INDUCTION AND ENHANCEMENT OF THE IMMUNE RESPONSE TO POLYSACCHARIDES WITH BACTERIAL LIPOPROTEINS

GOVERNMENT INTEREST

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

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of application Serial No. 08/472,640, filed June 7, 1995, which is a continuation-in-part of application serial No. 08/422,830, filed April 17, 1995.

FIELD OF THE INVENTION

The present invention relates to the use of bacterial lipoproteins in inducing humoral immunity in response to polysaccharide antigens and other T cell-independent antigens.

BACKGROUND

The cellular basis for induction of T cell-independent (TI) humoral immunity to bacterial organisms and their antigenic constituents is largely unknown, thus hampering efforts to develop sufficient defenses against bacterial infection. This poses serious problems, given the prevalence and significance of TI antigens from bacterial organisms such as *Haemophilus influenzae* type b polyribosyl-ribitolphosphate (PRP), Pneumococcal capsular polysaccharides (including type III), Group B Streptococcus serocharides, and *P. aeruginosa* capsular polysaccharides (including strain Fisher type 1).

This dearth of knowledge aside, immunologists do know that immunity requires the stimulation of B cell proliferation as well as Ig secretion. In related patent application Serial 08/315,492, filed September 30, 1994, the inventors previously demonstrated that B cells activated by αδ-dex, a construct that mimics the repetitive nature of the

type 2 class of T cell-independent antigens, required the presence of the cytokines IL-5, IL-3, GM-CSF, and/or IFN-γ to induce strong Ig secretory responses *in vitro*. The Ig-inducing activity of IL-3, GM-CSF, and IFN-γ, but not IL-5, required costimulation with IL-2. The implication of this work is that type 2 T cell-independent antigens may similarly not be able to stimulate Ig responses in the absence of cytokines.

The source of the cytokines which are required for immune responses to TI antigens is unknown but may be T cells, NK cells, monocytes, and other cytokine-producing cells. Immunocompromised patients, such as neonates, the elderly, those with HIV disease or patients undergoing chemotherapy, may not have the T cells or functional NK cells or monocytes that produce adequate amounts of cytokines for induction of optimal humoral immunity. Without additional help, these patients may not be able to mount a defense against TI antigens.

Moreover, the immune response of immunocompetent normal individuals to polysaccharide or other TI antigens is, in general, of low magnitude and low avidity. This reflects the absence of recruitment of T cell derived help. To date, the most effective way of generating an immune response to polysaccharide antigens has been to conjugate T cell epitopes to the polysaccharides (i.e., conjugate vaccines). These constructs, which stimulate T cell help, also enhance the response to the polysaccharide. While these conjugate vaccines provide benefit, those in the art recognize the many disadvantages associated with their use.

Even where individuals are able to mount an immune response, as for example after vaccination with a large inoculum of antigens, that response may require the coadministration of adjuvants. The most common adjuvants used in man are aluminum compounds (phosphate and hydroxide), such as alum. Alum, however, does not adjuvant all antigens (for reasons not entirely clear but perhaps due

to a charge effect) and both alum and other experimental adjuvants may cause inflammatory responses.

Thus, there is a need in the art for methods to induce immunoglobulins either to antigens that by themselves may not be able to induce adequate amounts of T cell help or cytokine-derived help, as for example, polysaccharides, as well as a need in the art to enhance the immune response in individuals that lack the ability to recruit this type of help. There is also a need in the art to enhance the immune responses to those antigens where currently available adjuvants are ineffective.

SUMMARY OF THE INVENTION

The present invention addresses these needs by providing a method of inducing the immune response to polysaccharides and other TI antigens by the coadministration of either microbial or synthetic lipoproteins. This coadministration includes injection of lipoproteins together with an antigen or a vaccine, or covalently attached to the antigen or the vaccine, as well as injection of the synthetically-derived active moiety of lipoproteins either together with antigen or covalently attached to the antigen. The lipoproteins of the present invention also provide a method of enhancing the immune response. In a preferred embodiment, the lipoprotein of the invention is lipoprotein D.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph depicting the over 25-fold enhancements in ^{3}H-TdR incorporation observed with combined αδ-dex + lipo-D stimulation, relative to that seen using αδ-dex alone.

FIG. 2 provides two graphs on the induction of Ig secretion by small resting B cells in the absence (Expt. A) or the presence (Expt. B) of αδ-dex, showing that lipo-D alone failed to stimulate significant Ig secretion but that the combined action of lipo-D and αδ-dex led to an over 10,000-fold induction in Ig secretion.

FIG. 3 is a series of charts demonstrating that lipo-D costimulates both IgM secretion and proliferation by αδ-dex-activated sort-purified B cells in a manner similar to that observed for the non-sorted B cell-enriched population.

FIG. 4 is a chart demonstrating that lipo-D costimulates IgA class switching to a degree similar to that seen with LPS.

FIG. 5 is a graph depicting the costimulation of IgM secretion by αδ-dex-activated-cells with lipo-OSPA.

FIG. 6 sets forth the anti-polysaccharide response (Fig. 6a) and the anti-protein response (Fig. 6b) between vaccines based on two different proteins, tetanus toxoid and lipoprotein D.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of inducing and enhancing the immune response to TI antigens by the coadministration of lipoproteins. As used herein, the immune response is the body's production of immunoglobulins, or antibodies, in response to a foreign entity. Inducing the immune response refers to establishing an immune response that did not previously exist whereas enhancing an immune response refers to optimizing or increasing a preexisting immune response.

As noted above, the foreign entity of interest in the present invention is the thymus cell (or T cell) -independent antigen or TI antigen. The TI antigen can induce an immune response by activating B cells directly without the apparent participation of T cells. Conversely, the thymus dependent antigen (TD) requires T cell help for antibody synthesis.

