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Molecular Adjuvants and Immunomodulators: New Approaches to Immunization

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INTRODUCTION

The use of adjuvants to aid in the vaccination of human beings stabilized approximately a half century ago with the incorporation of bacterial vaccines into aluminum hydroxide gels. Their efficacy along with minimal side effects dampened any impetus to substitute other adjuvant compounds. Nevertheless, substances that amplified humoral and cell-mediated immunity continued to be developed and characterized in animals, and these contributed greatly to the magnification and subsequent understanding of heretofore complex, subliminal, immunological events.

Recently, knowledge of the epitopes defining antigenicity in microorganisms has attained a degree of sophistication that should permit use in vaccines of well-defined synthetic structures devoid of potentially harmful contaminants. Such low-molecular-weight epitopes, however, are poor antigens generally, and this fact has stimulated the search for equally well-defined synthetic adjuvants necessary for amplification of the weak antigenicity of these epitopes. The current surge in our knowledge of adjuvants in the United States was stimulated in large part by an Office of Naval Research program that supported and brought together investigators concerned with developing new approaches to vaccine formulations, the results of which were published in 1987 (97). Accordingly, this review will discuss selected adjuvant models that recently have shown reasonable promise for optimizing the immune response of human beings to microbial vaccines. In addition, prophylactic treatment of animals with a number of immunomodulators, independent of antigen, has been shown to result in a remarkable increase in ability to ward off subsequent challenge with multiple 50% lethal doses (LD_{50}) of a wide range of bacteria, viruses (97), and some parasites (see below). This phenomenon also will be explored.

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LPS

General Characteristics

The early finding that the immune response to vaccines containing diphtheria and tetanus toxoids was elevated when *Haemophilis pertussis* or other gram-negative organisms were included (38) inspired studies to isolate the responsible factor (86, 147). They revealed the lipopolysaccharide (LPS)-protein complexes (endotoxins) extracted from the outer membrane of gram-negative bacteria to be remarkably potent adjuvants. Initial studies showed that the nitrogen content could be reduced to as low as 0.6% without loss of adjuvant action, indicating that the protein moiety was not essential for adjuvanticity (61). However, in later studies, the endotoxin-associated protein has been found to possess inherent adjuvant action (128). Both humoral and cell-mediated immunity (58, 104) could be elevated markedly by LPS. However, the profound toxicity of these endotoxins precluded their use in human vaccines (85). Within the last decade, rapid progress has been made in our knowledge of these extraordinary compounds. It has resulted in identification of lipid A as the active component (37), as well as in structural elucidation (123, 133) and synthesis of this adjuvant-active structure (52, 53), along with multiple analogs (46, 65, 75). This has led to an understanding of structure-function relationships such that a separation of toxic from beneficial properties has now been accomplished.

Multiple reviews covering earlier studies of the adjuvant action of LPS exist (54, 57, 58, 104, 146). In brief, heightened interest in understanding the molecular structure of this fascinating molecular complex with many diverse properties was initiated following the isolation and characterization of LPS by the hot phenol-water extraction method of Westphal and Luederitz (148). In particular, the isolation of endotoxins from polysaccharide-deficient mutant organisms resulted in knowledge that the adjuvant action, as well as many of the other endotoxic properties, lay in the structurally highly conserved

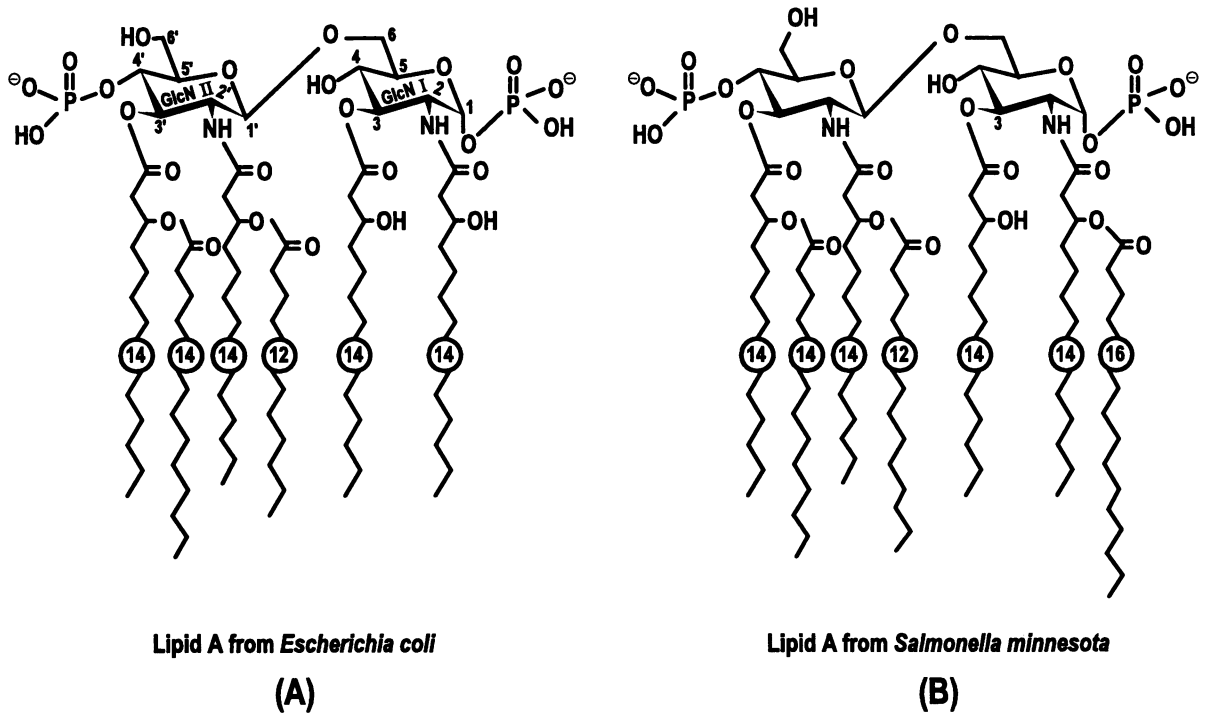


FIG. 1. Chemical structure of synthetic lipid A from *E. coli* (A) and *S. minnesota* (B). In native LPS the polysaccharide chains occur via KDO linked to the hydroxyl group at C-6′ of the nonreducing glucosamine unit. The numbers in circles indicate the number of carbon atoms in acyl chains.

