcc caggccgact gggctgccaa gaagctggta tgggtgcctt ccgacaagag
 301 tggctttgag ccagccagcc tcaaggagga ggtgggcgaa gaggccatcg tggagctggt
 361 ggagaatggg aagaaggtga aggtgaacaa ggatgacatc cagaagatga acccgcccaa
 421 gttctccaag gtggaggaca tggcagagct cacgtgcctc aacgaagcct cggtgctgca
 481 caacctcaag gagcgttact actcagggct catctacacc tattcaggcc tgttctgtgt
 541 ggtcatcaat ccttacaaga acctgcccat ctactctgaa gagattgtgg aaatgtacaa
 601 gggcaagaag aggcacgaga tgccccctca catctatgcc atcacagaca ccgcctacag
 661 gagtatgatg caagaccgag aagatcaatc catcttgtgc actggtgaat ctggagctgg
 721 caagacggag aacaccaaga aggtcatcca gtatctggcg tacgtggcgt cctcgcacaa
 781 gagcaagaag gaccagggcg agctggagcg gcagctgctg caggccaacc ccatcctgga
 841 ggccttcggg aacgccaaga ccgtgaagaa tgacaactcc tcccgcttcg gcaaattcat
 901 tcgcatcaac tttgatgtca atggctacat tgttggagcc aacattgaga cttatctttt
 961 ggagaaatct cgtgctatcc gccaagccaa ggaagaacgg accttccaca tcttctatta
1021 tctcctgtct ggggctggag agcacctgaa gaccgatctc ctgttggagc cgtacaacaa
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1141 ccaggagacc atggaggcca tgaggattat gggcatccca gaagaggagc aaatgggcct
1201 gctgcgggtc atctcagggg ttcttcagct cggcaacatc gtcttcaaga aggagcggaa
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1381 ttacgtccag aaggcgcaga ctaaagagca ggctgacttt gccatcgagg ccttggccaa
1441 ggcgacctat gagcggatgt tccgctggct ggtgctgcgc atcaacaagg ctctggacaa
1501 gaccaagagg cagggcgcct ccttcatcgg gatcctggac attgccggct tcgagatctt
1561 tgatctgaac tcgtttgagc agctgtgcat caattacacc aatgagaagc tgcagcagct
1621 cttcaaccac accatgttca tcctggagca ggaggagtac cagcgcgagg gcatcgagtg
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1741 aggccccccg ggcattctgg ccctgctgga cgaggagtgc tggttcccca aagccaccga
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1861 caagcagctg aaggacaaag ctgatttctg cattatccac tatgccggca aggtggatta
1921 caaagctgac gagtggctga tgaagaacat ggatcccctg aatgacaaca tcgccacact
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2221 ggccggcaag ctggacccgc atctcgtgct ggaccagctg cgctgcaacg gtgttctcga
2281 gggcatccgt atctgccgcc agggcttccc caacagggtg gtcttccagg agtttcggca
2341 gagatatgag atcctgactc caaactccat tcccaagggt ttcatggacg ggaagcaggc
2401 gtgcgtgctc atgataaaag ccctggagct cgacagcaat ctgtaccgca ttggccagag
2461 caaagtcttc ttccgtgccg gtgtgctggc ccacctggag gaggagcgag acctgaagat
2521 caccgacgtc atcatagggt tccaggcctg ctgcaggggc tacctggcca ggaaagcatt
2581 tgccaagcgg cagcagcagc ttaccgccat gaaggtcctc cagcggaact gcgctgccta
2641 cctgaagctg cggaactggc agtggtggcg gctcttcacc aaggtcaagc cgctgctgca
2701 ggtgagccgg caggaggagg agatgatggc caaggaggag gagctggtga aggtcagaga
2761 gaagcagctg gctgcggaga acaggctcac ggagatggag acgctgcagt ctcagctcat
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2881 tgaggagctc cgggcccgcc tgaccgccaa gaagcaggaa ttagaagaga tctgccatga
2941 cctagaggcc agggtggagg aggaggagga gcgctgccag cacctgcagg cggagaagaa
3001 gaagatgcag cagaacatcc aggagcttga ggagcagctg gaggaggagg agagcgcccg
3061 gcagaagctg cagctggaga aggtgaccac cgaggcgaag ctgaaaaagc tggaggagga
3121 gcagatcatc ctggaggacc agaactgcaa gctggccaag gaaaagaaac tgctggaaga
```

3181 cagaatagct gagttcacca ccaacctcac agaagaggag gagaaatcta agagcctcgc
3241 caagctcaag aacaagcatg aggcaatgat cactgacttg gaagagcgcc tccgcaggga
3301 ggagaagcag cgacaggagc tggagaagac ccgccggaag ctggagggag actccacaga
3361 cctcagcgac cagatcgccg agctccaggc ccagatcgcg gagctcaaga tgcagctggc
3421 caagaaagag gaggagctcc aggccgccct ggccagagtg gaagaggaag ctgcccagaa
3481 gaacatggcc ctcaagaaga tccgggagct ggaatctcag atctctgaac tccaggaaga
3541 cctggagtct gagcgtgctt ccaggaataa agctgagaag cagaaacggg accttgggga
3601 agagctagag gcgctgaaaa cagagttgga ggacacgctg gattccacag ctgcccagca
3661 ggagctcagg tcaaaacgtg agcaggaggt gaacatcctg aagaagaccc tggaggagga
3721 ggccaagacc cacgaggccc agatccagga gatgaggcag aagcactcac aggccgtgga
3781 ggagctggcg gagcagctgg agcagacgaa gcgggtgaaa gcaaacctcg agaaggcaaa
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3961 caagttcaac gagggagagc gcgtgcgcac agagctggcc gacaaggtca ccaagctgca
4021 ggtggagctg gacaacgtga ccgggcttct cagccagtcc gacagcaagt ccagcaagct
4081 caccaaggac ttctccgcgc tggagtccca gctgcaggac actcaggagc tgctgcagga
