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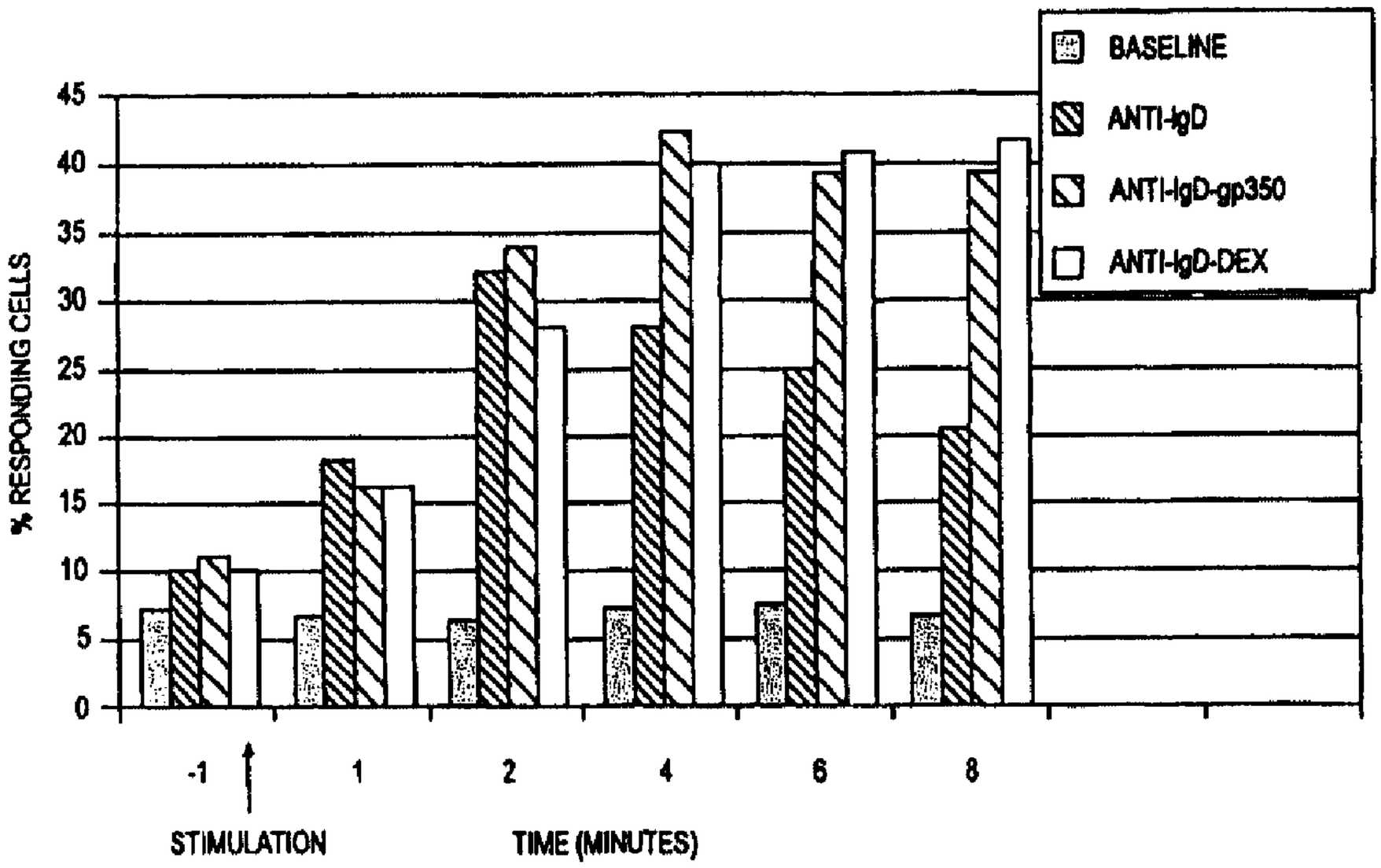
(72) Inventeurs/Inventors:
MOND, JAMES J., US;
LEES, ANDREW, US

(73) Propriétaire/Owner:
HENRY M. JACKSON FOUNDATION FOR THE ADVANCEMENT OF MILITARY MEDICINE, US

(74) Agent: KIRBY EADES GALE BAKER

(54) Titre : AUGMENTATION DE L'ACTIVATION DES CELLULES B ET DE LA SECRETION D'IMMUNOGLOBULINE PAR CO-STIMULATION DE RECEPTEURS DE L'ANTIGENE ET D'EBV GP350/220

(54) Title: ENHANCEMENT OF B CELL ACTIVATION AND IMMUNOGLOBULIN SECRETION BY CO-STIMULATION OF RECEPTORS FOR ANTIGEN AND EBV GP350/220



(57) Abrégé/Abstract:
The present invention provides vaccine adjuvants comprising the Epstein Barr Virus glycoprotein 350/220 or naturally occurring variants thereof, a fusion protein comprising EBV Gp350/220 sequence which binds to the CR2 receptor, or a synthetically-derived fragment of Gp350/220 which retains the ability to bind to the CR2 receptor. The present invention further provides immunostimulatory compositions comprising an EBV Gp350/220 adjuvant sequence that binds the CR2 complex and at least one antigen of interest other than Gp350/220. Co- administration of the adjuvant with an antigen of interest, other than an antigen comprising EBV 350/220 sequence, enhances the immunogenicity of the antigen. In a preferred embodiment, the adjuvant is directly or indirectly covalently bound to an antigen of interest to form an immunogenic composition. In a most preferred embodiment of the composition, antibodies are elicited against at least one Gp350/220 epitope and against at least one epitope of the antigen.

Abstract

The present invention provides vaccine adjuvants comprising the Epstein Barr Virus glycoprotein 350/220 or naturally occurring variants thereof, a fusion protein comprising EBV Gp350/220 sequence which binds to the CR2 receptor, or a synthetically-derived fragment of Gp350/220 which retains the ability to bind to the CR2 receptor. The present invention further provides immunostimulatory compositions comprising an EBV Gp350/220 adjuvant sequence that binds the CR2 complex and at least one antigen of interest other than Gp350/220. Co-administration of the adjuvant with an antigen of interest, other than an antigen comprising EBV 350/220 sequence, enhances the immunogenicity of the antigen. In a preferred embodiment, the adjuvant is directly or indirectly covalently bound to an antigen of interest to form an immunogenic composition. In a most preferred embodiment of the composition, antibodies are elicited against at least one Gp350/220 epitope and against at least one epitope of the antigen.

Enhancement of B Cell Activation and Immunoglobulin Secretion by Co-stimulation Of Receptors for Antigen and EBV Gp350/220

GOVERNMENT INTEREST

The invention described herein may be manufactured, licensed and used for governmental purposes without the payment of any royalties to us thereon.

FIELD OF THE INVENTION

The present invention relates to the use of Epstein Barr Virus glycoprotein 350/220 (Gp350/220), and naturally-occurring or synthetically-derived fragments of Gp350/220 which retain the ability to bind to the CR2 receptor on B cells. The invention also relates to non-complement derived peptides that bind to the CR2 receptor as well complement-derived peptides and the hexapeptide LYNVEA. These proteins, peptides, and fragments can be used as vaccine adjuvants and as adjuvanting components of immunostimulatory compositions and vaccines. The invention also relates to the use of non-complement derived peptides that bind to the CR2 receptors, as well as complement derived peptides and the hexapeptide LYNVEA.

BACKGROUND

Complement is the name given to a series of some 20 proteins which are activated by microbial invasion to form an important line of defense against infection. The most well-recognized complement functions are those leading to the osmotic lysis and/or phagocytosis of invading bacteria or parasites. Components in the cell

walls of infectious organisms trigger the complex and interconnected pathways of the complement enzyme cascade. During this process the most abundant component, C3, is converted into an enzymatically active form and ultimately cleaved into a number of fragments such as C3a and a series of phagocytosis-promoting peptides including C3b and related peptides, iC3b, C3dg.

C3a is an anaphylatoxin which triggers mast cells and basophils to release a host of chemotactic and inflammatory factors which both contribute to the activation of neutrophils and other phagocytic cells, and concentrate these cells at the site of microbial infection. C3b becomes covalently linked to the surface of the invading organism. The bound C3b interacts with the CR1 (CD35) receptors on the surface of the phagocytic cells. This interaction induces the activated phagocytes to engulf the microbes, which are then fused with cytoplasmic granules and destroyed. The destruction of invading microorganisms by phagocytic cells is an important part of cellular immunity.

More than two decades ago, researchers found that C3 peptides can stimulate resting B cells, thus suggesting that this complement component may also play a role in the humoral immune system. Hartmann, Transplant. Rev. 23:70-104 (1975); and Hartman and Bokisch, J. Exp. Med. 142:600-610 (1975). It is now recognized that these stimulated B cells produce antibacterial antibodies that assist with the process of phagocytosis. Phagocytes are most effective in combating bacteria when the bacteria are coated with antibodies. This effect, termed opsonization, is particularly important in combating encapsulated bacteria which are generally resistant to phagocytosis. It has been suggested that the generation of opsonizing antibodies is favored by the association of bacterial surface antigens with C3 peptides. In other words, the association of C3 on the bacterial surface stimulates B cells to produce anti-bacterial antibodies. Thus, C3 not only stimulates phagocytosis directly, but also stimulates B cells to produce antibodies that bind to the invading microorganism and further promote phagocytosis.