lipid moiety, termed lipid A (36, 122). As such, lipid A received concerted efforts to elucidate its structure. The structure was established first for enterobacterial lipid A's of *Salmonella typhimurium* (133), *Escherichia coli* (51, 153), *Salmonella minnesota* (123), and *Proteus mirabilis* (127). The successful synthesis of *E. coli* lipid A was described in 1985 (52), and its structure, together with that of synthetic *S. minnesota* lipid A, is shown in Fig. 1. This synthesis allowed multiple changes in architecture in constructing synthetic lipid A partial structures and analogs. As detailed below, the adjuvant action of lipid A or LPS was readily duplicated by several of these synthetic structures with markedly diminished toxicity. Thus, their applicability to human vaccination is indicated.

Multiple facets of the immune system are influenced both in vivo and in vitro by the toxic native LPS. These have been reviewed extensively (57, 104, 105) and establish that LPS can act in vitro directly on each of the three cells making up the immunocompetent islet responsible for generating immunity: i.e., the macrophage, thymus-derived (T) cells, and bone marrow-derived (B) cells. However, in recent years it has become clear that an analysis of the stimulatory action of LPS on the immune system in vivo becomes an analysis of the function of the multiple cytokines demonstrated to be released by cells stimulated with LPS (142). Sequential tracing of the triggering and subsequent events instigated by LPS or lipid A in vivo, however, becomes difficult with the knowledge that they can bind to and affect many different cells that secrete different cytokines (91). Individual cytokines, in turn, can influence more than one cell type (41). In addition, both in vitro and in vivo tests described conditions wherein LPS can either enhance or suppress the response to antigens (58, 137, 139). Consequently, the cell population(s) initially triggered by this adjuvant to start the cytokine cascade leading to a heightened immune response could be singular or multiple.

The increased expression of humoral or cell-mediated immunity was found to be effectively accomplished also by lipid A (21), as well as by certain synthetic derivatives of diminished toxicity resulting from substitution of fatty acids and deletion of phosphate groups (73). Several classic reports established that synthetic lipid A's mimicking those native to *E. coli*, designated 506 or LA-15-PP, and *S. minnesota*, designated 516 or LA-16-PP, were both excellent adjuvants (35, 73, 74). An example is illustrated in Table 1, and the data served to initiate the quest for manipulation of the structural moieties such that the adjuvant action would be retained but the toxicity would be eliminated. The structures of lipid A and its analogs are described in Fig. 2 and 3. Their effects on immunocompetent cell functions are summarized below.

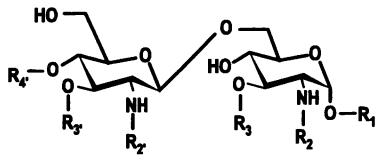
Structure-Function Relationships

When the basic *S. minnesota* lipid A was altered with respect to the number and position of the fatty acids and/or phosphate groups, a variety of compounds emerged, some with lessened toxicity which still retained adjuvanticity (Table 2). Notable among these was compound LA-18-PP, in which the fatty acids at R_3 and R_3' were changed from 3-OH-14:0 to 14:0. Activities

TABLE 1. Adjuvant action of synthetic lipid A's[a]

Compound	LD_{50} (ng)[b]	Log increase in anti-BSA titer[c]	IL-1 (U)
LA-15PP (*E. coli* 506)	13	6.6	38
LA-16PP (*S. minnesota* 516)	5	5.3	11

[a] Data taken from references 73 and 74.
[b] Determined in galactosamine-sensitized mice.
[c] BSA, bovine serum albumin.

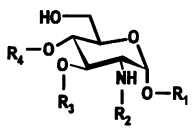


SYNTHETIC COMPOUND		Nature of: R_1	R_2	R_3	R_2'	R_3'	R_4'
Precursor Ia	LA-14-PP (406)	$PO(OH)_2$	3-OH-14:0	3-OH-14:0	3-OH-14:0	3-OH-14:0	$PO(OH)_2$
	LA-14-HP (405)	$PO(OH)_2$	3-OH-14:0	3-OH-14:0	3-OH-14:0	3-OH-14:0	H
	LA-14-PH (404)	H	3-OH-14:0	3-OH-14:0	3-OH-14:0	3-OH-14:0	$PO(OH)_2$
E. coli LipA	LA-15-PP (506)	$PO(OH)_2$	3-OH-14:0	3-OH-14:0	3-O(12:0)-14:0	3-O(14:0)-14:0	$PO(OH)_2$
	LA-15-PH (504)	H	3-OH-14:0	3-OH-14:0	3-O(12:0)-14:0	3-O(14:0)-14:0	$PO(OH)_2$
S. minnesota LipA	LA-16-PP (516)	$PO(OH)_2$	3-O(16:0)-14:0	3-OH-14:0	3-O(12:0)-14:0	3-O(14:0)-14:0	$PO(OH)_2$
	LA-17-PP	$PO(OH)_2$	14:0	14:0	14:0	14:0	$PO(OH)_2$
	LA-17-PH	H	14:0	14:0	14:0	14:0	$PO(OH)_2$
	LA-18-PP	$PO(OH)_2$	3-OH-14:0	14:0	3-OH-14:0	14:0	$PO(OH)_2$
	LA-18-PH	H	3-OH-14:0	14:0	3-OH-14:0	14:0	$PO(OH)_2$

FIG. 2. Chemical structure of the partial structures and analogs of synthetic lipid A's.

retained were interleukin-1 (IL-1) release and stimulation of delayed-type hypersensitivity (73).