4141 ggagaaccgg cagaagctga gcctgagcac caagctcaag caggtggagg acgagaagaa
4201 ttccttccgg gagcagctgg aggaggagga ggaggccaag cacaacctgg agaagcagat
4261 cgccaccctc catgcccagg tggccgacat gaaaaagaag atggaggaca gtgtggggtg
4321 cctggaaact gctgaggagg tgaagaggaa gctccagaag gacctggagg gcctgagcca
4381 gcggcacgag gagaaggtgg ccgcctacga caagctggag aagaccaaga cgcggctgca
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4561 gtatgcagag gagcgcgacc gggctgaggc ggaggcccga gagaaggaga ccaaggctct
4621 gtcgctggcc cgggccctgg aggaagccat ggagcagaag gcggagctgg agcggctcaa
4681 caagcagttc cgcacggaga tggaggacct tatgagctcc aaggatgatg tgggcaagag
4741 tgtccacgag ctggagaagt ccaagcgggc cctagagcag caggtggagg agatgaagac
4801 gcagctggaa gagctggagg acgagctgca ggccaccgaa gatgccaagc tgcggttgga
4861 ggtcaacctg caggccatga aggcccagtt cgagcgggac ctgcagggcc gggacgagca
4921 gagcgaggag aagaagaagc agctggtcag acaggtgcgg gagatggagg cagagctgga
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5101 gctgcggaag ctgcaggccc agatgaagga ctgcatgcgc gagctggatg acacccgcgc
5161 ctctcgtgag gagatcctgg cccaggccaa agagaacgag aagaagctga agagcatgga
5221 ggccgagatg atccagttgc aggaggaact ggcagccgcg gagcgtgcca agcgccaggc
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5341 ggcgttagag gagaagcggc gtctggaggc ccgcatcgcc cagctggagg aggagctgga
5401 ggaggagcag ggcaacacgg agctgatcaa cgaccggctg aagaaggcca acctgcagat
5461 cgaccagatc aacaccgacc tgaacctgga gcgcagccac gcccagaaga acgagaatgc
5521 tcggcagcag ctggaacgcc agaacaagga gcttaaggtc aagctgcagg agatggaggg
5581 cactgtcaag tccaagtaca aggcctccat caccgccctc gaggccaaga ttgcacagct
5641 ggaggagcag ctggacaacg agaccaagga gcgccaggca gcctgcaaac aggtgcgtcg
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5761 gcagtacaag gaccaggccg acaaggcatc tacccgcctg aagcagctca agcggcagct
5821 ggaggaggcc gaagaggagg cccagcgggc caacgcctcc cgccggaaac tgcagcgcga
5881 gctggaggac gccactgaga cggccgatgc catgaaccgc gaagtcagct ccctaaagaa
5941 caagctcagg cgcggggacc tgccgtttgt cgtgccccgc cgaatggccc ggaaaggcgc
6001 cggggatggc tccgacgaag aggtagatgg caaagcggat ggggctgagg ccaaacctgc
6061 cgaataagcc tcttctcctg cagcctgaga tggatggaca gacagacacc acagcctccc
6121 cttcccagac cccgcagcac gcctctcccc accttcttgg gactgctgtg aacatgcctc
6181 ctcctgccct ccgccccgtc cccccatccc gtttccctcc aggtgttgtt gagggcattt
6241 ggcttcctct gctgcatccc cttccagctc cctcccctgc tcagaatctg ataccaaaga
6301 gacagggccc gggcccaggc agagagcgac cagcaggctc ctcagccctc tcttgccaaa
6361 aagcacaaga tgttgaggcg agcagggcag gccccggggg aggggccaga gttttctatg
6421 aatctatttt tcttcagact gaggcctttt ggtagtcgga gcccccgcag tcgtcagcct
6481 ccctgacgtc tgccaccagc gcccccactc ctcctccttt ctttgctgtt tgcaatcaca
6541 cgtggtgacc tcacacacct ctgccccttg ggcctcccac tcccatggct ctgggcggtc

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6601 cagaaggagc aggccctggg cctccacctc tgtgcagggc acagaaggct ggggtggggg
6661 gaggagtgga ttcctcccca ccctgtccca ggcagcgcca ctgtccgctg tctccctcct
6721 gattctaaaa tgtctcaagt gcaatgcccc ctcccctcct ttaccgagga cagcctgcct
6781 ctgccacagc aaggctgtcg gggtcaagct ggaaaggcca gcagccttcc agtggcttct
6841 cccaacactc ttggggacca aatatattta atggttaagg gacttgtccc aagtctgaca
6901 gccagagcgt tagaggggcc agcggccctc ccaggcgatc ttgtgtctac tctaggactg
6961 ggcccgaggg tggtttacct gcaccgttga ctcagtatag tttaaaaatc tgccacctgc
7021 acaggtattt ttgaaagcaa aataaggttt tctttttccc cctttcttgt aataaatgat
7081 aaaattccga gtctttctca ctgcctttgt ttagaagaga gtagctcgtc ctcactggtc
7141 tacactggtt gccgaattta cttgtattcc taactgtttt gtatatgctg cattgagact
7201 tacggcaaga aggcattttt ttttttaaa ggaaacaaac tctcaaatca tgaagtgata
7261 taaaagctgc atatgcctac aaagctctga attcaggtcc cagttgctgt cacaaaggag
7321 tgagtgaaac tcccacccta ccccctttt tatataataa aagtgcctta gcatgtgttg
7381 cagctgtcac cactacagta agctggttta cagatgtttt ccactgagca tcacaataaa
7441 gagaaccatg tgctaaaaaa aaaaaaaaaa aaaa  (SEQ ID NO: 49)
```

(B)

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   1 maqqaadkyl yvdknfinnp laqadwaakk lvwvpsdksg fepaslkeev geeaivelve
  61 ngkkvkvnkd diqkmnppkf skvedmaelt clneasvlhn lkeryysgli ytysglfcvv
 121 inpyknlpiy seeivemykg kkrhempphi yaitdtayrs mmqdredqsi lctgesgagk