There are two known classes of TI antigens. Type 1 antigens, such as bacterial lipopolysaccharides, may activate B cells "polyclonally," that is, regardless of the antigen specificity of the B cell. Type 2 TI antigens are characterized by their linear nature and spaced highly repetitive determinants. Such antigens bind to antigenspecific B cells by cross-linking the Ig receptors on

This Sheet Has Been Replaced

the surface of the B cell, a process known as membrane (m)Ig-mediated signaling.

Much has been learned about mIg-mediated signaling in response to TI antigens based on a polyclonal *in vitro* model developed by the inventors. The inventors synthesized dextran-conjugated anti-IgD antibodies (αδ-dex) in order to simulate the repeating epitope nature of polysaccharides. αδ-Dex cross-links mIg in a multivalent fashion and induces potent and sustained B cell signaling. αδ-Dex, which stimulates proliferation by small resting B cells, fails to induce Ig secretion in the absence of exogenous cytokines, and recent studies show that TI antigens similarly cannot induce Ig secretion in the absence of such cytokines.

The requirement for cytokines may hamper the treatment of those who need it most: the immunocompromised patients. Such patients, who lack functional T cells, cannot produce cytokines and thus are at risk for infection by clinically relevant TI antigens such as polysaccharides derived from *Haemophilus influenzae* type b polyribosyl-ribitol-phosphate (PRP), *S. Pneumonia*, Group B Streptococcus, *N. meningitides*, *Salmonella*, *P. aeruginosa* mucoexopolysaccharides, and *P. aeruginosa* (including strain Fisher type 1). The coadministration of lipoprotein of this invention, however, results in B cell proliferation and Ig secretion even in the absence of cytokines, as set forth in detail below.

Lipoproteins have been previously shown to deliver non-mIg-mediated signals to B cells. Melchers, et al., 49 J. Exp. Med. (1975) 142:473. Prior studies on the B cell activating properties of lipoproteins, however, employed heterogenous populations of lymphoid cells in various stages of *in vivo*-preactivation and cultured at relatively high cell densities which tend to facilitate interactions of B cells with other cell types. Because these cells were not fractionated according to density and, hence, prior activation state, these studies left unresolved whether lipoproteins acted directly at the level of the resting B cell, whether additional cell types

played key roles in their action, and how lipoprotein-mediated signaling integrated functionally with other B cell stimuli, including mIg-mediated TI-2-like stimuli also present in bacterial cell walls.

In contrast to these other studies, the work that led to this invention used highly-enriched and sort-purified resting murine B cells. With this specific population of B cells, the data indicate that lipoproteins must act in concert with other stimuli to induce strong proliferative and Ig secretory responses. These data are consistent with a recent study using >98% pure, small resting human B cells, in which synthetic lipopeptide significantly enhanced proliferation and Ig secretion only when acting in concert with anti-CD40 antibody.

The lipoproteins of the present invention may be either of microbial origin or may be synthetic lipoproteins. The microbial lipoproteins are generally components of bacterial cell walls and include, but are not limited to, the distinct lipoproteins that have been identified in the cell walls of different bacteria. Erdile, L., et al., Inf. and Imm. (1993) 61:81. The lipoproteins of the current invention may also be derived from the genes encoding them, such as lipoprotein-D from *Haemophilus influenzae* and lipoprotein-OSPA from *Borrelia burgdorferi*. Id. and see Song et al., Infect. & Immun. (1995) 63(2):696.

The lipoproteins of the present invention also include synthetic lipid moieties, as typified by Pam_3Cys, that are structurally similar to the amino terminus of bacterial lipoproteins. Klein, B., et al., Immunology (1987) 61:29. When these synthetic lipid moieties are conjugated to a small peptide, they can mimic the B cell-activating properties of these molecules. Further, removal of this lipid moiety from bacterial lipoproteins renders them non-functional.

The lipoproteins of the claimed invention also include fragments or sections thereof that impart the proliferation and Ig secretion actions observed with lipoprotein D.

As set forth in the Examples, in contrast to previous studies, neither lipoprotein-D, lipoprotein-OSPA, nor Pam_3Cys by themselves stimulate significant proliferation or Ig secretion. However, in combination with TI-like, multivalent antigen receptor cross-linking, these molecules costimulate striking inductions of Ig secretion and marked enhancements in cellular proliferation in the absence of exogenous cytokines. Moreover, these act directly at the level of the B cell without a requirement for recruitment of non-B cell types. These data suggest an additional, novel pathway for induction of specific, T cell-independent humoral immunity in response to bacterial challenge.

The lipoproteins of the claimed invention may be coadministered with the antigen in any way familiar to those of ordinary skill in the art. For example, the lipoproteins may be simply co-injected with the antigen or bound directly to the antigen. Any form of chemical binding, including covalent, is within the scope of this invention. A preferred method of covalent conjugation is set forth in application Serial No. 08/482,661, filed June 7, 1995, which is a continuation-in-part of application Serial No. 08/408,717, filed March 22, 1995, which is a continuation-in-part of application Serial No. 07/124,491, filed September 23, 1993, the so-called "CDAP" conjugation method, the disclosures of which are specifically incorporated herein by reference. The invention also encompasses fusion proteins comprised of lipoproteins and the antigen of interest and/or also injection of the DNA from which these fusion proteins were derived.

In addition to an antigen, the lipoproteins of the claimed invention may be administered with a vaccine, such as the dual conjugate vaccines of application Serial No. 08/468,060, filed June 6, 1995 (a continuation-in-part of application Serial No. 08/402,565, filed March 13, 1995) and the dual conjugate vaccines of application Serial No. 08/444,727, filed May 19, 1995 (a continuation of 08/055,163, filed February 10, 1993), the disclosures of which are

specifically incorporated herein by reference. As with the antigen, the lipoproteins may be co-administered with the vaccine in any way familiar to those of ordinary skill in the art. As above, the lipoproteins may be simply co-injected with the vaccine or bound directly to the vaccine by, for example, the CDAP method mentioned above, although any form of chemical binding is within the scope of this invention.