A precursor of lipid A (compound 406 or LA-14-PP) possesses the phosphates at R_1 and R_4' but only 3-hydroxy fatty acid residues at positions R_2, R_3, R_2', and R_3'. This compound also was toxic in D-galactosamine-treated mice but less so in nonsensitized animals (73). It was an excellent adjuvant to antibody formation as well as a strong mitogen. It was also capable of activating macrophages (34, 73, 129), although it was incapable of inducing cytokine release (IL-1) from human peripheral monocytes (94). When the phosphorylation pattern was changed on this precursor molecule by substitution of hydrogen for a phosphate group at either R_4' or R_1 (compounds 405 and 404), toxicity was apparent but reduced, as was adjuvant action.



SYNTHETIC COMPOUND	Nature of: R_1	R_2	R_3	R_4
Lipid X LA-11-HP (401)	$PO(OH)_2$	3-OH-14:0	3-OH-14:0	H
Lipid Y LA-13-HP (408)	$PO(OH)_2$	3-O(16:0)-14:0	3-OH-14:0	H
GLA-57	H	3-O(12:0)-14:0	14:0	$PO(OH)_2$
GLA-27	H	3-O(14:0)-14:0	14:0	$PO(OH)_2$
GLA-58	H	3-O(16:0)-14:0	14:0	$PO(OH)_2$
GLA-59	H	3-O(14:0)-14:0	3-OH-14:0	$PO(OH)_2$
GLA-60	H	3-OH-14:0	3-O(14:0)-14:0	$PO(OH)_2$

FIG. 3. Chemical structure of the monosaccharide partial structures and analogs of synthetic lipid A's.

Of considerable interest is the adjuvant activity in the absence of overt toxicity present in lipid Y, the reducing monosaccharide unit of *S. minnesota* lipid A (35, 74). Contrariwise, lipid X, the reducing monosaccharide unit of *E. coli* lipid A, was not lethal and did not display any adjuvant activity. As Fig. 3 shows, lipid X contains 3-OH-14:0 groups at R_2 and R_3, phosphate at R_1, and H at R_4.

With respect to analogs of the nonreducing monosaccharide region of lipid A, Kumazawa et al. (76) have shown excellent adjuvant and mitogenic activities to be associated with insertion of 3-O(14:0)-14:0 at position R_2, a phosphate at R_4, and a 3-OH-14:0 at R_3. In a later study, a derivative carrying 3-OH(14:0)-14:0 at R_2 and a 3-OH-14:0 group at R_3 (designated glycolipid A-59, GLA-59) as well as a derivative reversing the position of these two fatty acids (designated GLA-60) showed reduced toxicity and strong adjuvant action on antibody synthesis and macrophage activities (77). Introducing two 3-acyloxyacyl groups [3-O(14:0)-14:0] at positions R_2 and R_3 (compound GLA-47) resulted in a nontoxic compound that exhibited good adjuvanticity on antibody formation but no effect on macrophage activity or IL-1 secretion. On the other hand, substituting a 14:0 fatty acid for 3-O(14:0)-14:0 at R_3 (compound GLA-27) resulted in an excellent nontoxic adjuvant that also activated macrophages and induced IL-1 release (130).

Consequently, manipulation of the phosphate or the fatty acid content or their position appears to affect the toxicity and adjuvant action. The 3-acyloxyacyl groups present in both *E. coli* and *Salmonella* lipid A appear nonessential for adjuvant action since compound 406, which is devoid of these, was a good immunostimulant. Introduction of 14:0 at all four fatty acid positions of the lipid A backbone (with retention of the phosphate) diminished toxicity considerably but retained adjuvanticity. Similarly, compound LA-18-PP with 14:0 located at positions R_3 and R_3' and 3-OH-14:0 at R_2 and R_2' was a good adjuvant, exhibiting reduced toxicity.

As is the case with LPS, the temporal sequence of events accentuated by lipid A in vivo is governed by multiple steps

TABLE 2. Biological properties of synthetic lipid A analogs[a]

Compound[b]	LD_{50} (ng)[c]	Fever (no. of rabbits positive/no. tested)	Anti-BSA titer[d]	% Cytotoxicity[e]	Macrophage activation[f]	IL-1 (U)
LA-17PP	100	3/3	11	83	3.5	12
LA-17PH		2/3	6	ND	4.8	15
LA-18PP	126	0/3	10	83	5.2	11
LA-18PH		0/3	6	ND	4	5

[a] Data taken from reference 73.
[b] For structure, see Fig. 2.
[c] Determined in galactosamine-sensitized mice.
[d] Log anti-bovine serum albumin (BSA) hemagglutination titer.
[e] Percent killing of L929 cells by serum from *M. bovis* BCG-primed mice elicited by compound. ND, not determined.
[f] Superoxide anion generation by 1 μg of compound in guinea pig peritoneal macrophages (stimulation index).

that are regulated by cytokine release and the emergence of their receptors and is still conjectural (41). Identification of the initial cell triggered by LPS or lipid A would aid considerably in clarifying their modus operandi. Whether a single entity is provoked by lipid A or LPS to usher in the cascade of cellular activation and cytokine release or multiple cells are stimulated and each contributes independently to the heightened immune response of the host is a key question. Conditions under which a selective pathway might be triggered in vivo need to be defined.