 181 tentkkviqy layvasshks kkdqgelerq llqanpilea fgnaktvknd nssrfgkfir
 241 infdvngyiv ganietylle ksrairqake ertfhifyyl lsgagehlkt dlllepynky
 301 rflsnghvti pgqqdkdmfq etmeamrimg ipeeeqmgll rvisgvlqlg nivfkkernt
 361 dqasmpdnta aqkvshllgi nvtdftrgil tprikvgrdy vqkaqtkeqa dfaiealaka
 421 tyermfrwlv lrinkaldkt krqgasfigi ldiagfeifd lnsfeqlcin ytneklqqlf
 481 nhtmfileqe eyqregiewn fidfgldlqp cidliekpag ppgilallde ecwfpkatdk
 541 sfvekvmqeq gthpkfqkpk qlkdkadfci ihyagkvdyk adewlmknmd plndniatll
 601 hqssdkfvse lwkdvdriig ldqvagmset alpgafktrk gmfrtvgqly keqlaklmat
 661 lrntnpnfvr ciipnhekka gkldphlvld qlrcngvleg iricrqgfpn rvvfqefrqr
 721 yeiltpnsip kgfmdgkqac vlmikaleld snlyrigqsk vffragvlah leeerdlkit
 781 dviigfqacc rgylarkafa krqqqltamk vlqrncaayl klrnwqwwrl ftkvkpllqv
 841 srqeeemmak eeelvkvrek qlaaenrlte metlqsqlma eklqlqeqlq aetelcaeae
 901 elrarltakk qeleeichdl earveeeeer cqhlqaekkk mqqniqelee qleeeesarq
 961 klqlekvtte aklkkleeeq iiledqnckl akekklledr iaefttnlte eeekskslak
1021 lknkheamit dleerlrree kqrqelektr rklegdstdl sdqiaelqaq iaelkmqlak
1081 keeelqaala rveeeaaqkn malkkirele sqiselqedl eserasrnka ekqkrdlgee
1141 lealkteled tldstaaqqe lrskreqevn ilkktleeea ktheaqiqem rqkhsqavee
1201 laeqleqtkr vkanlekakq tlenergela nevkvllqgk gdsehkrkkv eaqlqelqvk
1261 fnegervrte ladkvtklqv eldnvtglls qsdsksskslt kdfsalesql qdtqellqee
1321 nrqklslstk lkqvedekns freqleeeee akhnlekqia tlhaqvadmk kkmedsvgcl
1381 etaeevkrkl qkdleglsqr heekvaaydk lektktrlqq elddllvdld hqrqsacnle
1441 kkqkkfdqll aeektisaky aeerdraeae areketkals laraleeame qkaelerlnk
1501 qfrtemedlm sskddvgksv helekskral eqqveemktq leeledelqa tedaklrlev
1561 nlqamkaqfe rdlqgrdeqs eekkkqlvrq vremeaeled erkqrsmava arkklemdlk
1621 dleahidsan knrdeaikql rklqaqmkdc mrelddtras reeilaqake nekklksmea
1681 emiqlqeela aaerakrqaq qerdeladei anssgkgala leekrrlear iaqleeelee
1741 eqgntelind rlkkanlqid qintdlnler shaqknenar qqlerqnkel kvklqemegt
1801 vkskykasit aleakiaqle eqldnetker qaackqvrrt ekklkdvllq vdderrnaeq
1861 ykdqadkast rlkqlkrqle eaeeeaqran asrrklqrel edatetadam nrevsslknk
1921 lrrgdlpfvv prrmarkgag dgsdeevdgk adgaeakpae  (SEQ ID NO: 50)
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Figure 13

(A)

```
   1 gtctttcctg ggagatgggc gcgcaaaccg accagtgggt ctgggggcgg cagtgatggg
  61 cgtggagatg gcccaatgag ggtgggagtg ggtggggcag gcgcgagcag cagtgctaaa
 121 ggagcccggc ggaggcagcg gtgggtttgg aattgagacg ctggatctgt ggtcgctgct
 181 ggggacgtgt gccggcgcca ccatcttcgg ctgaagaggc aattactttt gggtccttct
 241 gtttacaatg gcccagagaa ctggactgga ggatcccgag aggtatctct ttgtggacag
 301 ggctgtcatc tacaaccctg ccactcaagc tgactggaca gctaaaaagc tggtgtggat
 361 tccatcggaa cgccatggtt ttgaggcagc tagtattaaa gaagagcggg gcgatgaggt
 421 tatggtggag ctggcagaga atgggaagaa agcaatggtc aacaaagatg acattcagaa
 481 gatgaaccca ccaaagttct ccaaggtgga ggatatggca gagctgacat gcttgaacga
 541 agcctctgtc ttacataatt tgaaggaccg ctactattca ggacttatct atacttactc
 601 tggactcttc tgtgtggtga taaatcctta caagaacctt ccaatttact ctgagaatat
 661 tattgaaatg tatagaggga agaaacgcca tgagatgcca ccacacatct acgccatatc
 721 agagtctgct tacagatgca tgcttcaaga tcgtgaggac cagtcaattc tatgcacggg
 781 tgaatcgggt gccgggaaga cagaaaatac caagaaagtc attcagtacc ttgcccacgt
 841 tgcttcttct cacaaaggaa gaaaggacca taatattcct ggggaacttg aacggcagct
 901 tttacaagca aatccaattc tggaatcctt tggaaatgcg aagactgtga aaaatgataa
 961 ctcatctcgc tttggcaagt ttatccggat caactttgat gtaactggct atattgttgg
1021 ggccaacatt gaaacatacc ttctggaaaa gtctcgtgct gttcgtcaag ctaaagatga
1081 gcgtacattt catatctttt atcagttgct ctctggagca ggggaacacc tgaaatccga
1141 cttactcctg gaaggtttca acaactacag attcctctcc aatggctata ttcctattcc
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1441 gcccaggatc aaggttggcc gggattacgt acagaaagcc cagaccaaag agcaggctga
1501 ttttgcagtg gaagcattgg caaaagctac ctatgagcgg ttgtttcgct ggctcgttca
1561 ccgcatcaat aaagcgctgg ataggaccaa acgccaggga gcttccttca ttgggatcct
1621 ggatattgct ggttttgaaa tttttgagct gaactccttc gagcagctgt gcatcaacta
1681 caccaacgag aagctgcagc agctgttcaa ccacaccatg ttcatcctgg agcaggagga
1741 gtaccagcga gagggcatcg agtggaactt tatcgacttc ggcctggacc tgcagccctg