When used as a vaccine, the lipoproteins of the claimed invention may be administered by any method familiar to those of ordinary skill in the art, but are preferably administered by intravenous, intramuscular, intranasal, oral, and subcutaneous injections. The dosage can be readily determined by those of ordinary skill in the art, but an acceptable range is .01 µg to 100 µg per inoculum. Secondary booster immunizations may be given at intervals ranging from one week to many months later. Similar approaches can be used in T cell depleted animals or humans.

For use of lipoprotein D as an adjuvant, typical doses may range from 0.1 to 100 µg per inoculum and may be given at the same site as the antigen or vaccine, or at a different site of injection.

Additional studies have shown that lipidation of protein D is important in enhancing its effectiveness as a carrier molecule, at least in mice and possibly in other species as well. Thus, protein D is not as effective as lipoprotein D in enhancing anti-polysaccharide responses when injected into mice as a protein-polysaccharide conjugate.

The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention.

Example 1

Materials and Methods

Mice. Female DBA/2 mice were obtained from the National Cancer Institute (Frederick, MD) and were used at 7-10 weeks of age. The experiments were conducted according to the

principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare Publ No. (National Institutes of Health) 78-23.

Culture medium. RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), L-glutamine (2 mM), 2-mercaptoethanol (0.05 mM), penicillin (50 µg/ml) and streptomycin (50 µg/ml) were used for culturing cells.

Reagents. αδ-Dex was prepared by conjugation of Hδ^a/1 (monoclonal mouse IgG2b (b allotype) anti-mouse IgD (a allotype)) to a high molecular weight dextran (2 x 10^6 M.W.) as previously described in Pecanha, L., et al., J. Immunol. (1991) 146:833. Approximately 9 Hδ^a/1 antibodies were conjugated to each dextran molecule. Pam_3Cys (S-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-cysteine) was obtained from Boehringer Mannheim Biochemica. A stock solution was prepared by dissolving 1 mg of Pam_3Cys in 1 ml of 95% ethanol, and stored at -20°C until used. Murine rIFN-γ prepared from Chinese hamster ovary cells, was obtained from Genentech (South San Francisco, CA). Murine recombinant IL-1, IL-2, IL-4, and IL-5 were obtained from Dr. Stephanie Vogel (USUHS, Bethesda, MD), Dr. Maurice Gately (Hoffman-La Roche, Nutley, NJ), Dr. Alan Levine (Searle, St. Louis, MO), and Dr. Richard Hodes (NIH, Bethesda, MD), respectively. Recombinant human TGF-β2 was obtained from Wendy Waegell (Celtrix Pharmaceuticals, Santa Clara, CA). Recombinant murine IL-3 and GM-CSF were purchased from Pharmingen. FITC-anti-CD3ε mAb (2C11) and FITC-rat IgG1 anti-mouse IgA mAb were purchased from Pharmingen (San Diego, CA). PE-labelled affinity-purified polyclonal goat anti-mouse IgM antibody was purchased from Southern Biotechnology Associates (Birmingham, AL). Monoclonal rat IgG2b anti-mouse $Fc_\gamma RII$ (2.4G2) was purified from ascites.

Preparation and culture of B cells. Enriched populations of B cells were obtained from spleen cells from which T cells

were eliminated by treatment with rat anti-Thy-I, anti-CD4, and anti-CD8 monoclonal antibodies, followed by monoclonal mouse anti-rat Igκ and complement. Cells were fractionated on the basis of their density over discontinuous Percoll gradients (Pharmacia, Piscataway, NJ) consisting of 70, 65, 60, and 50% Percoll solutions (with densities of 1.086, 1.081, 1.074, and 1.062 g/ml, respectively). The high density (small, resting) cells were collected from the 70 to 65% interface and consisted of ~90% B cells. Unless otherwise indicated these cells were used in the studies reported herein. Highly purified B cells were obtained by electronic cell sorting of membrane (m)IgM+CD3-cells on an EPICS Elite cytometer (Coulter Corp, Hialeah, FL) after staining T-depleted, small, resting spleen cells with FITC-anti-CD3ε + PE-anti-IgM antibodies. Sorted cells were immediately reanalyzed and found to be consistently >99% B cells. Functional assays were carried out in either 96- or 24 well flat-bottom Costar plates (Costar, Cambridge, MA). Cultured-cells were incubated at 1 x 10^5 cells/ml in a total volume of 200 µL (96-well plate) or 1 ml (24-well plate) at 37°C in a humidified atmosphere containing 6% CO_2.

Measurement of DNA synthesis. DNA synthesis was determined by ^{3}H-TdR uptake (2µCi/well; 6.7 Ci/nmol; 1mCi = 37 GBq; ICN, Irvine, CA) over a 4 hr period. Cells were harvested (PHD cell harvester, Cambridge Technology, Watertown, MA) onto glass fiber filters and [^{3}H]TdR incorporation was determined by liquid scintillation spectrometry.

Quantitation of secreted Ig isotype concentrations in culture SN. Ig isotype concentrations were measured by an ELISA assay. For determination of concentrations of secreted IgM, IgG3, (IgG1, IgG2b, IgG2a), and IgA in cult··re SN, Immulon 2, 96-well flat-bottomed ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with unlabelled affinity-purified polyclonal goat anti-mouse IgM, IgG3, IgG, and IgA antibodies (Southern Biotechnology