The B cell stimulatory property of the C3 peptides does not require the entire molecule, but is contained in a short sequence containing the hexapeptide LYNVEA.

Lambris et al., Proc. Natl. Acad. Sci. USA, 82:4235-39 (1985); and Frade et al., BBRC 188:833-42 (1992). Notably, C3 molecules, and shorter peptides containing the hexapeptide sequence are only stimulatory as multimers, indicating that cross-linking of the C3 receptor is necessary for B cell proliferation. Servis and Lambris, J. Immunol. 142:2207-12 (1989); and Tsokos et al., J. Immunol. 144:1640-45 (1990). Because many molecules of C3 can bind to a single bacterium, this condition is easily satisfied in vivo.

More recent work indicates that the immunostimulatory effect of cross-linking the C3 receptor on the B cell is mediated by lowering the activation threshold for stimulation of the antigen receptor. When the C3 receptor is cross-linked, either less antigen or antigen with a lower affinity for the antigen receptor on a B cell is required for B cell stimulation. Mongini et al., J. Immunol. 159:3782-91 (1997).

Both B cells and phagocytic cells express CR1 receptors on their cell surface. However, unlike phagocytes, B cells also express the structurally related CR2 receptors (CD21). Cross-linking of CR2 molecules on the B cell surface appears to be directly responsible for the stimulatory effect of C3d, C3dg, C3bi and iC3b peptides. Reviewed in Frade, Seminars in Immunology 2:159-64 (1990). Moreover, CR1, CR2, and another protein, CD19, appear to be associated on the B cell surface. Agents which cross-link any member of this complex result in an enhanced B cell response. This signal may be provided by multimeric C3 peptides, or by antibodies directed against one or more of these associated proteins. Nemerow et al., J. Immunol. 135:3068-73 (1985); and Kozono et al., J. Immunol. 160:1565-72 (1998); Carter and Fearson, Science 256:105-07 (1992).

Indications that crosslinking of CR2 molecules promotes B cell activation have led to the use of C3d sequences as an adjuvant. Dempsey and coworkers demonstrated that a recombinant fusion protein of hen egg lysozyme containing one copy of C3d did not appreciably change the immunogenicity of the lysozyme. However, the fusion of two or three copies of the C3d peptide increased the level of anti-lysozyme antibodies by 1000- and 10,000-fold, respectively. Dempsey et al., Science, 271:348-50 (1996).

In addition to binding complement components, the CR2 receptor has also been identified as the receptor for the B-cell lymphotropic Epstein-Barr Virus (EBV). Fingeroth et al., Proc. Natl. Acad. Sci. USA 81:4510-14 (1984); and Frade et al., Proc. Natl. Acad. Sci. USA 82:1490-93 (1985). EBV has long been recognized as a B cell mitogen and polyclonal activator of antibody synthesis. In vivo, primary EBV infection is characterized by non-specific hypergammaglobulinemia. In vitro, EBV transformed B cells secrete Ig. See review, Giovana and Blaese, Adv. Immunol. 37:99-149 (F.J. Dixon ed., 1985).

EBV infects over 95% of the world population and is best known as the causative agent for infectious mononucleosis. Moreover, EBV is also strongly associated with a host of pathologies including endemic Burkit's lymphoma, undifferentiated nasopharyngeal carcinoma, X-linked proliferative disorder (XLPD), hairy cell leukemia, post-transplant lymphoproliferative disorders, and some types of Hodgkin's lymphoma, T cell lymphomas, and gastric carcinomas. In addition, unusual EBV-derived tumors are frequently found in immunosuppressed patients, including those infected with the AIDs virus. Consequently, investigators have long sought a safe and effective vaccine to prevent EBV infection. The EBV infection process is initiated by the binding of the major EBV outer membrane glycoprotein, Gp350/220, to CR2. This interaction stimulates phagocytosis or fusion of the virus with the B cell membrane which allows the viral genome to enter the cytoplasm. Tanner et al., Cell 50:2-3-213 (1987). Interestingly, some evidence suggests that C3d and Gp350/220 bind to different sites on the CR2 receptor. Barel et al., J. Immunol. 141:1590-1595 (1988). Viral entry is via the Gp350/220 protein and most of these vaccines have focused on blocking the infection process by eliciting anti-Gp350/220 antibodies. See reviews, Morgan, Vaccine, 10:563-571 (1992); and Spring et al., J. Natl. Cancer Ctr. 88:1436-41 (1996). Of course, these vaccines are designed solely to elicit antibodies against Gp350/220.

Thus, there remains a need for safe and effective adjuvants directed at activating B cells through the CR2 receptor complex.

SUMMARY OF THE INVENTION

The present invention addresses these needs by providing vaccine adjuvants which bind to the CR2 complex. These adjuvants include an Epstein Barr Virus glycoprotein 350/220 or naturally occurring variant thereof, a fusion protein comprising EBV 350/220 sequence sufficient to bind the CR2 receptor, or a recombinant or synthetically-derived fragment of Gp350/220 which retains the ability to bind to the CR2 receptor. The adjuvants of the invention also include non-complement derived peptides that bind to the CR2 receptor as well as complement derived peptides and those related to the hexapeptide LYNVEA. Co-administration of the adjuvant with an antigen of interest, which is other than an antigen comprising EBV 350/220 sequence, enhances the immunogenicity of the antigen. In a preferred embodiment, the adjuvant is directly or indirectly covalently bound to an antigen of interest, to form an immunogenic composition. In a preferred embodiment of the composition, antibodies are elicited against at least one Gp350/220 epitope and against at least one epitope of the antigen.

Certain exemplary embodiments provide a use of: at least one Epstein-Barr virus (EBV) Gp350/220 molecule covalently conjugated to at least one protein, peptide, polypeptide, or polysaccharide comprising an antigenic epitope other than an epitope of EBV Gp350/220, wherein the EBV Gp350/220 molecule binds to a CR2 receptor (CR2); such that the EBV Gp350/220 molecule and the protein, peptide, polypeptide, or polysaccharide are on the same molecule; for the preparation of a medicament for promoting a humoral immune response with respect to the antigenic epitope other than an epitope of EBV Gp350/220.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 Figure 1 illustrates the Epstein-Barr virus major outer envelope glycoprotein Gp350/220. Panels 1A and 1B show the sequence of Gp350 and Gp220, respectively.

Fig. 2 Figure 2 illustrates the percentage of purified peripheral B cells responding to anti-IgD, anti-IgD--Gp350 and anti-IgD--dextran.

Fig. 3 Figure 3 illustrates the percentage of purified peripheral B cells responding to various concentrations of anti-IgD--Gp350.

DETAILED DESCRIPTION OF THE INVENTION

The present invention addresses the need in the art for safe and effective adjuvants, immunogenic compositions, and vaccines by recognizing the adjuvanting effects of Gp350/220. Prior uses of the protein focused solely on the generation of anti-Gp350/220 antibodies and failed to extend beyond this use. Thus, although

EBV infection appears to be a non-specific activator of B cells, the present invention targets that stimulatory effect to antigen-specific B cells by coupling the B cell activating activity of EBV Gp350/220 to one or more copies of an antigen of interest. Using such a composition, a B cell that bears surface Ig specific for the antigen may be simultaneously stimulated through the antigen receptor and through Gp350/220-mediated cross-linking of the CR2 receptor. This co-stimulation will result in the increased production of antibodies specific for the antigen of interest. Thus, this invention presents Gp350/220 as an adjuvant for the generation, stimulation, or enhancement of antigen specific immune responses or as an adjuvanting component of an immunostimulatory composition.

In one embodiment of this invention, one or more moieties comprising at least one antigenic epitope other than Gp350/220 are directly or indirectly conjugated to EBV Gp350/220. Preferably, the moiety is incorporated into an existing EBV vaccine. Antibodies are thus raised against both the EBV Gp350/220 component and against at least one additional epitope of the moiety. In the course of EBV infection, it is likely that multiple copies of Gp350/220 in the viral membrane cross-link CR2 receptors on the B cell surface. Thus, in a preferred embodiment, the Gp350/220 component contains multiple copies of Gp350/220 sequence to promote CR2 cross-linking.

In another embodiment, at least one copy of Gp350/220 is directly or indirectly conjugated to a moiety containing at least one antigenic epitope. In a preferred embodiment, two or more copies of Gp350/220 are directly or indirectly conjugated to the moiety. It is also preferred that the moiety or moieties present multiple copies of at least one antigenic epitope. In each case, the Gp350/220 sequences function as an adjuvant to increase the immunogenicity of the moiety. A moiety may be any antigenic component including haptens, T cell-dependent (TD) antigens, Type 2 T cell-independent (TI-2) antigens, the definitions of which are well known in the art and are described in Roit, Essential Immunology, (1994) Blackwell Scientific Publications; and Paul, Fundamental Immunology, (1989) Raven Press.

A moiety may be a simple chemical compound; a polysaccharide, including bacterial polysaccharides; a naturally occurring, recombinant, or synthetic protein, polypeptide, or peptide; a synthetic peptide; a recombinant fusion protein; or a chemical or enzymatic fragment of any of the preceding. A moiety may include epitopes specific for other EBV antigens. In a preferred embodiment, the moieties are not specific to EBV but elicit antibodies against other infectious diseases, allergens, tumor antigens, or conditions which respond to immune stimulation.