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3121 actggatgag gaggaggggg cccggcaaaa gctgcagctg gagaaggtga cagcagaggc
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3181 taaaatcaag aagatggaag aggaggttct gcttctcgaa gaccagaatt ccaaatttat
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6901 ccccgggtgg cagccatcac agtaacttcc aggtggtctc ctgagtgtct ggcttgataa
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7321 tctataacaa cttaatgtat taagttctga ctgtgcttca tatcatgtac ctctctagtg
7381 aagtagatgc gcaaacattc agtgacagca aatcagtgtt agtgacaagc cccgaccgtg
7441 gcgatgtgct ggaaaacacg gacctttggg gttaaaagct ttaacatctg tgaggaagaa
7501 ctggtcacat gggtttggaa tctttgattt cccctgtatg aattgtactg gctgttgacc
7561 accagacacc tgactgcaaa tatcttttct tgtattccca tatttctaga caatgatttt
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(B)

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 181 gagktentkk viqylahvas shkgrkdhni pgelerqllq anpilesfgn aktvkndnss
 241 rfgkfirinf dvtgyivgan ietylleksr avrqakdert fhifyqllsg agehlksdll
 301 legfnnyrfl sngyipipgq qdkdnfqetm eamhimgfsh eeilsmlkvv ssvlqfgnis
 361 fkkerntdqa smpentvaqk lchllgmnvm eftrailtpr ikvgrdyvqk aqtkeqadfa
 421 vealakatye rlfrwlvhri nkaldrtkrq gasfigildi agfeifelns feqlcinytn
 481 eklqqlfnht mfileqeeyq regiewnfid fgldlqpcid lierpanppg vlalldeecw
 541 fpkatdktfv eklvqeqgsh skfqkprqlk dkadfciihy agkvdykade wlmknmdpln
 601 dnvatllhqs sdrfvaelwk dvdrivgldq vtgmtetafg sayktkkgmf rtvgqlykes
 661 ltklmatlrn tnpnfvrcii pnhekragkl dphlvldqlr cngvlegiri crqgfpnriv
 721 fqefrqryei ltpnaipkgf mdgkqacerm iraleldpnl yrigqskiff ragvlahlee
 781 erdlkitdii iffqavcrgy larkafakkq qqlsalkvlq rncaaylklr hwqwwrvftk
 841 vkpllqvtrq eeelqakdee llkvkekqtk vegeleemer khqqlleekn ilaeqlqaet
 901 elfaeaeemr arlaakkqel eeilhdlesr veeeeernqi lqnekkkmqa hiqdleeqld
 961 eeegarqklq lekvtaeaki kkmeeevlll edqnskfike kklmedriae cssqlaeeee
1021 kaknlakirn kqevmisdle erlkkeektr qelekakrkl dgettdlqdq iaelqaqvde
1081 lkvqltkkee elqgalargd detlhknnal kvarelqaqi aelqedfese kasrnkaekq
1141 krdlseelea lkteledtld ttaaqqelrt kreqevaelk kaledetknh eaqiqdmrqr
1201 hataleelse qleqakrfka nleknkqgle tdnkelacev kvlqqvkaes ehkrkkldaq
1261 vqelhakvse gdrlrvelae kanklqneld nvstlleeae kkgikfakda aglesqlqdt
1321 qellqeetrq klnlssrirq leeeknslqe qqeeeeeark nlekqvlalq sqladtkkkv
1381 dddlgtiesl eeakkkllkd vealsqrlee kvlaydklek tknrlqqeld dltvdldhqr
1441 qivsnlekkq kkfdqllaee kgisaryaee rdraeaeare ketkalslar aleealeake
1501 eferqnkqlr admedlmssk ddvgknvhel ekskraleqq veemrtqlee ledelqated
1561 aklrlevnmq amkaqferdl qtrdeqneek krlllkqvre leaelederk qralavaskk
1621 kmeidlkdle aqieaankar devikqlrkl qaqmkdyqre leearasrde ifaqskesek
1681 klksleaeil qlqeelasse rarrhaeqer deladeians asgksallde krrleariaq
1741 leeeleeeqs nmellndrfr kttlqvdtln telaaersaa qksdnarqql erqnkelkak
1801 lqelegavks kfkatisale akigqleeql eqeakeraaa nklvrrtekk lkeifmqved
1861 errhadqyke qmekanarmk qlkrqleeae eeatranasr rklqreldda teaneglsre
1921 vstlknrlrr ggpisfsssr sgrrqlhieg aslelsdddt esktsdvndt qppqse
(SEQ ID NO: 52)
```

Figure 14

(A)

```
   1 actgaggcgc tggatctgtg gtcgcggctg gggacgtgcg cccgcgccac catcttcggc
  61 tgaagaggca attgcttttg gatcgttcca tttacaatgg cgcagagaac tggactcgag
 121 gatccagaga ggtatctctt tgtggacagg gctgtcatct acaaccctgc cactcaagct
 181 gattggacag ctaaaaagct agtgtggatt ccatcagaac gccatggttt tgaggcagct
 241 agtatcaaag aagaacgggg agatgaagtt atggtggagt tggcagagaa tggaaagaaa
 301 gcaatggtca acaaagatga tattcagaag atgaacccac ctaagttttc caaggtggag
 361 gatatggcag aattgacatg cttgaatgaa gcttccgttt tacataatct gaaggatcgc
 421 tactattcag gactaatcta tacttattct ggactcttct gtgtagttat aaacccttac
 481 aagaatcttc caatttactc tgagaatatt attgaaatgt acagagggaa gaagcgtcat
 541 gagatgcctc cacacatcta tgctatatct gaatctgctt acagatgcat gcttcaagat
 601 cgtgaggacc agtcaattct ttgcacgggt gagtcaggtg ctgggaagac agaaaatata
 661 aagaaagtta ttcagtacct tgcccatgtt gcttcttcac ataaaggaag aaaggaccat
 721 aatattcctg gggaacttga acggcagctt ttgcaagcaa atccaattct ggaatcattt
 781 ggaaatgcga agactgtgaa aaatgataac tcatctcgtt ttggcaaatt tattcggatc
 841 aactttgatg taactggcta tatcgttggg gccaacattg aaacatacct tctggaaaag