Associates, Birmingham, AL), respectively. Plates were then washed, blocked with FBS-containing buffer, and incubated with various dilutions of culture SN and standards. After washing, plates were incubated with alkaline phosphatase-conjugated affinity-purified, polyclonal goat anti-mouse IgM, IgG3, IgG1, IgG2b, IgG2a, and IgA antibodies (Southern Biotechnology Associates) as indicated, washed again, and a fluorescent product was generated by cleavage of exogenous 4-methyl umbilliferyl phosphate (Sigma) by the plate-bound alkaline phosphatase-conjugated antibodies. For determination of IgE concentrations, a similar procedure was followed except that plates were coated with monoclonal rat IgG2a anti-mouse IgE (clone EM95) [purified from ascites, obtained from Dr. Fred Finkelman, USUHS, Bethesda, MD], followed by samples and standards, then affinity-purified polyclonal rabbit anti-mouse IgE (obtained from Dr. Ildy Katona, USUHS, Bethesda, MD), then alkaline phosphatase-conjugated affinity-purified polyclonal goat anti-rabbit IgG (Southern Biotechnology Associates). Fluorescence was quantitated on a 3M FluoroFAST 96 fluorometer (Mountainview, CA) and fluorescence units were converted to Ig concentrations by interpolation from standard curves that were determined with known concentrations of purified myeloma Ig. Each assay system showed no significant cross-reactivity or interference from other Ig isotypes (IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA) found in the culture supernatants.

Flow cytometric analysis. Cells were first incubated for 20 min with 5 µg/ml final concentration of rat IgG2b anti-FcγRII mAb (2.4G2) to prevent cytophilic binding of FITC-rat IgG1 anti-mouse IgA mAb which was subsequently added at 10 µg/ml final concentration for an additional 30 min. All steps were carried out at 4°C. Fluorescence analysis was accomplished on a FACScan (Becton Dickinson, Mountain View, CA) using logarithmic amplification. Only viable cells, identified on the basis of their characteristic forward and side scatter profiles and their exclusion of propidium iodide (Sigma), were analyzed.

Example 2

Lipo-D by itself is an ineffective mitogen for resting B cells but is markedly synergistic with mIg signaling

Previous reports indicated that lipoproteins, including lipoprotein-D (lipo-D), by themselves stimulated substantial B cell proliferation and Ig secretion. These studies used heterogeneous populations of lymphoid cells typically cultured at relatively high cell density (1×10^6 cells/ml). Thus, they left unanswered whether additional cell types and the state of B cell activation were important parameters in mediating these lipoprotein effects. The inventors thus tested the effects of lipo-D on a highly-enriched population of small resting B cells cultured at relatively low cell density (1×10^5 cells/ml). In contrast to the findings of previous reports, resting B cells proliferated weakly or not at all in response to lipo-D, added from 0.02 to 5 µg/ml (Fig. 1). Concentrations of lipo-D up to 40 µg/ml were also ineffective (data not shown). By contrast, B cells proliferated vigorously in response to LPS (data not shown).

The inventors had previously demonstrated that αδ-dex is an *in vitro* model for mIg-dependent TI-2 immunity as mediated by bacterial polysaccharides, and stimulates B cell proliferation, but not Ig secretion, in the absence of additional stimuli. To test the hypothesis that bacteria express constituents that could deliver both antigen-specific and non-specific stimuli directly to B cells for induction of humoral immunity, the inventors determined the effects of combined stimulation by optimal and suboptimal concentrations of αδ-dex with varying concentrations of lipo-D for B cell mitogenesis, as measured by ^{3}H-TdR incorporation. Whereas lipo-D by itself was relatively ineffective, it was markedly synergistic with αδ-dex for induction of proliferation. Over 25-fold enhancements in ^{3}H-TdR incorporation were observed with combined αδ-dex + lipo-D stimulation, relative to that seen using αδ-dex alone (Fig. 1). As little as 0.3 ng/ml of αδ-

dex, which by itself was relatively ineffective at stimulating proliferation, strongly costimulated proliferation when combined with 1 µg/ml of lipo-D. By contrast, up to 30 µg/ml of unconjugated bivalent anti-IgD antibody failed to costimulate proliferation with lipo-D (data not shown) indicating that multivalent mIg crosslinkage was required for this effect. The inventors had previously reported that unconjugated anti-IgD antibody was nevertheless capable of activating B cells as evidenced by its ability to upregulate MHC class II molecule expression and increase B cell size.

Example 3

Combined stimulation with lipo-D and αδ-dex leads to a striking induction of Ig secretion in the absence of exogenous cytokines

The next experiment determined whether lipo-D induced Ig secretion by small resting B cells in the presence or absence of αδ-dex. Lipo-D alone (0.2-5 µg/ml, Fig. 2, Expt A; 5-20 µg/ml, Fig. 2, Expt B) failed to stimulate significant Ig secretion. As reported previously, αδ-dex by itself was also an ineffective inducer of Ig synthesis by resting B cells. However, the combined action of lipo-D and αδ-dex led to an over 10,000-fold induction in Ig secretion in the absence of exogenous cytokines (Fig. 2). 5 µg/ml of lipo-D and 0.3 ng/ml of αδ-dex were found to be optimal for costimulation of Ig secretion. Higher and lower concentrations of lipo-D and αδ-dex were relatively inhibitory and ineffective, respectively.

Example 4

Lipo-D acts directly on the B cell to costimulate proliferation and Ig secretion

To determine whether lipo-D acts directly on the resting B cell to costimulate proliferation and Ig secretion in combination with αδ-dex, the inventors obtained a highly purified population of resting B cells (>99% mIgM+CD3-) through the method of electronic cell sorting of small T cell-

depleted spleen cells stained with PE-anti-IgM + FITC-anti-CD3. Any residual large, activated B cells were further eliminated on the basis of their characteristic forward scatter profile. As indicated in Fig. 3, lipo-D costimulated both proliferation and IgM secretion by αδ-dex-activated sort-purified B cells in a manner similar to that observed for the non-sorted B cell-enriched population. Thus, lipo-D acts directly at the level of the B cell to mediate these effects.