It is well established that multi-epitope antigens are more stimulatory than univalent antigens. This increased immunogenicity appears to result from the ability of multivalent antigens to promote more effective cross-linking of the antigen receptor. Thus, for the purpose of this invention, it is highly preferred that the moiety, or antigen of interest, either contain multiple copies of an antigenic epitope, or be presented as part of a larger construct containing multiple copies of the antigen. For in vitro use, the multi-epitopic moiety may be an antigen analog, such as anti-IgD or anti-IgM coupled to dextran, first described in Brunswick et al., J. Immunol 140:3364 (1988).

In one embodiment, at least one, and preferably two or more copies of Gp350/220 are conjugated to a polysaccharide-TD antigen composition such as those described in W.E. Dick and M. Beurret, *Conjugate Vaccines, in* Contrib. Microbiol. Immunol. Vol. 10, pp. 48-114, (J.M. Cruse & R.E. Lewis Jr. eds., 1989), or to any of dual conjugate compositions of Lees *et al.*, Vaccine 1160-66 (1994); U.S. Patent No. 5,585,100 (Mond and Lees); and U.S. Patent 5,955,079 (Mond and Lees).

The necessary sequence for CR2 binding is contained within the amino acid sequence of the EBV envelope glycoprotein Gp350 (also known as Gp340) and the related splice variant Gp220 (Beisel et al., J. Virol. 54, 665-674 (1985)), examples of which are presented herein as Figures 1A and 1B, respectively. Additional sequences are known in the art.

For the purpose of this invention, Gp350/220 further refers to non-complement derived peptides or other molecules which bind to CR2, block the binding of EBV

Gp350/220 to CR2, or both. Preferably, Gp350/220 refers to any polypeptide sequence containing EBV Gp350/220 amino acid sequence, a fragment, variant, derivative, or analog thereof, wherein at least a portion of the Gp350/220, Gp350/220 fragment, variant, derivative, or analog sequence which binds to the human CR2 B cell receptor. Such sequence may be contained in a full length protein, a recombinant or synthetic polypeptide or peptide containing Gp350/220 sequence, a recombinant fusion protein, or a chemical or enzymatically-derived fragment of any of the preceding. Although the CR2 binding regions of Gp350/220 have not been investigated, such identification may be made by those of ordinary skill in the art. In addition, portions of the Gp350/220 protein between amino acids 21-26, or between amino acids 372-378, have been suggested to contain sequences necessary for CR2 binding. Tanner et al., Cell 203-213 (1987); and Nemerow et al., 61:1416-20 (1987).

A Gp350/220 polypeptide "variant" as referred to herein means a naturally-occurring or synthetically programmed polypeptide substantially identical to either the Gp350 or Gp220 polypeptides (e.g., SEQ ID Nos: 1 and 2), but which has an amino acid sequence different from that of Gp350 or Gp220 because of one or more deletions, insertions or substitutions. Some Gp350/220 variant sequences have already been identified by sequencing the DNA of different strains of EBV, and are readily available to one of ordinary skill in the art. The variant amino acid sequence preferably is at least 60%, 65%, 70%, or 80%, identical to a Gp350/220 polypeptide amino acid sequence of SEQ ID Nos. 1 or 2, more preferably at least 85% identical, still more preferably at least 90% identical, and most preferably at least 95% identical. The percent identity can be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by *Devereux et al.* (Nucl. Acids Res. 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of *Needleman and Wunsch* (J. Mol. Biol. 48:443, 1970), as revised by *Smith and Waterman* (Adv. Appl. Math 2:482, 1981). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing

a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of *Gribskov and Burgess*, Nucl. Acids Res. 14:6745, 1986, as described by Schwartz and Dayhoff, eds., Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Variants can comprise conservatively substituted sequences, meaning that a given amino acid residue is replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known. Naturally occurring Gp350/220 variants are also encompassed by the invention. Examples of such variants are proteins that result from alternate mRNA splicing events, from proteolytic cleavage of the Gp350/220 polypeptides, and allelic variants of Gp350/220 polypeptide. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the Gp350/220 polypeptides.

Variants and derivatives of Gp350/220 polypeptides can be obtained by mutation of nucleotide sequences encoding Gp350/220 polypeptides. Alterations of the amino acid sequence can occur naturally, or be accomplished by any of a number of conventional methods. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene wherein predetermined codons can be

altered by substitution, deletion or insertion. Exemplary methods of making the alterations set forth above are disclosed by *Walder et al.* (Gene 42:133, 1986); *Bauer et al.* (Gene 37:73, 1985); *Craik*, (BioTechniques, January 1985, 12-19); *Smith et al.* (Genetic Engineering: Principles and Methods, Plenum Press, 1981); *Kunkel* (Proc. Natl. Acad. Sci. USA 82:488, 1985); *Kunkel et al.* (Methods in Enzymol. 154:367, 1987); and U.S. Patent Nos. 4,518,584 and 4,737,462.

Gp350/220 polypeptides can be modified to create Gp350/220 polypeptide derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, polyethylene glycol (PEG) groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of Gp350/220 polypeptides can be prepared by linking the chemical moieties to functional groups on Gp350/220 polypeptide amino acid side chains or at the N-terminus or C-terminus of a Gp350/220 polypeptide or the extracellular domain thereof. Other derivatives of Gp350/220 polypeptides within the scope of this invention include covalent or aggregative conjugates of Gp350/220 polypeptides or peptide with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate can contain a signal or leader polypeptide sequence (e.g. the α-factor leader of *Saccharomyces*) at the N-terminus of a Gp350/220 polypeptide. The signal or leader peptide co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall. Gp350/220 polypeptide conjugates can comprise peptides added to facilitate purification and identification of Gp350/220 polypeptides. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in *Hopp et al.*, Bio/Technology 6:1204, 1988.

For the purpose of this invention, Gp350/220 also refers to Gp350/220 analogs which are defined as CR2-binding molecules other than antibodies or portions of complement C3. Such molecules may be selected from naturally-occuring proteins or be totally synthetic. One of skill in the art recognizes that

numerous methods are available for selecting molecules which bind to a known receptor. For example, purified or recombinant CR2 could be used as a probe to detect binding in a phage expression library. Clones expressing a protein which binds to CR2 could be isolated and sequenced. Any clones which do not correspond to C3 products would be selected and tested for B cell stimulatory properties. Alternatively, additional naturally-occurring or synthetic sequences which bind CR2 may be selected by functional selection method of Menzel et al., U.S. Pat. No. 5,521,066.

In addition, Gp350/220 analogs may be obtained using the principles of rational drug design. Such a design would comprise the steps of determining the three-dimensional structure of that portion of the CR2 polypeptide which binds to Gp350/220, analyzing the three-dimensional structure for the likely binding site of Gp350/220 or the C3b peptide, synthesizing a molecule that is predicted to bind to a predictive reactive site, and determining the binding and adjuvanting activating activity of the molecule.

Epstein-Barr virus infects over 95% of the world population and is best known as the causative agent for infectious mononucleosis. Moreover, EBV is also strongly associated with a host of pathologies including endemic Burkit's lymphoma, undifferentiated nasopharyngeal carcinoma, X-linked proliferative disorder (XLPD), hairy cell leukemia, post-transplant lymphoproliferative disorders, and some types of Hodgkin's lymphoma, T cell lymphomas, and gastric carcinomas. In addition, unusual EBV-derived tumors are frequently found in immunosuppressed patients, including those infected with the AIDs virus. Consequently, investigators have long sought a safe and effective vaccine to prevent EBV infection. Because viral entry via the Gp350/220 protein is an essential step in viral infection, most of these vaccines have focused on blocking the infection process by eliciting anti-Gp350/220 antibodies. See reviews, Morgan, Vaccine, 10:563-571 (1992); and Spring et al., J. Natl. Cancer Ctr. 88:1436-41 (1996). Of course, these vaccines are designed solely to elicit antibodies against Gp350/220.

The invention also relates peptides that block the binding of the Gp350/220 peptides of the invention. In yet another embodiment, the invention relates to non-complement derived peptides that bind to the CR2 receptor as well as complement-derived peptides and peptides based on the hexapeptide LYNVEA, as well fragments, variants, derivatives, and analogs thereof.

The adjuvants and immunogenic compositions may be produced using recombinant techniques. The production and expression of recombinant proteins and fusion proteins is well known in the art and can be carried out using conventional procedures, such as those in Sambrook *et al.* Molecular Cloning: A Laboratory Manuals, Vols. 1-3, (2d ed. 1989), Cold Spring Harbor Laboratory Press. The production and purification of recombinant Gp350/220 is known in the art. Tanner et al., Cell 203-213 (1987). Gp350/220 fusion proteins can also be designed by fusing Gp350/220 polypeptides which retain CR2 binding activity to sequences encoding another polypeptide to aid in the purification of the Gp350/220 sequence. An example of such a fusion is a fusion of sequences encoding a Gp350 polypeptide to sequences encoding the product of the malE gene of the pMAL-c2 vector of New England Biolabs, Inc., or to a hexahistidine sequence. Such fusions allow for affinity purification of the fusion protein. In addition, methods for removing the non-Gp350 sequences from the fusion protein after purification are well known in the art. The adjuvant or composition may also be expressed in transgenic plants or plant products. The adjuvant or composition may then be administered orally as part of the plant or plant product, or be purified from the plant or plant product prior to administration.