 901 tctcgtgctg ttcgtcaagc aaaagatgaa cgtacttttc atatctttta ccagttgtta
 961 tctggagcag gagaacacct aaagtctgat ttgcttcttg aaggatttaa taactacagg
1021 tttctctcca atggctatat tcctattccg ggacagcaag acaaagataa tttccaggag
1081 accatggaag caatgcacat aatgggcttc tcccatgaag agattctgtc aatgcttaaa
1141 gtagtatctt cagtgctaca gtttggaaat atttctttca aaaaggagag aaatactgat
1201 caagcttcca tgccagaaaa tacagttgcg cagaagctct gccatcttct tgggatgaat
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1321 caaaaagccc agaccaaaga acaggcagat tttgcagtag aagcattggc aaaagctacc
1381 tatgagcggc tctttcgctg gctcgttcat cgcatcaata aagctctgga taggaccaaa
1441 cgtcagggag catctttcat tggaatcctg gatattgctg gatttgaaat ttttgagctg
1501 aactcctttg aacaactttg catcaactac accaatgaga agctgcagca gctgttcaac
1561 cacaccatgt ttatcctaga acaagaggaa taccagcgcg aaggcatcga gtggaacttc
1621 atcgatttcg ggctggatct gcagccatgc atcgacctaa tagagagacc tgcgaaccct
1681 cctggtgtac tggccctttt ggatgaagaa tgctggttcc ctaaagccac agataaaacc
1741 tttgttgaaa aactggttca agagcaaggt tcccactcca agtttcagaa acctcgacaa
1801 ttaaaagaca aagctgattt ttgcattata cattatgcag ggaaggtgga ctataaggca
1861 gatgagtggc tgatgaagaa tatggacccc ctgaatgaca acgtggccac ccttttgcac
1921 cagtcatcag acagatttgt ggcagagctt tggaaagatg tggaccgtat cgtgggtctg
1981 gatcaagtca ctggtatgac tgagacagct tttggctccg catataaaac caagaagggc
2041 atgtttcgta ccgttgggca actctacaaa gaatctctca ccaagctgat ggcaactctc
2101 cgaaacacca accctaactt tgttcgttgt atcattccaa atcacgagaa gagggctgga
2161 aaattggatc cacacctagt cctagatcag cttcgctgta atggtgtcct ggaagggatc
2221 cgaatctgtc gccagggctt ccctaaccga atagttttcc aggaattcag acagagatat
2281 gagatcctaa ctccaaatgc tattcctaaa ggttttatgg atggtaaaca ggcctgtgaa
2341 cgaatgatcc gggctttaga attggaccca aacttgtaca gaattggaca gagcaagata
2401 tttttcagag ctggagttct ggcacactta gaggaagaaa gagatttaaa aatcaccgat
2461 atcattatct tcttccaggc cgtttgcaga ggttacctgg ccagaaaggc ctttgccaag
2521 aagcagcagc aactaagtgc cttaaaggtc ttgcagcgga actgtgccgc gtacctgaaa
2581 ttacggcact ggcagtggtg gcgagtcttc acaaaggtga agccgcttct acaagtgact
2641 cgccaggagg aagaacttca ggccaaagat gaagagctgt tgaaggtgaa ggagaagcag
2701 acgaaggtgg aaggagagct ggaggagatg gagcggaagc accagcagct tttagaagag
2761 aagaatatcc ttgcagaaca actacaagca gagactgagc tctttgctga agcagaagag
2821 atgagggcaa gacttgctgc taaaaagcag gaattagaag agattctaca tgacttggag
2881 tctagggttg aagaagaaga agaaagaaac caaatcctcc aaaatgaaaa gaaaaaaatg
2941 caagcacata ttcaggacct ggaagaacag ctagacgagg aggaaggggc tcggcaaaag
3001 ctgcagctgg aaaaggtgac agcagaggcc aagatcaaga agatggaaga ggagattctg
3061 cttctcgagg accaaaattc caagttcatc aaagaaaaga aactcatgga agatcgcatt
3121 gctgagtgtt cctctcagct ggctgaagag gaagaaaagg cgaaaaactt ggccaaaatc
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3241 actcgtcagg aactggaaaa ggccaaaaga aaactcgacg gggagacgac cgacctgcag
3301 gaccagatcg cagagctgca ggcgcagatt gatgagctca agctgcagct ggccaagaag
3361 gaggaggagc tgcagggcgc actggccaga ggtgatgatg aaacactcca taagaacaat
3421 gcccttaaag ttgtgcgaga gctacaagcc caaattgctg aacttcagga agactttgaa
3481 tccgagaagg cttcacggaa caaggccgaa aagcagaaaa gggacttgag tgaggaactg
3541 gaagctctga aaacagagct ggaggacacg ctggacacca cggcagccca gcaggaacta
3601 cgtacaaaac gtgaacaaga agtggcagag ctgaagaaag ctcttgagga ggaaactaag
3661 aaccatgaag ctcaaatcca ggacatgaga caaagacacg caacagccct ggaggagctc
3721 tcagagcagc tggaacaggc caagcggttc aaagcaaatc tagagaagaa caagcagggc
3781 ctggagacag ataacaagga gctggcgtgt gaggtgaagg tcctgcagca ggtcaaggct
3841 gagtctgagc acaagaggaa gaagctcgac gcgcaggtcc aggagctcca tgccaaggtc
3901 tctgaaggcg acaggctcag ggtggagctg gcggagaaag caagtaagct gcagaatgag
3961 ctagataatg tctccaccct tctggaagaa gcagagaaga agggtattaa atttgctaag
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4081 cgccagaaac taaacctgag cagtcggatc cggcagctgg aagaggagaa gaacagtctt
4141 caggagcagc aggaggagga ggaggaggcc aggaagaacc tggagaagca agtgctggcc
4201 ctgcagtccc agttggctga taccaagaag aaagtagatg acgacctggg aacaattgaa
4261 agtctggaag aagccaagaa gaagcttctg aaggacgcgg aggccctgag ccagcgcctg
4321 gaggagaagg cactggcgta tgacaaactg gagaagacca agaaccgcct gcagcaggag
4381 ctggacgacc tcacggtgga cctggaccac cagcgccagg tcgcctccaa cttggagaag