Stimulation of IL-1

A cytokine mandatory for generating many immunologic reactions is a protein with hormone-like characteristics termed IL-1 which is secreted by several cell types and has two major isoforms, IL-1α and IL-1β. The biological effects of IL-1 are multifaceted and include stimulation of T and B cells to differentiate along the pathway leading to antibody synthesis as well as activation of the acute-phase response (72). A major probe used in stimulating IL-1 release has been LPS. Recently, the structural requirements for IL-1 release from human peripheral monocytes have been studied, using LPS, lipid A, and their partial structures (93, 94). LPS from both smooth (S) and rough (R) forms of bacteria were strong inducers of IL-1, as expected. Lipid A from both *Escherichia* and *Salmonella* spp. also stimulated good activity but to a lesser extent. Of interest was the capability of lipid A partial structures with a marked diminution in toxicity to evoke IL-1 synthesis as well. On the other hand, precursor molecules such as lipid X and compound 406 exhibited little or no effect on IL-1 synthesis (93, 94). The presence of 3-acyloxyacyl residues was necessary for IL-1 induction.

Contrariwise, in a comparative study by Cavaillon et al. (15) of the relative ability of LPS and lipid A to generate cell-associated and secreted IL-1 in adherent murine peritoneal macrophages, a much higher effectiveness of native LPS was noted. Although lipid A isolated from *Bordetella pertussis* induced significant levels of cell-associated IL-1, little IL-1 release was noted. Synthetic *E. coli* lipid A (compound 506) also exhibited a much lesser capacity than LPS to induce the secretion of IL-1. Similar findings were reported in studies of adherent human monocytes (88). Since lipid A lacks the 3-deoxy-D-manno-octulosonic acid (KDO)-containing oligosaccharide moiety, an essential role for the latter was postulated for the release of IL-1. In support, these authors reported that exogenous sialogangliosides incubated with LPS blocked LPS-induced IL-1 production. Nevertheless, as discussed above, synthetic lipid A readily induced IL-1 release from human peripheral monocytes at levels of 5×10^{-6} to 5×10^{-3} μg of lipid A per ml. In addition, KDO-containing oligosaccharides [Hep-α (1.3) Hep-α (1.5)-KDO] and the synthetic α-methyl ketoside of KDO [α(2-4)-KDO] were capable of effecting IL-1 secretion but at much higher concentrations (1 to 10 μg), confirming earlier results (93).

Lasfargues and Chaby (87) reported that lipid X as well as lipid A elicited IL-1 production. In addition, synthetic propyl-2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-4-*O*-phosphono-6-*O*-tetradecanoyl-β-D-glycopyronoside (glycolipid M9) induced IL-1 secretion in murine and human monocytes more efficiently than LPS or lipid A, whereas several synthetic derivatives of KDO failed in this respect. A monosaccharide derivative of the basic structure of lipid A also has been shown by Charon et al. to induce IL-1 (16). Dijkstra et al. (31) reported that the incorporation of lipid A (from an Rc mutant) or monophosphoryl lipid A into liposomes reduced by 12,000-fold their IL-1-inducing capacity, suggesting that a direct interaction of the lipid A with the macrophage membrane was required for this property. The macrophage cell line RAW 264.7 was used as the target.

Thus, the release of IL-1 appears capable of being initiated optimally by LPS, to a lower extent by lipid A, and by some partial structures as well as synthetic monosaccharide analogs of lipid A.

Stimulation of NSR to Microbial Infection

Many of the properties of IL-1 are shared by tumor necrosis factor alpha (TNF) (41), which can be induced to peak nanomolar concentrations in serum 2 h after LPS injection into normal rabbits, mice, and humans, in whom it produces severe wasting along with hyperlipidemia (66). TNF is thought to be a major mediator in producing endotoxin shock. However, it has also been implicated as a mediator in the extraordinary beneficial effect of LPS in inducing nonspecific resistance (NSR) to infection by a variety of microorganisms when LPS is given prior to challenge. This seeming paradox may be dose related; i.e., high amounts of TNF are damaging, while lower amounts are stimulatory to the immune system. Thus, Parant et al. (116) found passively transferred TNF-rich serum to offset lethal challenge of mice with *Klebsiella pneumoniae*. Later studies showed that human recombinant TNF had the capacity to protect against *Klebsiella*, *Listeria*, and *Streptococcus* spp. (114). Similarly, LPS-induced IL-1α has been implicated as a mediator of NSR through its capacity to elevate bactericidal properties of macrophages via an increased respiratory burst (101). Also a candidate for contributing to NSR is interferon gamma (IFN-γ), which can be elicited by LPS (30, 39, 111, 136). Other cytokines released secondarily by TNF and IL-1 may be contributory. Consequently, multiple mediators from both macrophages and T cells may be responsible in

combination for this potentially important prophylactic treatment.

Of obvious importance is whether LPS analogs of diminished toxicity can produce the same NSR effect. Although the initial studies of Lam et al. (84) showed that an injection of synthetic lipid X (2 μg per mouse) protected mice significantly against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*, recent experiments with a synthetic and highly purified preparation by Aschenauer et al. (5), as well as Lam et al. (82), have demonstrated that lipid X is devoid of immunostimulatory activity, although it blocks endotoxin-induced in vitro and in vivo activities. The latter observation is consistent with the finding by Proctor et al. (118) that lipid X could protect mice against the lethality induced by intravenous injection of endotoxin. It was most effective when given near the time of endotoxin injection.