The invention also encompasses recombinant nucleic acid vectors, such as plasmids and recombinant viral vectors, that direct the expression of Gp350/220 sequences. The construction and expression of recombinant nucleic acid vectors is well known in the art and includes those techniques contained in Sambrook *et al.* Molecular Cloning: A Laboratory Manual, Vols. 1-3, (2d ed. 1989), Cold Spring Harbor Laboratory Press. Such nucleic acid vectors may be contained in a biological vector such as viruses and bacteria, preferably in a non-pathogenic or attenuated

microorganism, including attenuated viruses, bacteria, parasites, and virus-like particles. In one embodiment, the nucleic acid vector directs the expression of Gp350/220 in a biological vector, preferably on the surface of a bacterium, or as part of a viral capsid or envelope. Administration of the biological vector to a patient enhances the immune response to bacterial, viral, or parasitic antigens. Alternatively, Gp350/220 may be expressed as a fusion protein along with at least one antigenic moiety. In a preferred embodiment, the fusion protein is expressed on the surface of bacteria, virus, parasite, or particle to allow for effective antigen presentation. Administration of the biological vector to a patient will result in an enhanced immune response to at least one epitope of the moiety. Similarly, plasmid and viral nucleic acid vectors may be used to direct Gp350/220 or Gp350/220 fusion protein expression in yeast or other eukaryotic cells.

In one embodiment, Gp350/220 is expressed on the surface of mammalian tumor cells. These cells are used to elicit antibodies against tumor-specific antigens. In another embodiment, mammalian host cells are programed to express Gp350/220 as fusion protein along with at least one antigenic moiety. The host then elicits an immune response to at least one epitope of the moiety.

The adjuvants of the invention may be co-administered with at least one antigenic moiety. In a preferred embodiment, the adjuvants are preferably conjugated to the antigenic moiety to form an immunogenic (immunostimulatory) composition. The adjuvant or immunogenic composition may also be conjugated to additional immunostimulatory components. These immunostimulatory components, such as immunomodulators and/or cell targeting moieties, may further enhance the immune response. These entities are co-administered, and preferably chemically conjugated to the adjuvant or immunogenic composition. Such entities may include, for example, (1) detoxified lipopolysaccharides or derivatives, (2) muramyl dipeptides, (3) carbohydrates, lipids, and peptides that may interact with cell surface determinants to target the construct to immunologically relevant cells, (4) interleukins, including IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF, TGF-β and IFN-γ; (5) one or more universal T cell elements (TCE); (6); CD40 ligand; and (7) antibodies that

may interact with cell surface components. In one embodiment, the adjuvanting activity or immunogenicity of the composition may be enhanced by the co-administration or conjugation of an adjuvanting lipoprotein, as described in the co-pending application: *Induction and Enhancement of the Immune Response to Type 2 T Cell-independent Antigens Conjugated to Lipid or Lipid-containing Moieties* of Mond and Snapper, corresponding to International Patent Application PCT/US99/05647 published as International Patent Publication WO 99/47168.

Any form of conjugation is within the scope of this invention. Methods of conjugation are well known to those of ordinary skill in the art, and include the heteroligation techniques of Brunswick et al., J. Immunol., 140:3364 (1988); Wong, S.S., Chemistry of Protein Conjugates and Crosslinking, CRC Press, Boston (1991); Brenkeley et al., "Brief Survey of Methods for Preparing Protein Conjugates With Dyes, Haptens and Cross-Linking Agents", Bioconjugate Chemistry, 3, No. 1 (Jan. 1992); and Hermanson, G.T., Bioconjugate Techniques, Academic Press, San Diego (1996).

A preferred method of covalent conjugation is via CDAP (1-cyano-4-"dimethylamino"-pyridinium tetrafluoroborate) activation of the polysaccharide, set forth in applications Serial No. 08/482,616, and 08/482,666, filed June 7, 1995, (08/482,616 being now abandoned), which are continuation-in-part applications of application Serial No. 08/408,717, filed March 22, 1995, and issued July 29, 1997, as U.S. Patent No. 5,651,971, which correspond to U.S. Patents 5,693,326, 5,849,301, and 5,651,971.

The adjuvants and immunogenic compositions of the invention may be considered pharmaceutical compositions in that they elicit a biological effect on the immune system. When the pharmaceutical composition of the invention contains

antigen and is to be administered to an organism, preferably suspended, dissolved, compounded, or encapsulated, in a pharmaceutically acceptable carrier, vehicle, or diluent, it may be referred to as a vaccine. The adjuvants and immunogenic compositions of the claimed invention may be applied to isolated B cells in vitro as a pharmaceutical composition or administered directly to the patient as a vaccine.

The invention also relates to the treatment of a patient, or for the benefit of a patient, by administration of an adjuvanting amount of the adjuvant together with an antigen, or administration of an immunostimulatory amount of the compositions of the vaccine.

A patient is hereby defined as any person or non-human animal in need of immune stimulation, or to any subject for whom treatment may be beneficial, including humans, and non-human animals. Such non-human animals to be treated include all domesticated and feral vertebrates which contain receptors for EBV Gp350/220, in particular, non-human primates such as tamarins. Notably, mice do not normally express CR2, and therefore do not respond to the adjuvanting effect of Gp350/220. However, the creation of transgenic mice which express CR2 on their B cells is within the skill of those in the art. Such CR2 transgenic mice would therefore constitute patients for the purpose of this invention. One of skill in the art will, of course, recognize that the choice of antigens will depend on the disease or condition to be vaccinated against in a particular system.

An immunostimulatory amount refers to that amount of vaccine that is able to stimulate the immune response. As used herein, the immune response is defined as a set of biological effects leading to the body's production of immunoglobulins, or antibodies, in response to a foreign entity. Thus, the immune response refers to the activation of B cells, in vivo or in culture, through stimulation of B cell surface Ig receptor molecules. The measurement of the immune response is within the ordinary skill of those in this art and includes the determination of antibody levels using methods described in the series by P. Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theory of Enzyme Immunoassays, (Burdon & van Knippenberg eds., 3rd ed.,1985) Elsevier, New York; and Antibodies:

A Laboratory Manual, (Harlow & Lane eds., 1988), Cold Spring Harbor Laboratory Press; as well as procedures such as countercurrent immuno-electrophoresis (CIEP), radioimmunoassay, radio-immunoprecipitation, enzyme-linked immuno-sorbent assays (ELISA), dot blot assays, and sandwich assays, see U.S. Patent Nos. 4,376,110 and 4,486,530. Measurement of the immune response also includes detection or determination of B cell activation events that may precede antibody production, or signal an increase in antibody production. Such measurements include, B cell proliferation assays, phosphorylation assays, assays of intracytoplasmic free calcium concentration, and other methods of determining B cell activation known in the art. Representative assays are provided in Mongini et al., J. Immunol. 159:3782-91 (1997); Frade, et al., BBRC 188:833-842 (1992); Tsokos et al., J. Immunol. 144:1640-1645 (1990); Delcayre et al., BBRC 159:1213-1220 (1989); and Nemerow et al., J. Immunol. 135:3068-73 (1985).

The practice of the invention includes promoting, enhancing or stimulating an immune response. These actions refer to establishing an immune response that did not previously exist; to optimizing or increasing a desired immune response; to establishing or increasing a secondary response characterized by increased isotype switching, memory response, or both; to providing a statistically increased immunoprotective effect against a pathogen; to generating an equivalent or greater humoral immune response, or other measure of B cell activation, from a reduced or limiting dose of antigen; to generating an increased humoral immune response, or other measure of B cell activation, in response to an equivalent dose of antigen; or to lowering the affinity threshold for B cell activation in vivo or in vitro.

Preferably, an immunostimulatory amount refers to that amount of vaccine that is able to stimulate an immune response in a patient which is sufficient to prevent, ameliorate, or otherwise treat a disease or condition. Similarly, an adjuvanting amount is that amount of adjuvant which, when administered with an antigen, enhances the specific immune response to the antigen. Treatment may be defined as promoting, enhancing, or stimulating an immune response against a

moiety or antigen in a patient, or for the benefit of a patient. Such treatment may be for any purpose including experimental, prophylactic, or ameliorative.

Treatment comprises administering the pharmaceutical composition by any method familiar to those of ordinary skill in the art, including intravenous, intraperitoneal, intracorporeal injection, intra-articular, intraventricular, intrathecal, intratonsillar, intramuscular, subcutaneous, topically, intranasally, intravaginally, or orally. The preferred methods of administration are intravenous, intramuscular, intranasal, oral, and subcutaneous injections. The composition may also be given locally, such as by injection into the particular area, either intramuscularly or subcutaneously. The immunological composition may be administered in a slow-release form such as slow-release capsules, pellets, osmotic delivery devices, or pumps.

Secondary booster immunizations may be given at intervals ranging from one week to many months later. The dosage of the primary and secondary inocula can be readily determined by those of ordinary skill in the art, but an acceptable range is 0.01 µg to 100 µg per inoculum.