4441 aagcagaaga agtttgacca gctgttagca gaagagaaga gcatctctgc tcgctatgcc
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4561 gcccgggccc tcgaggaagc cctggaggcc aaggaggagt ttgagaggca gaacaagcag
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4681 gaacttgaaa aatccaaacg ggccctagag cagcaggtgg aggaaatgag gacccagctg
4741 gaggagctgg aagacgaact ccaggccacg gaagatgcca agcttcgtct ggaggtcaac
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5221 gagagagatg agctggcgga cgagatcacc aacagcgcct ctggcaagtc cgcgctgctg
5281 gatgagaagc ggcgtctgga agctcggatc gcacagctgg aggaggagct ggaagaggag
5341 cagagcaaca tggagctgct caacgaccgc ttccgcaaga ccactctaca ggtggacaca
5401 ctgaacgccg agctagcagc cgagcgcagc gccgcccaga agagtgacaa tgcacgccag
5461 caactggagc ggcagaacaa ggagctgaag gccaagctgc aggaactcga gggtgctgtc
5521 aagtctaagt tcaaggccac catctcagcc ctggaggcca agattgggca gctggaggag
5581 cagcttgagc aggaagccaa ggaacgagca gccgccaaca aattagtccg tcgcactgag
5641 aagaagctga aagaaatctt catgcaggtt gaggatgagc gtcgacacgc ggaccagtat
5701 aaagagcaga tggagaaggc caacgctcgg atgaagcagc ttaaacgcca gctggaggaa
5761 gcagaagaag aagcgacgcg tgccaacgca tctcggcgta aactccagcg ggaactggat
5821 gatgccaccg aggccaacga gggcctgagc cgcgaggtca gcaccctgaa gaaccggctg
5881 aggcggggtg gccccatcag cttctcttcc agccgatctg gccggcgcca gctgcacctt
5941 gaaggagctt ccctggagct ctccgacgat gacacagaaa gtaagaccag tgatgtcaac
6001 gagacgcagc caccccagtc agagtaaagt tgcaggaagc cagaggaggc aatacagtgg
6061 gacagttagg aatgcacccg gggcctcctg cagatttcgg aaattggcaa gctacgggat
6121 tccttcctga aagatcaact gtgtcttaag gctctccagc ctatgcatac tgtatcctgc
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6241 taaacagatt gtttcatcat tgcatctatt ttccatatag tcatcaagag accattttat
6301 aaaacatggt aagacccttt ttaaaacaaa ctccaggccc ttggttgcgg gtcgctgggt
6361 tattggggca gcgccgtggt cgtcactcag tcgctctgca tgctctctgt catacagaca
6421 ggtaacctag ttctgtgttc acgtggcccc cgactcctca gccacatcaa gtctcctaga
6481 ccactgtgga ctctaaactg cacttgtctc tctcatttcc ttcaaataat gatcaatgct
6541 atttcagtga gcaaactgtg aaaggggctt tggaaagagt aggaggggtg ggctggatcg

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 6601 gaagcaacac ccatttgggg ttaccatgtc catcccccaa ggggggccct gcccctcgag
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 6781 ttccagatgg tctcccgaac cgcctggctc tgccggcgcc ctcctcacgt caggagcaag
 6841 cagccgtgga cccctaagcc gagctggtgg aaggcccctc cctgtcgcca gccgggccct
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 6961 gtccgaggct tgttccaagt ttgtcaatga ggtttatgga gcctccagaa cagatgccat
 7021 cttcctgaat gttgacatgc cagtgggtgt gactccttca ttttttcctc tcccttccct
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 7141 acattacgga aagtgcctta gacactacag tactaagaca atgttgaata tatcattcgc
 7201 ctctataaca atttaatgta ttcagttttg actgtgcttc atatcatgta cctctctagt
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 7321 ctctgcaatg tgcttgaaaa cacaaacctt ttgggttaaa agctttaaca tctattagga
 7381 agaatttgtc ctgtgggttt ggaatcttgg attttccccc tttatgaact gtactggctg
 7441 ttgaccacca gacacctgac cgcaaatatc ttttcttgta ttcccatatt tctagacaat
 7501 gatttttgta agacaataaa tttattcatt atagatattt gcgcctgctc tgtttacttg
 7561 aagaaaaaag cacccgtgga gaataaagag acctcaataa acaagaataa tcatgtgaa
(SEQ ID NO: 53)
```

(B)

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    1 maqrtgledp erylfvdrav iynpatqadw takklvwips erhgfeaasi keergdevmv
   61 elaengkkam vnkddiqkmn ppkfskvedm aeltclneas vlhnlkdryy sgliytysgl
  121 fcvvinpykn lpiyseniie myrgkkrhem pphiyaises ayrcmlqdre dqsilctges
  181 gagktentkk viqylahvas shkgrkdhni pgelerqllq anpilesfgn aktvkndnss
  241 rfgkfirinf dvtgyivgan ietylleksr avrqakdert fhifyqllsg agehlksdll
  301 legfnnyrfl sngyipipgq qdkdnfqetm eamhimgfsh eeilsmlkvv ssvlqfgnis
  361 fkkerntdqa smpentvaqk lchllgmnvm eftrailtpr ikvgrdyvqk aqtkeqadfa
  421 vealakatye rlfrwlvhri nkaldrtkrq gasfigildi agfeifelns feqlcinytn
  481 eklqqlfnht mfileqeeyq regiewnfid fgldlqpcid lierpanppg vlalldeecw
  541 fpkatdktfv eklvqeqgsh skfqkprqlk dkadfciihy agkvdykade wlmknmdpln
  601 dnvatllhqs sdrfvaelwk dvdrivgldq vtgmtetafg sayktkkgmf rtvgqlykes
  661 ltklmatlrn tnpnfvrcii pnhekragkl dphlvldqlr cngvlegiri crqgfpnriv