Example 5

Activation of αδ-dex-stimulated B cells with lipo-D leads to predominant secretion of IgM with smaller amounts of mostly IgG3

T cell-independent humoral immune responses to bacteria often show a selective proclivity towards the production of IgM and IgG3. This experiment determined the Ig isotypic profile of Ig synthesized in response to lipo-D by αδ-dex-activated B cells. As indicated in Table 1, lipo-D induced mostly IgM secretion by αδ-dex-activated cells. The remainder of the secreted Ig was IgG (<1%), mostly IgG3. Thus, Ig isotype secretion in response to costimulation with lipo-D is similar to that obtained in B cells activated with LPS alone.

Table 1

Ig Secretion (ng/ml)

	IgM	IgG3	IgG1	IgG2b	IgG2a	IgE	IgA
Lipo-D+αδ-dex	31,900	255	12	13	<6	<1	<6

Lipo-D-mediated Ig isotype production. B cells were stimulated with 5 μg/ml of lipo-D in combination with 0.3 ng/ml of αδ-dex and the concentrations of various Ig isotypes in culture SN were measured 6 days later by ELISA.

Example 6

Lipo-D by itself is a relatively poor costimulator of cytokine-dependent Ig secretion

Ig secretion in response to αδ-dex activation requires the concomitant action of cytokines. Thus, IL-4 + IL-5 induce large Ig secretory responses in both αδ-dex-activated B cells. The inventors recently defined a second cytokine pathway for eliciting Ig secretory response which operates in αδ-dex-activated cells. Thus, IL-3, GM-CSF, and IFN-γ each synergize with IL-I + IL-2 for induction of Ig secretion by αδ-dex-activated sort-purified B cells. In B cell-enriched, but not sort-purified, cell cultures, IL-I + IL-2 by itself stimulates Ig secretion that is dependent upon secretion that is dependent upon the presence of AsGm-1$^+$ non-B, non-T cells. Thus, to determine the ability of lipo-D to costimulate cytokine-dependent Ig secretion, this experiment involved the addition of either IL-4 + IL-5 or IL-I + IL-2 and/or IL-3, GM-CSF, or IFN-γ to lipo-D-stimulated B cell-enriched cultures and the direct comparison of Ig secretion with analogous cultures stimulated with αδ-dex. As indicated in Table 2, αδ-dex strongly costimulated Ig secretion in response to both cytokine pathways described above. By contrast, lipo-D was a relatively poor costimulator of these Ig secretory responses. Specifically, IL-4 + IL-5 exhibited some Ig inducing activity in lipo-D-stimulated cells (over 8-fold compared to 1,600-fold using αδ-dex). In addition the combination of IL-1 + IL-2 + IL-3 also led to an over 4-fold induction in Ig secretion by lipo-D-activated cells compared to an over 380-fold induction using αδ-dex-activated cells. Thus, lipo-D by itself is a relatively poor costimulator of cytokine-dependent Ig secretion.

Table 2

IBM secretion(ng/ml)

	Medium	Lipo-D	αδ-dex
Medium	<6	420	72
IL-4+IL-5	565	3,625	117,000
IL-1+IL-2	<6	410	5,875
IL-3	<6	1,600	350
GM-CSF	7	600	435
IFN-γ	<6	470	130
IL-1,2+IL-3	12	1,950	27,500
IL-1,2+GM-CSF	<6	450	18,437
IL-1,2+IFN-γ	7	290	16,875

Lipo-D is a relatively poor costimulator of cytokine-mediated Ig secretion. B cells were stimulated in the presence or absence of lipo-D (5 μg/ml) or αδ-dex (3 ng/ml) with or without the indicated cytokines and IgM concentrations in culture SN were measured 6 days later by ELISA. Cytokines were added at initiation of culture, except IFN-γ which was added at 24 hours, at the following concentrations: IL-1 (150 U/ml), Il-2 (150 U/ml), IL-3 (100 U/ml), IL-4 (3,000 U/ml), IL-5 (150 U/ml), GM-CSF (100 U/ml), IFN-γ (10 U/ml).

Example 7

Lipo-D provides key signals for induction of Ig class switching

In related application Serial No. 08/400,322, filed March 8, 1995, the inventors recently established an *in vitro* model for induction of high-rate IgA class switching which required the combined action of αδ-dex with either LPS or CD40L in the presence of IL-4, IL-5, and the IgA switch factor, TGF-β. In this system all stimuli were required in order to obtain over 10% mIgA$^+$ cells, as assessed by flow cytometric analysis. In this experiment, the inventors determined whether lipo-D could replace LPS for costimulation of IgA class switching. In the absence of TGF-β, few if any mIgA$^+$ cells were detected, four days after initiation of culture (Fig. 5). Removal from culture of LPS led to a drop in %mIgA+ cells to ≤2% as previously reported (data not shown). However, replacement of LPS with lipo-D led to a

restoration in the IgA class switching response to over 8% mIgA+ cells. Thus, lipo-D provided a key signal for inducing IgA class switching in this system.

Example 8

Costimulation of proliferation and Ig secretion by lipo-D in αδ-dex-activated B cells appears to be a general property of bacterial lipoproteins

A series of analogous experiments were performed using another recombinant lipoprotein, lipo-OSPA from *Borrelia burgdorferi*, the causative agent of Lyme disease in humans as as well as a synthetic lipoprotein consensus structure, Pam_3Cys. As indicated in Figure 5, lipo-OSPA strongly costimulated IgM secretion by αδ-dex-activated-cells. Further, Pam_3Cys also strongly costimulated mitogenesis and Ig secretion. As with lipo-D, and in contrast to previous studies, neither lipo-OSPA nor Pam_3Cys by themselves significantly enhanced either proliferation or Ig secretion by small resting B cells but required coactivation with αδ-dex to mediate these affects. Thus, these data suggest that bacterial lipoproteins in general, which deliver non-specific signals to B cells, must act in concert with specific B cell activating signals such as that mediated by mIg cross-linking (multivalent antigen binding) in order to induce a strong, humoral immune response, without a requirement for recruiting ion-B cells.