Any pharmaceutically acceptable carrier can be employed for administration of the composition or vaccine of the invention. Carriers can be solids, powders or liquids, such as water, oils, including petroleum oil, animal oil, vegetable oil, peanut oil, soybean oil, mineral oil, sesame oil, and the like. With intravenous administration, sterile isotonic aqueous solutions are preferred carriers. Saline solutions, aqueous dextrose, and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, 18th Edition (A. Gennaro, ed., Mack Pub., Easton, Pa., 1990).

The immunological composition may also be formulated with solubilizing agents, emulsifiers, stabilizers, flavorants, and other components, including adjuvants. An adjuvant is herein defined as any composition which, when combined with an antigen, enhances the specific immune response to the antigen. Common

adjuvants include alum, Freund's, Titermax (CytRyx Corp.), RIBI T-700 adjuvant (RIBI Immunochemical), and STIMULON adjuvant QS 21 (Aquila Biopharm.).

The CR2 stimulatory activity of Gp350/220 sequences may be substituted with other molecules that bind to CR2 or to CR2-associated proteins such as CR1 and CD19. Specifically, these include: complement C3d, C3dg, C3bi, iC3b, and peptides thereof which contain the hexapeptide LYNVEA and bind to CR2, as well as antibodies directed against CR1, CR2, or CD19. However, the use of autologous sequences in a vaccine raises the possibility of eliciting an autoimmune response.

This scenario is of particular concern where the endogenous protein is highly stimulatory to B cells. Although the immune system normally (and necessarily) recognizes complement components as "self" antigens, the conjugation or fusion of C3d, or related peptides, to a foreign antigen presents this protein in an unusual context. Moreover, such constructs may display C3d epitopes that are rarely encountered in a natural setting. Indeed, elicitation of an autoimmune response against complement components may be particularly favored because multiple copies of these molecules are required to elicit an adjuvanting response, and this arrangement is not found in nature. In any event, presenting a host with an altered form of C3d raises the very real possibility of breaking tolerance to C3d and thereby inducing antibodies to a plethora of complement components derived from C3. As is readily appreciated by those in the art, the generation of antibodies against complement could result serious autoimmune pathologies.

In contrast, Gp350/220 sequences are highly preferred as adjuvants and adjuvanting components of immunostimulatory compositions, as compared to complement components, for the following additional reasons:

1) Antibodies raised against epitopes of the Gp350/220 adjuvant may themselves be beneficial in providing protection against EBV infection or infectivity.

2) Where the antigen linked to the complement component is of low molecular weight, the resulting construct would be of low molecular weight as well. The in vivo half-life of low molecular weight constructs is often short and this rapid elimination detracts from immunogenicity.

In contrast, compositions based on the larger Gp350/220 polypeptides will be expected to have a longer effective half-life than those based on C3d.

3) Effective antigen presentation depends on cross-linking of the antigen receptors on a B cell. Because more copies of antigen can be ligated to the larger Gp350/220 proteins than to C3b, constructs based on Gp350/220 will be more antigenic.

4) Antibodies raised against CR1, CR2, or CD19 are expensive and difficult to produce. Moreover, vaccination with antibody sequences can elicit undesirable immune responses, including autoimmune reactions.

5) The safety and efficacy of Gp350/220 vaccine components has already been examined, whereas the toxicity of C3 components is uncertain. Because complement activation triggers the acute inflammatory response, it is possible that complement-based adjuvants will stimulate inflammation.

6) It has been suggested in the field that C3d-fusion proteins are difficult to synthesize and purify, possibly due to problems in folding recombinantly produced C3d polypeptides.

7) Proper folding of C3d is critical to CR2 binding. In genetically engineered constructs with antigen, there may be antigens that distort the folding of C3d and reduce or eliminate its binding to the receptor. In contrast, folding of the CR2-binding domain in the larger Gp350/220 proteins is less likely to be disrupted by fusion with antigen.

The present invention is illustrated by the following Examples, which are not intended to be limiting in any way.

Example 1

Preparation of Conjugates and Controls

Reagents

Purified recombinant EBV Gp350 and Respiratory Syncitial Virus (RSV) glycoprotein FG were obtained from SmithKline Beecham Biologicals at 538 μg/ml and 319 μg/ml respectively. These proteins were then concentrated using a FILTRON Microsep concentrator to 1 mg/ml and 0.8 mg/ml, respectively. 5XHE is 75mM HEPES, 10mM EDTA, pH 7.3. SATA (N-hydroxysuccinimidyl S-acetylthioacetate from BioAffinity Systems), was prepared as a 10mM solution in dimethylformamide (DMF). The human IgD-specific monoclonal antibody δIA6.2 (25mg/ml in 150mM HEPES, 2mM EDTA, pH 7.3) was a gift from Dr. John Kearny of the University of Alabama, Birmingham, and is described in Halista et al., Ped. Res. 43:496-503 (1998). SIA (N-Hydroxysuccinimidyl iodoacetate from BioAffinity Systems) was 10mM in DMF, except for preparation of anti-Ig--Dex where it was 100mM in DMF. HEH is 0.5M hydroxylamine in 5XHE.

Gp350 and RSV FG were coupled to anti-IgD antibodies to create anti-IgD--Gp350 and anti-IgD--FG, as generally described in Lees et al., J. Immunol. 145:3594-3600 (1990). Briefly, 0.5mg (500 μl) of Gp350 was thiolated by mixing with 50μl of 10mM SATA in DMF to generate Gp350-SATA. Similarly, 0.5mg (625 μl) of FG was added to 25 μl 5XHE and 50 μl of 10mM SATA in DMF to generate FG-SATA. To iodacetylate the anti-IgD antibody 2mg (80μl) of δIA6.2 was added to 13.3μl of 10mM Iodoacetamide in water. After 10 minutes, 26.6μl of 10mM SIA in DMF was added to generate δIA6.2-SIA.

Each of the above reactions was allowed to proceed for ~2 hours at RT then dialyzed overnight against 10mM MES, 150mM NaCl, 2mM EDTA, pH 6. The SIA reaction was kept in the dark.

anti-Ig--Gp350 (anti-IgD--Gp350)

169μl (~0.5mg) of δIA6.2-SIA was added to 560μl (~0.5mg) Gp350-SATA and 80μl of HEH. The reaction was then concentrated to ~200μl using a FILTRON Microsep 10 and incubated overnight at 4°C in the dark. The reaction was then quenched by making 0.2mM in mercaptoethanol for 1 hour, followed by making 10mM in iodoacetamide. The conjugate was run over a 1X60 cm S400HR column equilibrated with PBS. The void volume fractions were pooled and sterile filtered through a Millipore 0.2μ Millex filter. The resulting anti-Ig--Gp350 preparation contained less than 5% unconjugated δIA6.2 as determined by HPLC analysis.

anti-Ig--FG (anti-IgD--FG)

169μl (~0.5mg) of δIA6.2-SIA was added to 890μl (~0.5mg) FG-SATA and 117μl of HEH. The reaction was then concentrated to ~200μl using a FILTRON Microsep 10 and incubated overnight at 4°C in the dark. The reaction was then quenched by making 0.2mM in mercaptoethanol for 1 hour, followed by making 10mM in iodoacetamide. The conjugate was run over a 1X60 cm S200HR column equilibrated with PBS. The void volume fractions were pooled and sterile filtered through a Millipore 0.2μ Millex filter. HPLC analysis was used to determine that the anti-Ig--FG preparation contained less than 20% unconjugated δIA6.2.

The protein concentrations of anti-Ig--Gp350 and anti-Ig--FG conjugates were estimated by OD_{280} using 1mg/ml/absorbance unit.

anti-Ig control (anti-IgD)

112μl (~0.33mg) of δIA6.2-SIA was added to 12.5μl of HEH. The reaction was incubated overnight at 4°C. The reaction was then quenched by making 0.2mM in mercaptoethanol for 1hour, followed by making 10mM in iodoacetamide. The reaction was then dialyzed against PBS to provide an anti-Ig control. The protein concentrations of the anti-Ig control was estimated by OD_{280} using 0.7 mg/ml/absorbance unit.

anti-Ig--Dextran (anti-IgD-Dex)

High molecular weight dextran T2000 (Pharmacia) was conjugated to δIA6.2 essentially as described in Lees et al., Vaccine 12:1160-66 (1994); and U.S. Patent No. 5,585,100 (Mond and Lees). AECM dextran was prepared by the method of Brunswick et al., J. Immunol. 140:3364 (1989) and fractionated on an S400HR column. The size-fractionated AECM dextran was suspended in saline to 15.5 mg/ml. DEX-SIA was generated by mixing 774μl of the AECM dextran with 100μl 5XHE and 100μl of 100mM was in DMF. In a separate reaction, 3mg of δIA6.2 (20mg/ml in PBS) was mixed with 50μl of 5XHE and 24μl of 10mM SATA in DMF. Each reaction was incubated for ~2hr at RT then dialyzed overnight against 10mM sodium acetate, 100mM NaCl, 2mM EDTA, pH 5, in the dark.