  721 fqefrqryei ltpnaipkgf mdgkqacerm iraleldpnl yrigqskiff ragvlahlee
  781 erdlkitdii iffqavcrgy larkafakkq qqlsalkvlq rncaaylklr hwqwwrvftk
  841 vkpllqvtrq eeelqakdee llkvkekqtk vegeleemer khqqlleekn ilaeqlqaet
  901 elfaeaeemr arlaakkqel eeilhdlesr veeeeernqi lqnekkkmqa hiqdleeqld
  961 eeegarqklq lekvtaeaki kkmeeeilll edqnskfike kklmedriae cssqlaeeee
 1021 kaknlakirn kqevmisdle erlkkeektr qelekakrkl dgettdlqdq iaelqaqide
 1081 lklqlakkee elqgalargd detlhknnal kvvrelqaqi aelqedfese kasrnkaekq
 1141 krdlseelea lkteledtld ttaaqqelrt kreqevaelk kaleeetknh eaqiqdmrqr
 1201 hataleelse qleqakrfka nleknkqgle tdnkelacev kvlqqvkaes ehkrkkldaq
 1261 vqelhakvse gdrlrvelae kasklqneld nvstlleeae kkgikfakda aslesqlqdt
 1321 qellqeetrq klnlssrirq leeeknslqe qqeeeeeark nlekqvlalq sqladtkkkv
 1381 dddlgtiesl eeakkkllkd aealsqrlee kalaydklek tknrlqqeld dltvdldhqr
 1441 qvasnlekkq kkfdqllaee ksisaryaee rdraeaeare ketkalslar aleealeake
 1501 eferqnkqlr admedlmssk ddvgknvhel ekskraleqq veemrtqlee ledelqated
 1561 aklrlevnmq amkaqferdl qtrdeqneek krllikqvre leaelederk qralavaskk
 1621 kmeidlkdle aqieaankar devikqlrkl qaqmkdyqre leearasrde ifaqskesek
 1681 klksleaeil qlqeelasse rarrhaeqer deladeitns asgksallde krrleariaq
 1741 leeeleeeqs nmellndrfr kttlqvdtln aelaaersaa qksdnarqql erqnkelkak
 1801 lqelegavks kfkatisale akigqleeql eqeakeraaa nklvrrtekk lkeifmqved
 1861 errhadqyke qmekanarmk qlkrqleeae eeatranasr rklqreldda teaneglsre
 1921 vstlknrlrr ggpisfsssr sgrrqlhleg aslelsdddt esktsdvnet qppqse
(SEQ ID NO: 54)
```

Figure 15

(A)

```
   1 ccttttctgt ccaggccgag gcctctggac cgccctgggc gccgaccatg gctgcagtga
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 241 ccctgcggga tgaaggggag gaggaggcag aagtggagct ggcggagagt gggcgccgcc
 301 tgcggctgcc cagggaccag atccagcgca tgaacccacc caagttcagc aaggcagaag
 361 atatggctga gctcacctgc ctcaacgagg cctcggtcct gcacaacctg cgagaacgct
 421 actactccgg gctcatttat acctactctg gcctcttctg tgtggtcatt aacccataca
 481 agcagctgcc catctacacg gaggccattg ttgaaatgta ccggggcaag aagcgccatg
 541 aggtgccacc tcacgtgtat gctgtgacgg agggcgcgta ccgcagcatg cttcaggatc
 601 gtgaggatca atccattctc tgcacgggag agtctggcgc tgggaagacg gagaacacca
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 781 ccaaccccat cctagaggcc tttggcaatg ccaagacagt gaagaacgac aactcttccc
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1261 gccgcctctt gggactcgga gtgaccgact tctccagagc ccttctcaca ccccgcatca
1321 aagtgggccg agattatgtt cagaaagcac aaaccaagga gcaggctgac tttgcgctgg
1381 aggctctggc caaagctacc tatgagcgcc tgttccgctg gctggttctg cggctcaacc
1441 gtgccctgga cagaagcccg cggcagggtg cctccttcct gggcatcctg gacatcgcgg
1501 gctttgagat cttccagctg aactccttcg agcagctgtg catcaactac accaacgaga
1561 agctacagca gctattcaac cacaccatgt tcgtgctgga gcaggaggag taccagcgag
1621 agggcatccc ctggaccttc ctagacttcg ggttggacct gcaaccttgc atcgacctca
1681 ttgagcgtcc ggccaaccct ccaggtctcc tggccctgct ggacgaggag tgctggttcc
1741 ccaaggccac ggacaagtct tttgtggaga aggtcgccca ggagcagggc agccacccca
1801 aattccagcg ccccaggaac ctgcgagatc aggccgactt cagcgtcctg cactatgccg
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(B)

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(C)

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(D)

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 601 ykasewlmkn mdplndnvaa llhqstdrlt aeiwkdvegi vgleqvsslg dgppggrprr
 661 gmfrtvgqly keslsrlmat lsntnpsfvr civpnhekra gkleprlvld qlrcngvleg
 721 iricrqgfpn rilfqefrqr yeiltpnaip kgfmdgkqac ekmiqaleld pnlyrvgqsk
 781 iffragvlaq leeerdlkvt diivsfqaaa rgylarrafq rrqqqqsalr vmqrncaayl
 841 klrnwqwwrl fikvkpllqv trqdevlqar aqelqkvqel qqqsarevge lqgrvaqlee
 901 ertrlaeqlr aeaelcseae etrarlaark qelelvvtel earvgeeeec srqlqsekkr
 961 lqqhiqeles hleaeegarq klqlekvtte akmkkfeedl llledqnskl skerrlleer
1021 laefssqaae eeekvkslnk lrlkyeatis dmedrlkkee kgrqeleklk rrldgessel
```

1081 qeqmveqkqr aeellaqlgr kedelqaall raeeeggara qllkslreaq aglaeaqedl
1141 eaervaraka ekqrrdlgee lealrgeled tldstnaqqe lrskreqevt elkkaleees
1201 rahevsmqel rqrhsqalve maeqleqarr gkgvwektrl sleaevselk aelsslqtsr