A number of other synthetic analogs of lipid A have been shown to elevate prophylactically resistance to challenge with a variety of infectious microorganisms. One of the most promising is a synthetic acylated glucosamine monosaccharide-1-phosphate, termed SDZ 953, representing only the reducing half of lipid A (83). In recent years (43), this nontoxic analog has been shown to dramatically protect several species against challenge with multiple LD_{50}s of *P. aeruginosa*, *E. coli*, and *S. aureus*. The authors advance the hypothesis that increased hematopoiesis and activation of macrophages account for the resistance. In addition, this partial lipid A structure induced tolerance to native endotoxins and blocked the toxicity of the latter (26). As such, it offers potential for inclusion in the therapeutic strategies for septic shock.

In this respect, an intriguing nontoxic diphosphoryl lipid A has been isolated from *Rhodopseudomonas sphaeroides* which was capable of blocking the induction of TNF (cachectin) and reversing the tolerance induced by toxic LPS from several genera (42). Its structure closely resembles that of the toxic isolates from *S. typhimurium* and *E. coli* (132), and the authors suggest that the inhibition may be due in the main to competitive binding to macrophages. This property, together with the compound's lack of toxicity, warrants its inclusion as a candidate for further study in alleviation of sepsis.

MPL

A successful approach to a formulation of lipid A with considerably reduced toxicity and retention of adjuvanticity was accomplished by Ribi and coworkers (120, 121). LPS initially was extracted from cell walls obtained from the rough (Re) mutants of *Salmonella* species. Treatment of this LPS with acid resulted in a mixture of structural homologs that varied in the number of acyl groups attached to the glucosamine disaccharide backbone. When compared with toxic lipid A, each of these homologs had lost the phosphate group at the C-1 position; accordingly, they were termed monophosphoryl lipid A (MPL). Bisphosphoryl lipid A and the endotoxin isolated from the Re mutant could be essentially quantitatively converted into MPL by acid treatment (119). Structurally, the fully acylated species of MPL correspond to the synthetic compound 504 (LA-15-PH) shown in Fig. 2. Recent modification of this structure deleted the 3-hydroxytetradecanoyl residue O-linked to the 3 position of the reducing-end glucosamine and led to further attenuation of endotoxic activity (107); this analog is now available commercially.

MPL proved to be nonlethal in doses of up to 15,000 μg in rabbits as contrasted with the lethal doses of 1 to 10 μg of native endotoxin. Similar findings were noted in guinea pigs, dogs, and horses (120). Phase I studies in human beings revealed no discernible toxicity at doses up to 100 $\mu g/m^2$ (143). Other parameters of endotoxicity (e.g., pyrogenicity, B-cell mitogenicity, and Shwartzman reaction) also were essentially lost (134). Yet the ability to regress certain tumors when administered together with a purified component of mycobacteria, trehalose dimycolate, was unimpaired. In addition, this combination expressed the property of elevating nonspecific resistance to a number of pathogens as well as increasing antibody synthesis to unrelated antigens (141).

An adjuvant to antibody formation, MPL elicited a 16-fold increase in enzyme-linked immunosorbent assay titers against ovalbumin, as well as a striking increase in antibody against two viral peptides when given together with trehalose dimycolate (120). Extension of these findings was reported in a number of studies (136, 137). Since adjuvants generally are most desirable in immunodeficient states, aged mice (17 to 24 months) were chosen as a model system. MPL was found capable of repairing the lesion(s) responsible for the immune deficiency in such mice. In studies with sheep erythrocytes as antigen, antibody-forming cells were restored to levels equal to or above those normally exhibited by young adult mice. Inasmuch as a monoclonal interferon (IFN-γ) antibody abrogated the adjuvant action, the nature of this immunodeficiency included IFN-γ levels below threshold requirements (136). A similar finding was observed with LPS, muramyl dipeptide, or polyribonucleotide complexes as adjuvant (111). Both the T-cell and macrophage were found to be targets of the MPL adjuvant action. Activity was a function of mediator release, with MPL inducing secretion of IFN-γ from T cells and IL-1 from macrophages. Although IL-6 was not measured in these studies, release of this cytokine by LPS has been documented (55). No evidence indicating that MPL increased IL-2 release was obtained (136). Thus, the higher titers resulting from MPL and LPS, as well as other adjuvants, may reflect a multifaceted stimulation of several cell types and release of multiple mediators acting in synergy. Support for this is indicated by the report of Kunimoto et al. (78) describing the synergistic elevation of immunoglobulin M (IgM) levels by IL-1 and IL-6, both mediators known to be released by LPS. A mechanistic model of how IFN-γ might mediate the adjuvant action of MPL has been described by Gustafson and Rhodes (39).

Baker et al., in a recent series of papers (6–8), obtained evidence for an effect of MPL on T-suppressor cells. With pneumococcal polysaccharide as antigen, MPL injection abrogated the tolerance induced by low doses of this antigen, which is well documented to be mediated by T-suppressor cells (7). Of importance was their finding that MPL was active as an adjuvant when given 2 days after pneumococcal polysaccharide to young (2- to 4-week-old) mice, which are normally unresponsive at that age (8). Consequently, MPL preparations may be feasible candidates for inclusion in polysaccharide vaccines, such as *Haemophilus influenzae*, to which infants are known to be poorly responsive.

The effect of MPL on the serum antibody response to pneumococcal polysaccharides types 3 and 14 in 6- to 8-week-old mice also was studied by Yin et al. (152). Both IgM and IgG levels were increased by this adjuvant when given 2 to 4 days after intraperitoneal injection of 25 μg of type 14 pneumococcal polysaccharide, thus confirming the postantigen temporal requirement of Baker et al. (8). MPL given on day 4 also reversed the specific unresponsiveness induced by a large dose (5 μg) of pneumococcal polysaccharide type 3.