Approximately 3 mg of the DEX-SIA and about 3 mg of the SATA-treated antibody were combined with 75μl of 5XHE containing 0.5M hydroxylamine. The reaction was allowed to proceed overnight at 4° in the dark. The reaction was then quenched by making 0.2mM in mercaptoethanol for 1 hour, followed by making 10mM in iodoacetamide to consume unreacted thiol groups. Unconjugated protein was removed by gel filtration on a 1X60 cm S400HR column equilibrated with PBS. The void volume fractions are pooled and sterile filtered through a Millipore 0.2μ Millex filter. Protein concentration was determined from OD_{280} using 0.71 mg/ml protein/absorbance unit. Dextran concentration was determined using the resorcinol assay of Monsigny et al., Anal. Chem 175:525 (1988). The protein/dextran ratio of the conjugate was determined to be about 1mg/mg.

anti-CR2 antibody

Anti-CR2-specific antisera HB5 was a kind gift from Dr. George Tsokos (Uniformed Services University of the Health Sciences, Bethesda, MD.).

Example 2

Demonstration of the Adjuvanting Effect of Gp350/220 Sequences

The enhanced immunostimulatory effect obtained by administering the compositions of the invention is demonstrated by the following in vitro model. B cells were purified from human peripheral human blood by standard techniques. The purified B cells were then cultured in microtiter plates at 200,000 cells per well in the presence or absence of various concentrations of Gp350, anti-Ig antibodies, FG, anti-Ig--dextran, or anti-Ig--Gp350 described in Example 1. Tritiated thymidine was added to the culture 48 hours after the presentation of the stimuli. 18 hours following the addition of tritiated thymidine, the cells were harvested and the amount of incorporated tritium was determined by liquid scintillation spectrometry.

Anti-Ig--Gp350 provides an in vitro model for the antigen-containing compositions of the invention wherein cross-linking of membrane bound Ig receptor by the anti-IgD antibodies simulates the cross-linking of Ig receptors by antigen. The RSV viral coat glycoprotein FG conjugated to anti-IgD was used as a control for the presentation of a similarly-sized protein that is not known to bind CR2. The amount of incorporated tritium reflects the proliferative activity of the cells. This in turn, provides a measure of the immunostimulatory effect of the tested compounds.

The data in Table I demonstrate that anti-Ig conjugated to Gp350 stimulated high levels of proliferation even at concentrations as low as .01 μg/ml. In contrast, anti-Ig conjugated to glycoprotein FG was not stimulatory.

This experiment demonstrates that Gp350/220 provides an excellent carrier for antigens of interest. The Gp350/220 sequences provide an adjuvanting effect which enhances immune responsiveness even at low antigen concentrations.

Table I

ENHANCED STIMULATORY ACTIVITY OF ANTI-Ig CONJUGATEDTO Gp350 ON HUMAN B LYMPHOCYTES

	Concentration of Stimuli (µg/ml)				
	0	10	1	0.1	.01
Stimuli	**Thymidine Incorporation (cpm)**				
(medium)	1615				
Gp350		3,154	1,626	2,314	2,612
anti-Ig--FG		6,196	4,353	2,238	ND
anti-Ig control		2,320	2,516	2,722	2,811
anti-Ig--dextran		20,699	19,292	26,826	27,830
anti-Ig--Gp350		19,223	19,047	20,673	20,949

Example 3

The Adjuvanting Effect of Gp350/220 Sequences is Attenuated by Anti-CR2 Antibodies

B cells were purified from human peripheral human blood and cultured in microtiter plates at 200,000 cells per well in the presence or absence of stimuli (anti-Ig--dextran or anti-Ig--Gp350) with or without anti-CR2 antibody HB5. Tritiated thymidine was added to the culture 72 hours after the presentation of the stimuli. 18 hours following the addition of tritiated thymidine, the cells were harvested and the amount of incorporated tritium was determined by liquid scintillation spectrometry.

The data in Table 2 shows that the stimulatory effect of anti-Ig--dextran is enhanced by the addition of antibodies specific for CR2. In contrast, the stimulatory effect of anti-Ig--Gp350 is attenuated by addition of anti-CR2, suggesting that the Gp350 moiety of anti-Ig--Gp350 acts through the CR2 complex.

Table 2

ANTIBODIES DIRECTED AGAINST CR2 ATTENUATE THE STIMULATORY ACTIVITY OF ANTI-IgD CONJUGATED TO Gp350

	Concentration of anti-CR2 antibody (µg/ml)			
	0	100	10	1
Stimuli (ug/ml)	Thymidine Incorporation (cpm)			
(medium)	915 +/- 157	184 +/- 31	318 +/- 72	468 +/- 89
anti-Ig--Gp350 (1)	8,472 +/- 1,228	1,013 +/- 83	1,126 +/- 81	2,370 +/- 16
anti-Ig--Gp350 (10)	19,759 +/- 1,710	1,007 +/- 547	2,606 +/- 331	7,275 +/- 1,362
anti-Ig--dextran (1)	2,184 +/- 262	25,706 +/- 324	14,453 +/- 900	4,135 +/- 76
anti-Ig--dextran (10)	13,195 +/- 1,325	16,066 +/- 2,485	29,325 +/- 2,390	20,968 +/- 1,277

Example 4

Gp350/220 Sequences Initiate an Extended B Cell Stimulatory Response

The percentage of cells responding to anti-Ig--Gp350 was determined using the indo-1 loading assay as described in Brunswick et al., Proc. Nat'l. Acad. Sci. USA 86:6724-28 (1989). Briefly, B cells, purified from human peripheral human blood, were loaded with indo-1 and stimulated by the addition of anti-Ig, anti-Ig--dex, and anti-Ig--Gp350 to a final concentration of 1.0 ug/ml. Calcium flux was measured and used to calculate the percentage of cells responding to the stimuli.

Figure 2 shows the percentage of cells responding over the course of the assay. Anti-Ig--Gp350 and anti-Ig--dex both activate approximately 40% of the B cells within four minutes of stimulation. This level remains relatively constant until at least eight minutes post-stimulation (the duration of the assay). In contrast, the stimulatory effect of unconjugated anti-Ig antibodies (anti-IgD) peaks at two minutes after stimulation and declines to 20% by eight minutes.

Example 5

Low Levels of Gp350/220 Sequences are required to Initiate an Extended B Cell Stimulatory Response

B cell sensitivity to stimulation by anti-Ig--Gp350 was analyzed using the indo-1 loading assay described in Example 4. Briefly, indo-1 loaded B cells were stimulated with various concentrations of anti-Ig--Gp350 and calcium flux was measured every minute for nine minutes. Figure 3 shows that as little as 0.1 ug/ml of anti-Ig--Gp350 generates a stable response in about one third of the isolated B cells.

The specification is most thoroughly understood in light of the teachings of the references citied within the specification. The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the

scope of the invention. The skilled artisan recognizes that many other embodiments are encompassed by the claimed invention and that it is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

SEQUENCE LISTING

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<120> ENHANCEMENT OF B CELL ACTIVATION AND IMMUNOGLOBULIN SECRETION BY CO-STIMULATION OF RECEPTORS FOR ANTIGEN AND EBV Gp350/220

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Pro Arg Pro Arg Tyr Asn Ala Thr Thr Tyr Leu Pro Pro Ser Thr Ser
565 570 575

Ser Lys Leu Arg Pro Arg Trp Thr Phe Thr Ser Pro Pro Val Thr Thr
580 585 590

Ala Gln Ala Thr Val Pro Val Pro Pro Thr Ser Gln Pro Arg Phe Ser
595 600 605

Asn Leu Ser Met Leu Val Leu Gln Trp Ala Ser Leu Ala Val Leu Thr
610 615 620

Leu Leu Leu Leu Leu Val Met Ala Asp Cys Ala Phe Arg Arg Asn Leu
625 630 635 640

Ser Thr Ser His Thr Tyr Thr Thr Pro Pro Tyr Asp Asp Ala Glu Thr
645 650 655

Tyr Val

<210> 3

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: illustrative hexapeptide

<400> 3

Leu Tyr Asn Val Glu Ala
1 5

CLAIMS

1. Use of:

at least one Epstein-Barr virus (EBV) Gp350/220 molecule covalently conjugated to at least one protein, peptide, polypeptide, or polysaccharide comprising an antigenic epitope other than an epitope of EBV Gp350/220, wherein said EBV Gp350/220 molecule binds to a CR2 receptor (CR2);

such that said EBV Gp350/220 molecule and said protein, peptide, polypeptide, or polysaccharide are on the same molecule;

for the preparation of a medicament for promoting a humoral immune response with respect to said antigenic epitope other than an epitope of EBV Gp350/220.

2. The use of claim 1 wherein the at least one Gp350/220 molecule comprises amino acids 21-26 of SEQ ID NO. 1 or SEQ ID NO. 2.

3. The use of claim 1 or 2 wherein the at least one Gp350/220 molecule comprises amino acids 372-378 of SEQ ID NO. 1 or SEQ ID NO. 2.

4. The use of any one of claims 1-3 wherein the at least one Gp350/220 molecule is a fragment, amino acid variant, or derivative of Gp350/220 that binds to a CR2 receptor.

5. The use of any one of claims 1-4 wherein the medicament comprises at least two Epstein-Barr virus (EBV) Gp350/220 molecules that bind to CR2.

6. The use of any one of claims 1-5 wherein the medicament comprises multiple copies of said EBV Gp350/220 molecule that bind to CR2.

7. The use of any one of claims 1-6 wherein the medicament comprises a multiplicity of said protein, peptide, polypeptide, or polysaccharide comprising an antigenic epitope other than an epitope of EBV Gp350/220.