Example 9

Lipoprotein D can enhance the anti-polysaccharide response when given together with polysaccharide-protein conjugates

Diphtheria toxoid pneumococcal polysaccharide (DT-Pn14) was injected at various doses in the, presence or absence of lipoprotein D. The addition of lipoprotein D induced a 5-10 fold greater anti-polysaccharide response than was seen with DT-Pn14 alone. Furthermore, while 0.01 µg of DT-Pn14 elicited

low, if any, detectable response, it induced a significant response when injected together with lipoprotein D. This demonstrates that lipoprotein D can be used as an adjuvant to enhance responses to polysaccharide antigens.

Table 3

Enhancement of anti-polysaccharide response by coinjection with lipoprotein D

	dose (ug)	injected lipo D	IgG Anti-Pn14 titer day 28
DT-Pn14	.1	-	1,318
	.01	-	20
DT-Pn14	.1	+	10,240
	.01	+	498

Groups of 5 DBA/2 mice were injected when DT-Pn14 in the presence or absence of lipoprotein D. Anti-Pn14 ELISA were measured 28 days later.

Example 10

Lipoprotein D can enhance anti-polysaccharide response in T-cell deficient animals

Mice were injected with 500 µg - 1.0 mg of an anti-CD4 antibody (GK1.5, obtained from ATCC) to induce T cell depletion. One day later, the mice were injected with 5.0 µg of either pneumococcal polysaccharide type 14-lipoprotein D ("PN14-LPD") or PN14 alone. Fourteen days later, IgG1 anti-PN14 responses were measured. As set forth below in Table 4, lipoprotein D conjugates stimulated high levels of anti-PN14 response in T cell depleted mice.

Table 4

Conjugate vaccine with lipoprotein D as a component stimulates anti-polysaccharide response in T cell depleted mice

Antigen (µg/mouse)		Anti-CD4	IgG1 anti-PN14 d14
PN14-LPD	(5.0)	+	5,834
PN14	(5.0)	+	< 10

Thus, not only does lipoprotein D enhance anti-polysaccharide responses in immunocompetent animals, but it also enhances anti-polysaccharide responses in T cell depleted animals. Accordingly, lipoprotein D may be a valuable tool to enhance anti-polysaccharide responses in T cell deficient individuals, such as those suffering from HIV.

Example 11

To assess the effect of the protein on the anti-polysaccharide response, different vaccines based on *Haemophilus influenzae* type b polyribosyl-ribitol-phosphate (PRP) were prepared. These "Hib vaccines" included either tetanus toxoid (TT) and lipoprotein D (LPD) as the source of T cell epitopes. A PRP-TT Hib vaccine was prepared using CNBr activation of the polysaccharide and PRP-TT and PRP-LPD Hib vaccines were prepared using CDAP activation of the polysaccharide.

Groups of 10 female 5 week old OFA rats were immunized twice subcutaneously 4 weeks apart with 1/4 of a H.D. of the vaccines, and bleedings were taken on day 28, 42, 56, 69 and 83.

Anti-PRR'P response evaluated by ELISA (coating with tyraminated-PRR'P). A non-parametric method called "Robust" was used for the comparison of the anti-PS titres induced by different preparations. HIB 001A44 served as reference.

Figure 6a depicts the anti-polysaccharide response. The lipoprotein D conjugate, the PRP-LPD, induced a comparable primary anti-PS response to that of the tetanus toxoid conjugate, PRP-TT but induced a much higher (> 10X) secondary anti-PS response than the tetanus toxoid-PS. As set forth in Figure 6b, the PRP-LPD conjugate induced a very low anti-lipoprotein D response.

The experiment also suggested that the anti-polysaccharide responses are similar 14 and 28 days after booster. At day 69, (41 days after the booster), the response began to decrease for some conjugates, including the lipoprotein D conjugate.

In addition, to assess the effect of combined vaccines, a DTPa.HB vaccine (Diphtheria, tetanus toxoid, acellular pertussis with Hepatitis B) was combined with the conjugates. The combination did not diminish the anti-polysaccharide response.

Example 12

The Antigenicity and Immunogenicity of Lipoprotein D Constructs

In this experiment, we evaluated the effect of the conjugation of lipoprotein D conjugated to Hib PRP and S. pneumoniae PS 14 and 6B.

The conjugates were prepared using the CDAP activation and coupling chemistry (different PS/protein ratios) described below. The conjugates were characterized *in vitro* for their antigenicity using anti-PS and anti-LPD antibodies and the amount of free PS was determined by immunoprecipitation. The immunogenicity of the conjugates was evaluated in a rat model and the protective efficacy of anti-PS antibodies induced in rats was evaluated in infant rats (protection against Hib) or in mice (protection against S. pneumoniae 6B).

Materials and Methods

The Hib PRP and S. pneumoniae polysaccharide 6B and 14 were extracted and purified from inactivated cell cultures. The purified material met the WHO and US specifications in

This Sheet Has Been Replaced



terms of residual protein, nucleic acid, endotoxin, structural sugars and molecular size distribution.

Haemophilus influenzae lipoprotein D was expressed in E. coli and purified using conventional column chromatography. The purity of the proteins was above 90% as assessed by different methods (SDS-PAGE,CE,HPLC)

The activated Hib PRP, S. pneumoniae polysaccharide 6B or S. Pneumoniae polysaccharide 14 were conjugated to lipoprotein D or tetanus toxoid. Two methods for conjugating the polysaccharide to the lipoprotein or tetanus toxoid are disclosed herein.

Coupling of LipoD to Pn14

A. Direct conjugation using CDAP

Pn14 is in saline @ 5 mg/ml on ice. CDAP @ 100 mg/ml in acetonitrile, 0.2 M TEA, 6 mg/ml lipoprotein D in 10 mM sodium phosphate, 0.2 M NaCl, 0.1% Empigen (a detergent from CalBiochem) pH 7.2, on ice.