Much effort is being generated to find newer adjuvant regimens active at promoting the antigenicity of weak tumor antigens. In this regard, MPL together with trehalose dimycolate has been shown to cause regression of experimental

tumors in several species (121). In order to exploit this potentially therapeutic adjuvant, Mitchell et al. (102) incorporated MPL together with the cell wall skeleton of *Mycobacterium phlei* (analogous to trehalose dimycolate) in a squalene water-in-oil emulsion as an adjuvant to autologous melanoma antigens for immunization of humans. Homogenates of two melanoma cell lines, originally started from biopsies of nodules from two different female patients, were mixed and used as antigen. An increase in the numbers of circulating precursor cells of melanoma-reactive, cytotoxic lymphocytes occurred and offered the strongest correlation with clinical responsiveness. No toxicity was observed. Since systemic injection of many cytokines results in diverse toxicities (41), local release of such remedial cytokines stimulated by adjuvants such as MPL may be a more desirable therapeutic approach.

MPL also retained the capacity to increase resistance nonspecifically against a variety of microorganisms. In studies with influenza virus A/PR/8/34, 100 μg of MPL in squalene water-in-oil emulsion protected 50% of the mice from aerosol challenge (141). MPL was also protective in infection models involving *Salmonella enteritidis* (141), *Listeria monocytogene* (141), *Toxoplasma gondii* (141), *E. coli* (141, 144), and *Staphylococcus epidermidis* (17).

POLYRIBONUCLEOTIDE COMPLEXES

In seeking to understand the mechanisms by which the endotoxic LPS increased antibody synthesis, a second distinctive group of synthetic molecules, the polyribonucleotide complexes, were studied. Initially, the cytotoxic action of LPS in vivo was hypothesized to cause the release of cellular nucleic acids which acted as trephones (nourishment) for immunocompetent cells. Consequently, nucleic acids per se were explored as possible endogenous host mediators of the potent adjuvant action of LPS (56). Support for this hypothesis was gained by the finding that stimulation of the immune response to unrelated antigens was a property of both endogenously and exogenously derived nucleic acids, as well as of the low-molecular-weight breakdown products isolated following treatment of nucleic acids with nucleases (100). Subsequently, Braun and colleagues, guided both by their previous extensive studies showing that oligonucleotides could stimulate division in bacterial cells and by their insight, postulated that mammalian antibody synthesis might require endogenous stimulatory molecules of a similar nature. They recognized and tested the potential of the then newly developed (1965) synthetic double-stranded polyribonucleotides (12). Their demonstration that poly(I) · poly(C) and poly(A) · poly(U) complexes were capable of increasing the numbers of antibody-forming cells four- to fivefold caused interest to focus rapidly on these synthetic complexes because of their defined structure and ready availability. Synthetic polynucleotides are formed following the action of the enzyme, polynucleotide phosphorylase, on the desired mononucleotide diphosphates. A double helix is formed when the polymerized single strands from opposite base pairs are mixed. Thus, poly(A) mixed with poly(U) forms the poly(A) · poly(U) complex. Similarly, poly(I) mixed with poly(C) forms poly(I) · poly(C). These compounds have been found to mimic the viral component responsible for induction of interferon (33).

Further, extensive studies have shown the polyribonucleotide complexes to be adjuvants in mice, rats, guinea pigs, chickens, rabbits, and humans and to differ from LPS in several parameters. Their enhancing activity for a wide range of thymus-dependent antigens has been established (56, 60). The signal from this adjuvant is apparently received very rapidly by the host cells inasmuch as the complexes are destroyed within 5 to 10 min by the nucleases in serum. These adjuvants have proved to be very effective T-cell stimulants for both T-helper (40) and suppressor cells (103), as well as cytotoxic cells (62). Like LPS and MPL, they show distinctive temporal requirements in that injection of this adjuvant together with an antigen increases antibody synthesis (56). In contrast, injection of poly(A) · poly(U) 1 or 2 days before antigen injection causes the activation of suppressor cells with a resulting diminution of antibody synthesis (103). Thus, these synthetic molecules can be considered true immunomodulators of the immune system. Complexing of the base pairs has been shown to be a requirement; single strands of the polymerized nucleotides are without activity in most instances.

Evidence that poly(A) · poly(U) functions as an adjuvant by causing T lymphocytes to hasten their secretion of T-helper factors has been gained. Such presumptive cytokines secreted under the influence of poly(A) · poly(U) have been shown to increase the number of antibody-forming cells in the spleen and were active in restoring the deficient immune response of aging mice and preventing the development of immune tolerance in normal mice. In addition, poly(A) · poly(U) has been shown to increase markedly the maturation of the immune system in the neonatal mouse (40). Also, human peripheral blood leukocytes have been shown to double the synthesis of immunoglobulin when stimulated by poly(A) · poly(U). Peripheral blood lymphocytes from patients with acquired agammaglobulinemia similarly showed elevated immunoglobulin synthesis under the influence of polynucleotides. However, such cells from patients with congenital agammaglobulinemia could not be elevated in their synthetic capacity by poly(A) · poly(U) (62). Recently, IFN-γ has been identified as one T-cell cytokine released by poly(A) · poly(U) inasmuch as its adjuvant action was reduced by one-half following addition of a monoclonal anti-IFN-γ antibody to the spleen cell culture system (111). Consequently, this adjuvant may function by causing the early release of IFN-γ from Th-1 cells, when it is given with antigen. Contrariwise, 24 to 48 h after poly(A) · poly(U) administration, Th-2 cells may switch on, releasing IL-4 and IL-10, which dampen the positive effects of IFN-γ to result in suppression.