8. The use of any one of claims 1-7 wherein said protein, peptide, polypeptide, or polysaccharide comprising an antigenic epitope other than an epitope of EBV Gp350/220 comprises multiple copies of said antigenic epitope other than an epitope of EBV Gp350/220.

9. The use of any one of claims 1-8 wherein said protein, peptide, polypeptide, or polysaccharide comprising an antigenic epitope other than an epitope of EBV Gp350/220 is selected from haptens, T cell-dependent antigens, and Type 2 T cell-independent antigens.

10. The use of any one of claims 1-9 wherein the medicament elicits antibodies for an allergen, a tumor antigen, or an infectious disease other than EBV.

11. The use of any one of claims 1-10 wherein the medicament further elicits antibodies for EBV.

12. The use of any one of claims 1-11 wherein the medicament further comprises a pharmaceutically acceptable carrier.

13. The use of any one of claims 1-12 wherein the conjugation is by 1-cyano-4-“dimethylamino”-pyridinium tetrafluoroborate (CDAP) chemistry.

14. The use of any one of claims 1-13 wherein the at least one protein, peptide, polypeptide, or polysaccharide comprising an antigenic epitope other than an epitope of EBV Gp350/220 and at least one EBV Gp350/220 molecule comprises a recombinant fusion protein.

15. The use of any one of claims 1-14 wherein at least two proteins, peptides, polypeptides, or polysaccharides each comprising an antigenic epitope other than an epitope of EBV Gp350/220 and at least one EBV Gp350/220 molecule comprises a recombinant fusion protein.

16. The use of any one of claims 1-15 wherein the medicament is in a form of a biological vector.

17. The use of claim 16 wherein the biological vector is selected from: a plant or plant product; bacterium; parasite; virus or virus-like particle; and a yeast, mammalian, or other eukaryotic cell.

18. The use of any one of claims 1-17 wherein said at least one EBV Gp350/220 molecule is expressed on the surface of a bacterium, viral capsid or envelope, parasite, or mammalian tumor cell.

19. The use of claim 18 wherein the medicament enhances the immune response to bacterial, viral, parasitic, or tumor-specific antigens.

20. The use of any one of claims 1-19 wherein said EBV Gp350/220 molecule is conjugated to said protein, peptide, polypeptide, or polysaccharide such that one or more natural, synthetic, or chemical molecule, spacer, linker, amino acid, polypeptide, protein, hapten, antigen, or polysaccharide intervenes between said EBV Gp350/220 molecule and said protein, peptide, polypeptide, or polysaccharide comprising said antigenic epitope other than an epitope of EBV Gp350/220.

21. The use of any one of claims 1-7 wherein each protein, peptide, polypeptide, or polysaccharide comprising an antigenic epitope other than an epitope of EBV Gp350/220 comprises a single copy of said antigenic epitope other than an epitope of EBV Gp350/220.

FIG. 1

AMINO ACID SEQUENCE OF EPSTEIN-BARR VIRUS Gp350/220
Beisel et al., J. Virol. 54, 665-674 (1985)
Translated from GENBANK accession number M10593

FIG. 1A

Epstein-Barr virus major outer envelope glycoprotein Gp350.

SEQ ID NO:1

1 meaallvcqy tiqslihltg edpgffnvei pefpfyptcn vctadvnvti nfdvggkkhq
61 ldldfgqltp htkavyqprg afggsenatn lfllellgag elaltmrskk lpinvttgee
121 qqvslesvdv yfqdvfgtmw chhaemqnpv ylipetvpyi kwdncnstni tavvraqgld
181 vtlplslpts aqdsnfsvkt emlgneidie cimedgeisq vlpgdnkfni tcsgyeshvp
241 sggiltstsp vatpipgtgy ayslrltprp vsrflgnnsi lyvfysgngp kasggdyciq
301 snivfsdeip asqdmptntt dityvgdnat ysvpmvtsed anspnvtvta fwawpnntet
361 dfkckwtlts gtpsgcenis gafasnrtfd itvsglgtap ktliitrtat natttthkvi
421 fskapesttt sptlnttgfa dpntttglps sthvptnlta pastGptvst advtsptpag
481 ttsgaspvtp spspwdngte skapdmtsst spvttptpna tsptpavttp tpnatsptpa
541 vttptpnats ptlgktspts avttptpnat sptlgktspt savttptpna tsptlgktsp
601 tsavttptpn atGptvgets pqanatnhtl ggtsptpvvt sqpknatsav ttgqhnitss
661 stssmslrps snpetlspst sdnstshmpl ltsahptgge nitqvtpasi sthhvstssp
721 eprpgttsqa sGpgnsstst kpgevnvtkg tppqnatspq apsgqktavp tvtstggkan
781 sttggkhttg hgartstept tdyggdsttp rprynattyl ppstssklrp rwtftsppvt
841 taqatvpvpp tsqprfsnls mlvlqwasla vltllllvm adcafrrnls tshtyttppy
901 ddaetyv

FIG. 1B

Epstein-Barr virus major outer envelope glycoprotein Gp220.

SEQ ID NO:2

1 meaallvcqy tiqslihltg edpgffnvei pefpfyptcn vctadvnvti nfdvggkkhq
61 ldldfgqltp htkavyqprg afggsenatn lfllellgag elaltmrskk lpinvttgee
121 qqvslesvdv yfqdvfgtmw chhaemqnpv ylipetvpyi kwdncnstni tavvraqgld
181 vtlplslpts aqdsnfsvkt emlgneidie cimedgeisq vlpgdnkfni tcsgyeshvp
241 sggiltstsp vatpipgtgy ayslrltprp vsrflgnnsi lyvfysgngp kasggdyciq
301 snivfsdeip asqdmptntt dityvgdnat ysvpmvtsed anspnvtvta fwawpnntet
361 dfkckwtlts gtpsgcenis gafasnrtfd itvsglgtap ktliitrtat natttthkvi
421 fskapesttt sptlnttgfa dpntttglps sthvptnlta pastGptvst advtsptpag
481 ttsgaspvtp spspwdngte stppqnatsp qapsgqktav ptvtstggka nsttggkhtt
541 ghgartstep ttdyggdstt prprynatty lppstssklr prwtftsppv ttaqatvpvp
601 ptsqprfsnl smlvlqwasl avltllllv madcafrrnl stshtyttpp yddaetyv

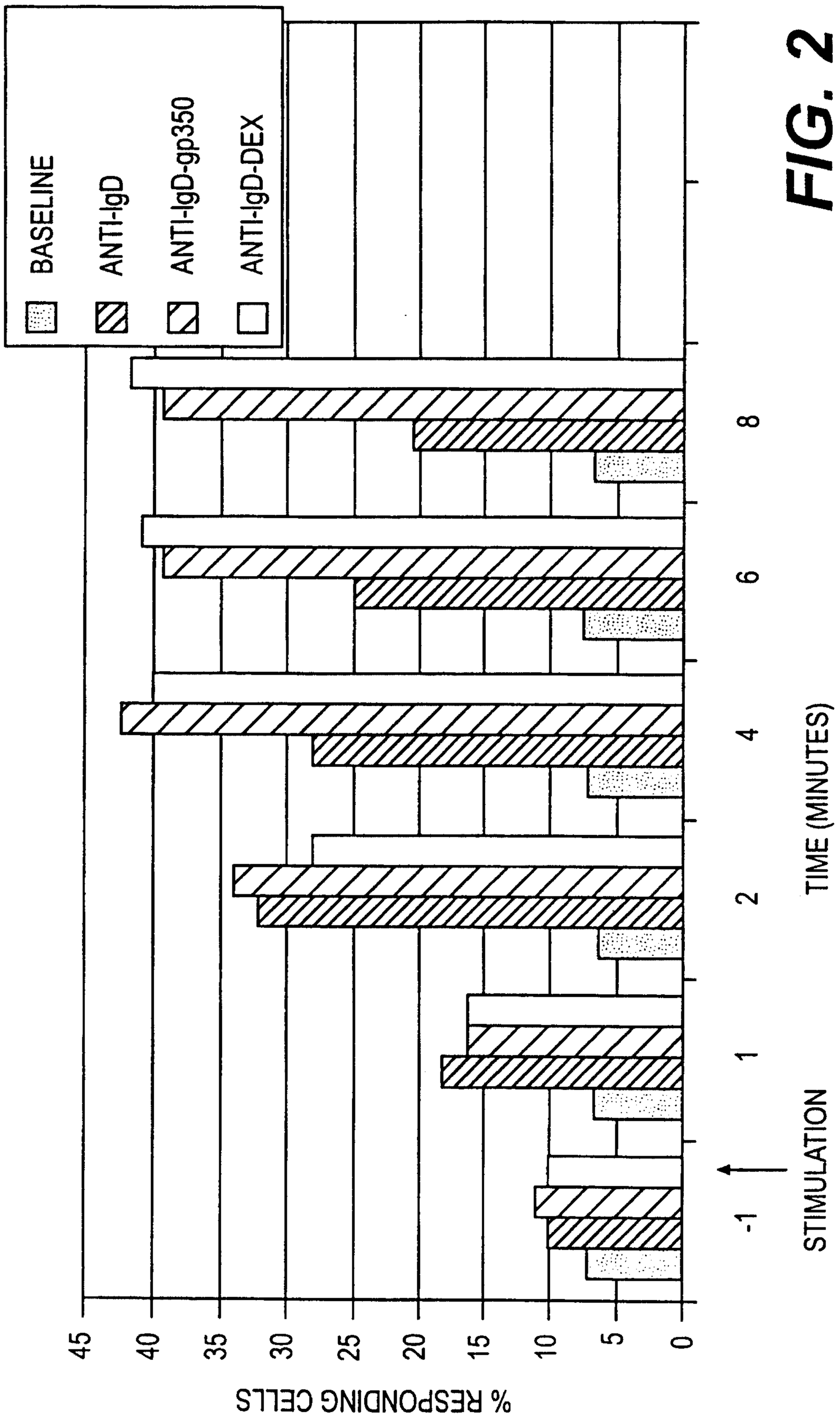

BASELINE
ANTI-IgD
ANTI-IgD-gp350
ANTI-IgD-DEX
8
6
4
2
1
-1
TIME (MINUTES)
STIMULATION
45
40
35
30
25
20
15
10
5
0
% RESPONDING CELLS