Activation and coupling are performed at 0-4°C. CDAP is slowly added to a stirred solution of Pn14 at a ratio of 0.75 mg CDAP/mg Pn14. At 30 seconds, the pH is raised to 10 with TEA (usually about 2x the volume of CDAP used) and maintained at pH 10 for a total of 2 minutes with TEA.

After a total of 2.5 minutes, the lipoprotein D is added to the activated Ps, while mixing, at ratio of 2.5 mg protein/mg Pn14. The pH should be in the range of 9-9.5.

After one hour, the reaction is quenched by the addition of one quarter volume of 1 M glycine @ pH 8. After an overnight incubation at 4°C, the conjugate is purified by passage over an S500HR (Pharmacia) gel filtration column.

The high molecular weight conjugate, containing protein and polysaccharide, is pooled and filtered through a 0.2 micron filter. Protein is determined using the Lowery assay, polysaccharide using a resorcinol assay.

This Sheet Has Been Replaced

B. Coupling via a spacer

Pn14 is activated with CDAP as above. At 2.5 minutes, one half volume of 0.5 M adipic dihydrazide at pH 8 is added. After one hour, the solution is exhaustively dialyzed into saline.

Hydrazide content is measured using TNBS, polysaccharide using a resorcinol assay.

Lipoprotein is coupled to the Pn14-hydrazide as described by Lees, et al., Vaccine, 1994, 12, 1160. In brief, the Pn14-Hydrazide is iodoacetylated with iodoacetyl N-hydroxysuccinimide (SIA, Sigma). The protein is thiolated with S-Acetylthioacetyl N-hydroxysuccinimide (SATA, Sigma). Following desalting and concentration using a Centricon 30 device, the two are combined and the pH raised to 7.5 using 1/9 volume of 0.75 M HEPES, 0.5 M hydroxylamine. After an overnight reaction at 4°C, the reaction is quenched with mercaptoethanol @ 0.2 mNM for one hour, followed by iodoacetamide @ 10 mM for 10 minutes. The conjugate is purified as described above. Those skilled in the art will recognize that other methods of conjugating protein to polysaccharide may also be practiced.

Experimental Results

1. ***Immunogenicity of conjugates in rats***

Groups of 10 OFA rats (female, 5-6 weeks old) were injected subcutaneously (200 µl) with an amount of conjugate corresponding to 2.5 µg of PS (for PRP) or ranging from 0.1 to 10 µg of PS (pneumococcal PS 6B or PS 14). The rats were boosted with the same amount of conjugates one month later and a blood sample was collected immediately before and 15 days after the booster for antibody analysis. The anti-LPD antibodies were measured by ELISA using protein D as coating antigen. Anti-PS antibodies were measured by ELISA using tyraminated PS for coating and, for anti-PS 6B or 14, absorption of anti-CPS antibodies was performed by addition of

This Sheet Has Been Replaced

CPS to the serum (1 mg/ml for anti-PS 14 and 5 mg/ml for anti-PS 6B). The detection of specific antibodies was made using an anti-rat conjugate labelled to peroxidase. For all the ELISA's, a reference serum was used and the titers were calculated in arbitrary units using the 4-parameter methods.

2. ***Passive protection experiments***

a) **Protection of infant rats against Hib challenge**

Infant OFA rats (4-5 days) were injected intra-peritoneally (IP) with 100 µl of serum dilutions (in PBS) containing anti-PRP antibodies. Twenty-four hours later, the rats were challenged by the IP route with about 5 x 10^4 colony forming units (CFU) of log phase Hib, strain Eagan. After 24 hours, the rats were killed and a blood sample was drawn from each rat. The bacteremia was measured by plating serial dilutions of the blood on chocolate agar and counting the colonies one day later. Rats with less than 10 CFU/100 µl of blood were considered protected.

b) **Protecting of mice against a lethal challenge by S. pneumoniae 6B**

Groups of 10 mice (outbred OF1, 6 weeks old, female mice) were injected intraperitonealiy with 10^3 CFU of S. pneumoniae bacteria type 6B, 24 hours after passive immunization with serum (100 µl of 1.5 dilution) containing anti-PS 6B antibodies. The control groups were injected with the serum of nonimmunized animals or animals immunized with PS 14 conjugates. The number of deaths in each group was recorded during the next 18 days.

The anti-PS response (anti-PRP, anti-PS6B) is higher (at least 10 times) when LPD is used instead of TT as the protein. Antibodies induced by PRP-LPD conjugates are at least equivalent or even better than antibodies induced by PRP-TT conjugates (at equivalent titer) in a passive protection model in infant rat.

This Sheet Has Been Replaced

Full protection was observed with anti-PS 6B-LPD conjugates in a passive mouse protection test.

Conclusion

These results demonstrate that LPD is a superior protein as compared to TT for the different capsular PS tested (PRP, PS 6B)

This Sheet Has Been Replaced

This Sheet Has Been Replaced

Table 6: **Evaluation of PRP conjugates obtained by the CDAP technology using TT or LPD as carrier.**

Conjugates	Antibodies at day 42 (rat)
	α-PS (γ/ml) (***)
PRP-TT	
C 294	5
C 295	18
PRP-LPD	
001	222
Exp. II	
PRP-LPD	
001	248
003	101
004	200

Non immunized rats have mean titers of 0.11 and 0.04 for anti-PS in Exp. I and II respectively.

Table 7:

Passive protection in infant rats challenged with Hib by serum from rats immunized with LPD-PRP conjugates

Serum from rats immunized with		Protection against bacteremia with			
		5 µg/ml	1µg/ml	0.2 µg/ml	-------
NaCl (control)		------	------	------	0/9
TT-PRP		8/9	0/10	0/10	------
LPD-PRP 003		9/9	4/10	0/8	------
LPD-PRP 004		10/10	4/8	0/10	------

Groups of 10 OFA rats (5 days old) were injected IP with 100 µl of serum and challenged 24 hrs later IP with 5.10^4 CFU/rat (Eagan strain).