Little, if any, toxicity has been displayed by poly(A) · poly(U). On the other hand, poly(I) · poly(C) has proven to be toxic to intestinal tissue, pyrogenic, and lethal for mice and to induce hemorrhagic necrosis in kidneys (117).

With the extensive documentation of the adjuvant action of poly(A) · poly(U) and its lack of demonstrable toxicity, this homoribopolymer was tested in human beings as an adjunct to surgery for breast cancer (79, 81). These trials were preceded by a demonstration of its efficacy in the treatment of spontaneous mammary tumors in mice and transplantable melanoma in hamsters (80). Treatment of women with 30 mg of poly(A) · poly(U) intravenously once a week for 6 weeks following surgery with or without cobalt therapy resulted in an increase in the actuarial survival rate of 71% in the adjuvant-treated group compared with 57% in the control group after 87 months. This increase was due in large part to a heightened survival in lymph node metastasis-positive patients. Since no significant toxicity was observed in these patients, poly(A) · poly(U) is undergoing further testing in clinical trials.

In a study of poly(A) · poly(U) action on tumors in rats, Nolibe and Thang (110) confirmed an increase in natural killer cell activity in peripheral blood leukocytes as well as lung intracapillary leukocytes. Of interest was their finding of a profound reduction in the number of pulmonary metastases in rats given poly(A) · poly(U) [or a mismatched analog of poly(I) · poly(C)] intraperitoneally twice a week over a 9-month period. Control rats

averaged 172 nodules versus 27 for rats given poly(A) · poly(U) and 19 for the mismatched poly(I) · poly(C). In addition, a marked regression of tumor mass resulted from the intraperitoneal injection of poly(I) · poly(C) together with IL-2, as reported by Iigo et al. (50). Poly(A) · poly(U), on the other hand, retarded the growth of the adenocarcinoma 755 tumor only slightly.

In addition to augmenting the specific immune response, the polyribonucleotide complexes, like lipid A, are effective at amplifying innate NSR against diverse microbial infections. For example, poly(A) · poly(U) injected 1 day before challenge protected 7-day-old mice against *P. aeruginosa* and 4-month-old mice against *Streptococcus pneumoniae*. The extensive early studies on this phenomenon have been reviewed (59). In addition, a striking protection against murine cytomegalovirus by these complexes has been reported recently (1, 90).

IMIQUIMOD

A recently described adjuvant, termed Imiquimod (R837), has been shown to be active against herpes simplex viruses (HSV). This immunomodulator is a quinolinamine derivative with the formula 1-(2-methyl propyl)-1H-imidazo (4,5-c) quinolin-4-amine which has no in vitro activity against HSV. Nevertheless, recent studies in guinea pigs have shown topical R-837 to be effective against HSV type 2 genital infection (10). Therapy initiated 12 to 36 h after infection reduced neural recovery of virus, as well as the total mean lesion score and the period of vaginal HSV shedding.

Imiquimod also proved to be an effective adjunct to an HSV glycoprotein subunit vaccine in guinea pigs (11). Inclusion of R837 at the time of vaccination followed by 5-day treatment regimens decreased the overall severity of the acute disease compared with immunization alone, with less virus shedding and fewer recurrent HSV lesion days.

MDPs AND ANALOGS

Freund's complete adjuvant, a water-in-oil emulsion in which water droplets containing antigen are emulsified in mineral oil containing killed mycobacteria or their cell walls, has been the adjuvant of choice for animal experimentation for many years. Its profound toxicity, however, has precluded its use in human beings. Consequently, much effort has been devoted to isolating the active component of the mycobacterium and understanding the physical attributes that contribute to the markedly enhanced immune response it induces. Studies culminated in a description of the adjuvant properties of a fraction isolated from the Wax D component of mycobacteria. The minimal structure capable of replacing the activity of the whole bacterium was published in 1974 (32) and was identified as *N*-acetylmuramyl-L-alanyl-D isoglutamine (muramyl dipeptide [MDP]); L-valine, L-serine, or L-threonine can substitute for L-alanine. In the ensuing years, the multiplicity of properties of this extraordinary molecule have been described in detail and reviewed extensively (2, 13, 19, 20, 89, 113, 115). This myriad of studies in animals has established that MDP exhibits a broad array of immunological effects, including (i) enhancement or suppression of antibody levels dependent on time of administration relative to antigen; (ii) increased cell-mediated immunity; (iii) increased nonspecific immunity to bacteria, viruses, fungi, and parasites; (iv) stimulation of natural resistance to tumors; (v) induction of autoimmune disease; (vi) increased cytokine release; (vii) increased IFN-γ; (viii) pyrogenicity; and (ix) somnogenic activity.

Since some toxicities (e.g., pyrogenicity, experimental autoimmune diseases, and uveitis) were associated with MDP, the search was extended to synthesize analogs devoid of such properties. On the hypothesis that the addition of fatty acids to MDP would aid attachment and subsequent integration of MDP into target cell membranes, lipophilic derivatives were synthesized in which fatty acids were attached to C-6 of the glucosamine (99) or to the C-terminal end of the peptide chain (115). Also, the addition of a third amino acid and L-alanine phosphatidylethanolamine to MDP (MTP-PE) has been found to be an effective adjuvant for delivery via liposomes (140). A low toxicity for humans of MTP-PE has been observed by Wintsch et al. (150).

An analog receiving major interest in the clinical arena involves attachment of a butyl ester group into MDP (*N*-acetylmuramyl-L-alanyl-D-glutamine-*n*-butyl ester, termed murabutide). This compound was devoid of the toxicities associated with MDP but retained full adjuvant properties as well as the ability to stimulate NSR to microbial infections (135). Following successful phase I and II clinical tests, it shows promise as an adjuvant for human vaccination.