1261 qegeqkrrrl esqlqevqgr ssdserarse aaeklqraqa elesvstals eaeskairlg
1321 kelssaesql hdtqellqee traklalgsr vraleaeaag lreqmeeevv areragrelq
1381 stqaqlsewr rrqeeeaavl eageearrra areaetltqr laekteaver lerarrrlqq
1441 elddatvdlg qqkqllstle kkqrkfdqll aeekaavlra vedrerieae grerearals
1501 ltraleeeqe areelerqnr alraeleall sskddvgknv helerarkaa eqaasdlrtq
1561 vteledelta aedaklrlev tvqalkaqhe rdlqgrddag eerrrqlakq lrdaeverde
1621 erkqralama arkklelele elkaqtsaag qgkeeavkql kkmqvqmkel wreveetrss
1681 rdemftlsre nekklkglea evlrlqeela asdrarrqaq qdrdemaeev asgnlskaat
1741 leekrqlegr lsqleeelee eqnnsellkd hyrklvlqve slttelsaer sfsakaesgr
1801 qqlerqiqel rarlgeedag ararqkmlia alesklaqae eqleqesrer ilsgklvrra
1861 ekrlkevvlq vdeerrvadq vrdqleksnl rlkqlkrqle eaeeeasraq agrrrlqrel
1921 edvtesaesm nrevttlrnr lrrgpltftt rtvrqvfrle egvasdeeea egaepgsapg
1981 qepeapppat pq (SEQ ID NO: 58)

Figure 16

(A)

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  61 ccatgtcggt gcccgggcgg aaggcgcccc ccaggccggg cccagtgccc gaggcggccc
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 181 ccccgcaggt ggagtggacg gcccggcgtc tcgtgtgggt gccttcggag cttcacgggt
 241 tcgaggcggc ggcgctgcgg gacgaaggcg aggaggaggc ggaggtggag ctggcggaga
 301 gcgggaggcg gctgcgactg ccgcgggacc agatccagcg catgaacccg cccaagttca
 361 gcaaggccga ggacatggcc gagctgacct gcctcaacga ggcctcggtc ctgcacaacc
 421 tccgggagcg gtactactcc ggcctcatct acacgtactc cggccttttc tgtgtggtca
 481 tcaacccgta caagcagctt cccatctaca cagaagccat tgtggagatg taccggggca
 541 agaagcgcca cgaggtgcca ccccacgtgt acgcagtgac cgaggggccc tatcggagca
 601 tgctgcagga tcgtgaggac cagtccattc tctgcactgg agagtctgga gctgggaaga
 661 cggaaaacac caagaaggtc atccagtacc tcgcccacgt ggcatcgtct ccaaagggca
 721 ggaaggagcc gggtgtcccc ggtgagctgg agcggcagct gcttcaggcc aaccccatcc
 781 tagaggcctt tggcaatgcc aagacagtga agaatgacaa ctcctcccga ttcggcaaat
 841 tcatccgcat caactttgat gttgccgggt acatcgtggg cgccaacatt gagacctacc
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1021 cccactaccg gttcctgacc aacgggccgt catcctctcc cggccaggag cgggaactct
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1141 tgctgcggat ggtctcagca gttctccagt ttggcaacat tgccttgaag agagaacgga
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1321 actatgtgca gaaagcccag actaaggaac aggctgactt cgcgctggag gccctggcca
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1561 tcttcaacca caccatgttc gtgctggagc aggaggagta ccagcgtgag ggcatccgct
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3121 ggccgagttc tcatcccagg cagctgagga ggaggagaag gtcaagagcc tcaataagct
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3181 acggctcaaa tatgaggcca caatcgcaga catggaggga ccgcctacgg aaggaggaga
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6781 ctggac  (SEQ ID NO: 59)
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(B)

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 121 vlhnlreryy sgliytysgl fcvvinpykq lpiyteaive myrgkkrhev pphvyavteg
 181 ayrsmlqdre dqsilctges gagktentkk viqylahvas spkgrkepgv pgelerqllq
 241 anpileafgn aktvkndnss rfgkfirinf dvagyivgan ietylleksr airqakdecs
 301 fhifyqllgg ageqlkadll lepcshyrfl tngpssspgq erelfqetle slrvlgfshe
 361 eiismlrmvs avlqfgnial krerntdqat mpdntaaqkl crllglgvtd fsralltpri
 421 kvgrdyvqka qtkeqadfal ealakatyer lfrwlvlrln raldrsprqg asflgildia
 481 gfeifqlnsf eqlcinytne klqqlfnhtm fvleqeeyqr egipwtfldf gldlqpcidl
 541 ierpanppgl lalldeecwf pkatdksfve kvaqeqgghp kfqrprhlrd qadfsvlhya
 601 gkvdykanew lmknmdplnd nvaallhqst drltaeiwkd vegivgleqv sslgdgppgg
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1561 lraqvteled eltaaedakl rlevtvqalk tqherdlqgr deageerrrq lakqlrdaev
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1681 trtsreeifs qnresekrlk gleaevlrlq eelaasdrar rqaqqdrdem adevangnls
1741 kaaileekrq legrlgqlee eleeeqsnse llndryrkll lqveslttel saersfsaka
1801 esgrqqlerq iqelrgrlge edagararhk mtiaaleskl aqaeeqleqe trerilsgkl
1861 vrraekrlke vvlqveeerr vadqlrdqle kgnlrvkqlk rqleeaeeea sraqagrrrl
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1981 gpspepegsp pahpq  (SEQ ID NO: 60)
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