The bacteremia was determined after 24 hrs.
Mice with <10 CFU/100 µl of blood were considered protected.
Controls have 10^3 - 10^6 CFU µl of blood.

This Sheet Has Been Replaced



Table 8: Evaluation of PS-6B conjugates obtained by the CDAP technology using TT or LPD as carrier.

Conjugates	Dose (γ)	Antibodies at day 42 (rat)
		α-PS (γ/ml)
Exp. I		
PS 6B-TT 1	0.1	97
	1	16
	10	21
2	0.1	140
	1	110
	30	30
Exp. II		
PS6B-LPD		
001	0.1	2839
	1	1671
	10	597
002	0.1	2941
	1	1831
	10	1125
Exp. III		
PS 68-LPD		
002	0.1	2027
	1	272
	10	2747

This Sheet Has Been Replaced



This Sheet Has Been Replaced

Table 9: Evaluation of PS-14 conjugates obtained by the CDAP technology using TT or LPD as carrier.

Conjugates	Antibodies at day 42 (rat)
	α-PS (γ/ml)
Exp. I	
PS 14-TT	24
	147
	373
	37
	71
	398
Exp. II	
unconj PS 14	
PS 14-LPD	
001	10
	55
	161
	24
	103
	144
Exp. III	
PS 14-LPD	
002	57
	146
	231

Table 10:

Passive protection in mice challenged with S. pneumonaie type 6B by serum from mice immunized with LPD-PS 6B conjugates

Serum from rats immunized with	N° of surviving mice at day		
	10	15	18
NaCl (control)	5/10	4/10	3/10
rat anti-PS 6B	7/10	6/10	6/10
rat anti-PS 6B-LPD 002	10/10	10/10	10/10
rabbit anti-PS 14-KLH	7/10*	3/10	3/10

*several mice are sick

5 weeks old female OFA mice were injected IP with 100 µl of serum diluted 5 fold and challenged 24 hours with 100 µl of Pn 6B strain (6/6B/52) passaged twice in mice. The mortality was recorded up to 18 days after challenge.

This Sheet Has Been Replaced

Example 13

To assess the effect of lipidation, rats were injected with 0.1 to 30 µg of a conjugate of lipoprotein D (Lipo D), protein D, or tetanus toxoid conjugated to pneumococcal polysaccharide 6B. Lipo D, Protein D, and pneumococcal polysaccharide 6B were prepared according to the methods as discussed above at pages 6 and 20-21. Tetanus toxoid is easily obtained from many sources known to those ordinarily skilled in the art. Conjugates were prepared as discussed above at pages 20-22. The protein D conjugates were prepared using the same methodology as that set forth for lipoprotein D conjugates.

Anti-polysaccharide responses were measured by ELISA 28 days after initial injection of the conjugate and 28 days after a booster injection. As set forth in Table 11, anti-polysaccharide responses were significantly higher in mice injected with the Lipo D-conjugate as compared to those injected with the protein D conjugate.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. 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.

This Sheet Has Been Replaced



Table 11

Immunogenicity of PS-6B Conjugates Obtained by the CDAP Technology Using TT, LPD or Protein D as the Protein Carrier

Conjugates	Dose (µg)	Antibody Titer (day 42) anti-PS (µg/ml)
Exp. I		
PS 6B-TT		
001	0.1	97
	1	16
	10	21
0002	0.1	140
	1	110
	30	30
Exp. II		
PS 6B-LPD		
001	0.1	2839
	1	1671
	10	597
0002	0.1	2941
	1	1831
	10	1125
Exp. III		
PS 6B-LPD		
0002	0.1	2027
	1	272
	10	2747
PS 6B-PD		
001	0.1	49
	1	5
	10	4

This Sheet Has Been Replaced

We claim:

1. A method of inducing an immune response to polysaccharides or other T cell-independent antigen in an individual, comprising:

administering the antigen, and

coadministering a lipoprotein.

2. The method of claim 1 wherein the lipoprotein is derived from a microorganism.

3. The method of claim 2 wherein the lipoprotein is lipoprotein D.

4. The method of claim 1 wherein the lipoprotein is a synthetic lipoprotein.

5. The method of claim 1 wherein the antigen is a polysaccharide derived from a bacteria selected from a group consisting of *Haemophilus influenzae* type b, *S. pneumonia*, Group B Streptococcus, *N. meningitidis*, *Salmonella*, and *P. aeruginosa* mucoexopolysaccharides.

6. A method of enhancing responses to polysaccharides or other T cell-independent antigens, comprising:

administering the antigen, and

coadministering a lipoprotein.

7. The method of claim 6, wherein the lipoprotein is derived from a microorganism.

8. The method of claim 6 wherein the lipoprotein is lipoprotein D.

9. The method of claim 6 wherein the lipoprotein is a synthetic lipoprotein.

10. The method of claim 6 wherein the antigen is a polysaccharide derived from a bacteria selected from a group consisting of *Haemophilus influenzae* type b, *S. pneumonia*, Group B Streptococcus, *N. meningitidis*, *Salmonella*, and *P. aeruginosa* mucoexopolysaccharides.

11. A vaccine comprising a clinically relevant polysaccharide covalently attached to lipoprotein D.

12. The vaccine of claim 11 wherein the polysaccharide is derived from a bacteria selected from a group consisting of

This Sheet Has Been Replaced

Haemophilus influenzae type b, *S. pneumonia*, Group B Streptococcus, *N. meningitidis*, *Salmonella*, and *P. aeruginosa* mucoexopolysaccharides.

13. A method for making the vaccine of claim 11 wherein the lipoprotein D is directly covalently attached to the polysaccharide.

14. The method of claim 13 wherein the covalent attachment is via CDAP activation of the polysaccharide.

This Sheet Has Been Replaced