FIG. 2

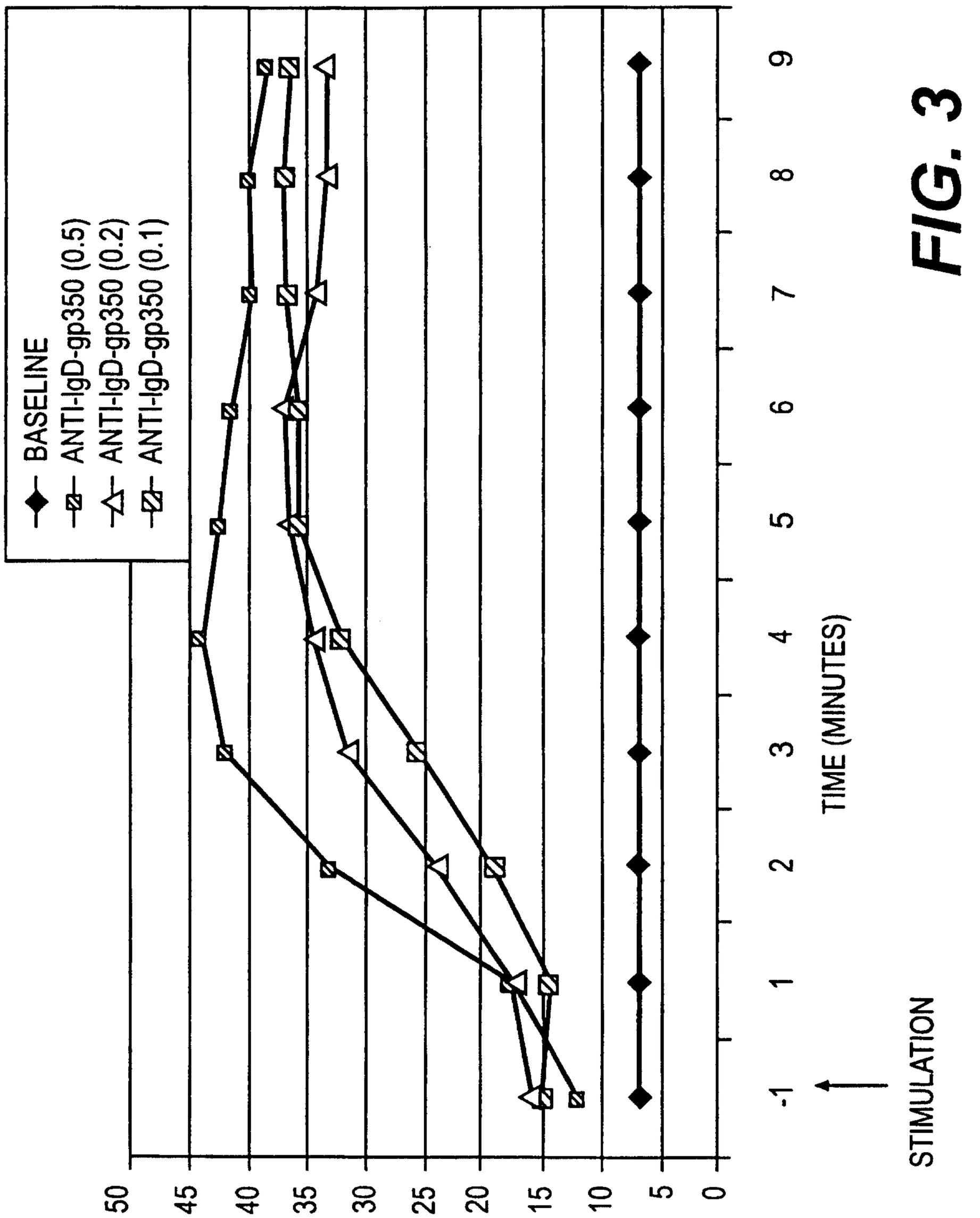
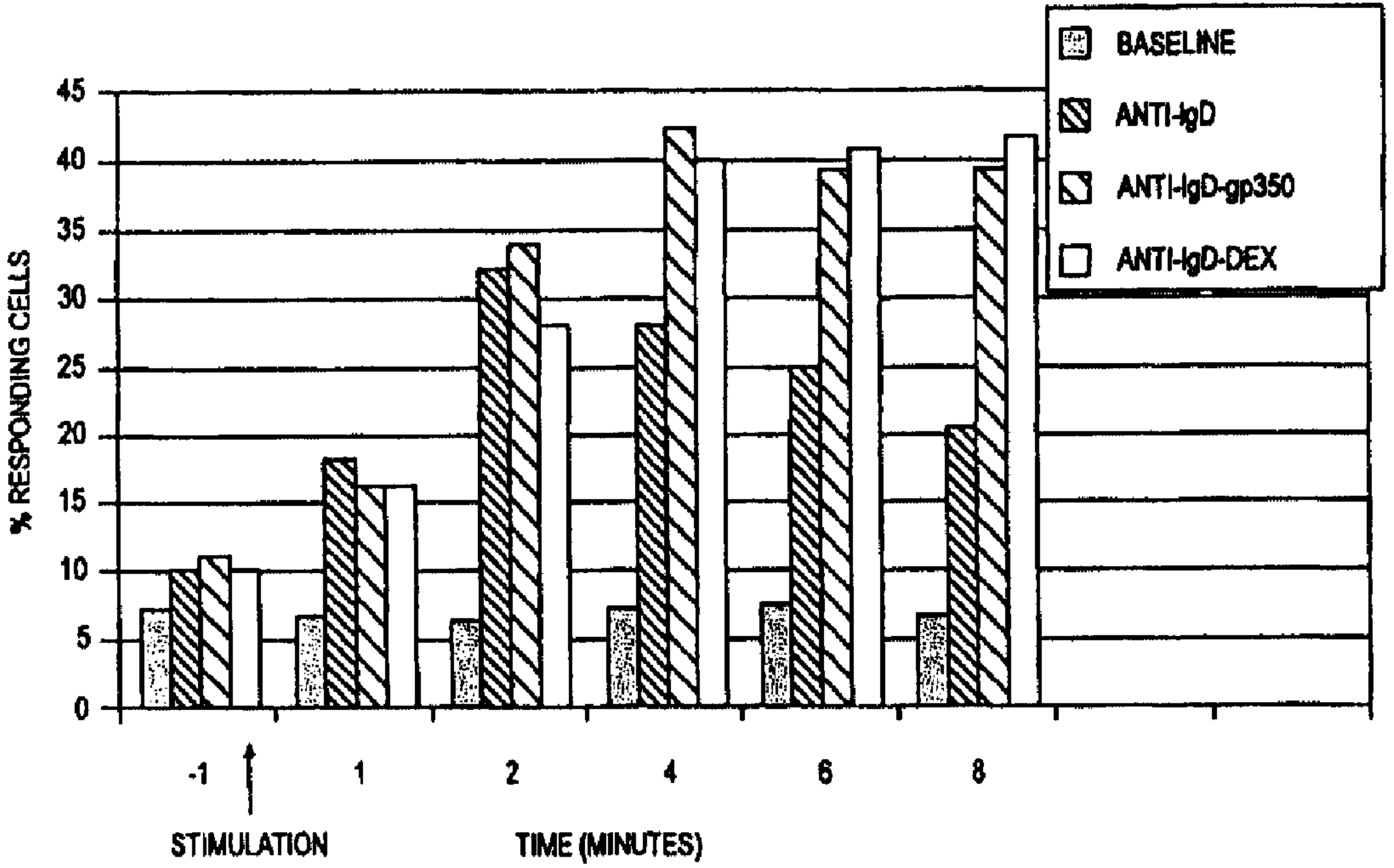

BASELINE
ANTI-IgD-gp350 (0.5)
ANTI-IgD-gp350 (0.2)
ANTI-IgD-gp350 (0.1)
% RESPONDING CELLS
50
45
40
35
30
25
20
15
10
5
0
STIMULATION
-1
1
2
3
4
TIME (MINUTES)
5
6
7
8
9

FIG. 3


BASELINE
ANTI-IgD
ANTI-IgD-gp350
ANTI-IgD-DEX
% RESPONDING CELLS
45
40
35
30
25
20
15
10
5
0
-1
1
2
4
6
8
STIMULATION
TIME (MINUTES)