The ability of MDP, when given prophylactically, to offset infections by a variety of microbial agents has been tested in a number of experimental disease models. Thus, MDP was found to protect mice against lethal infection by *Plasmodium berghei* with only minimal histopathological abnormalities produced (70, 71). This study was preceded by an earlier one in which MDP was shown to be a stimulant of both humoral and cell-mediated immunity. On the other hand, in this model phagocytosis was already stimulated maximally by the parasite alone (71). Extension of these studies to rhesus monkeys challenged with *Plasmodium knowlesi* resulted in some protection by a vaccine consisting of an aqueous suspension of MDP plus a whole-antigen preparation of the plasmodium (69). In addition, strong inhibition of the growth in vitro of *Plasmodium falciparum* was exhibited by sera of rabbits immunized with *Plasmodium falciparum* merozoite surface protein (gp 165), together with 6-*O*-2-tetradecylhexadecanoyl-MDP (termed B30) in liposomes, as well as by MDP-B30 plus LA-15-PH, a synthetic equivalent of monophosphoryl lipid A (47). However, Collins et al. (25) failed to induce protective immunity following vaccination with *Plasmodium falciparum* peptides fused with diphtheria toxoid with which MDP was combined. Conjugation of a plasmodial peptide to diphtheria toxoid with MDP was found by Lew et al. (92) to produce high levels of antibody with high avidity.

Allison and Byars have developed an adjuvant formulation consisting of *N*-acetylmuranyl-L-threonyl-D-isoglutamine in a squalane pluronic polymer emulsion (SAF) (2). It was effective in animals in increasing the response to influenza virus hemagglutinin, hepatitis B virus surface antigens, and HSV type 2. MDP also aided the immunogenicity of an inactivated whole simian immunodeficiency virus vaccine when given to rhesus macaques. No virus was recovered from three of three monkeys receiving MDP following intravenous challenge with simian immunodeficiency virus compared with one of three responding to the challenge alone (14). Murphey-Corb et al. (106) have reported the efficacy of threonyl MDP, as well as MDP alone, in enhancing the protection induced by a formalin-inactivated whole simian immunodeficiency virus vaccine against challenge with simian immunodeficiency virus in 9 of 10 rhesus monkeys.

Incorporation of MTP-PE into liposomes along with a glycoprotein from HSV type 1 resulted in an enhancement of proliferation of antigen-specific peripheral blood lymphocytes. This enhancement was related to a 75% suppression of virus activity (44). Similar results were obtained without liposomes by Sanchez-Pescador et al. (125).

An enhanced capacity of human polymorphonuclear leukocytes stimulated by MDP to kill *C. albicans* has been reported by Jupin et al. (63). An MDP analog (MDP-lys [L18]) was found to augment a live *S. enteritidis* vaccine in mice (112). Nonspecific enhancement of resistance against a polymicrobial intraperitoneal challenge consisting of *Streptococcus* (*Enterococcus*) *faecalis*, *E. coli*, and *Bacteroides fragilis* was documented by Cheadle et al. (18).

The usefulness of MDP as an adjuvant has been extended to induction of infertility in animals by Shaha et al. (126). Thus, 80% of rats immunized with *n*-MDP plus a 40-kDa antigen from human serum became infertile compared with 13% of controls. A thylated derivate of LPS combined with the antigen produced the same result. MDP had been shown by Sacco et al. (124) to enhance the in vitro contraceptive capacity of sera from female squirrel monkeys immunized with a glycoprotein from porcine zona pellucida.

Like LPS and the polyribonucleotide complexes, the many activities of MDP can be attributed to its ability to cause the release of multiple cytokines.

NONIONIC BLOCK COPOLYMERS

In 1981, a new approach to adjuvanticity was introduced by Hunter et al., who used nonionic block copolymer surfactants (49). These compounds are composed of two chains of hydrophobic polyoxyethylenes of various lengths combined with a block of hydrophobic polyoxypropylene and are administered with oil and squalene in an oil-in-water emulsion. They proved to be effective adjuvants in several animal species and are now available commercially. These copolymers are thought to function by binding protein antigens to the surface of oil drops, resulting in more effective antigen presentation. In addition, complement is activated, facilitating binding to antigen-presenting cells. Such preparations enhanced antibody titers to levels comparable to those reached when Freund's incomplete adjuvant was used and exhibited synergy when combined with LPS. Of importance, titers of IgG2a and IgG2b, which are associated with protection against infectious challenge, were heightened preferentially (48, 64, 131).

To obtain adjuvanticity against a broader array of antigens, similar polymers with emulsion-stabilizing silica particles were tested with water-in-oil emulsions in squalene (termed Titer Max). Titers against trinitrophenol conjugated to ovalbumin and luteinizing hormone-releasing hormone were compared in several animal species with several other commercially available adjuvants (9). Titer Max equalled or exceeded each of them under the conditions used. Consequently, such preparations have been combined with MTP in the adjuvant formulation adopted by Allison and Byars for testing in clinical trials (2).

SAPONINS

The bark of a South American tree, *Quillaja saponaria*, has been found to yield a class of compounds termed saponins or Quil A, which have been shown to be excellent adjuvants. Saponins are amphipathic, with a hydrophilic carbohydrate linked to a hydrophobic steroid or triterpene moiety. Their monomeric molecular weights range from 1,800 to 2,200, indicating 8 to 10 monosaccharide residues. They form the basis of immunostimulatory adjuvant complexes known as ISCOMS that generally are complexes of saponin with viral membrane proteins. Their detergent-like activity has been postulated to be responsible for their action on the immune response; however, undesirable side effects, including strong hemolytic activity, have prevented their extensive use to date. Although the initial adjuvant studies were carried out with relatively crude preparations, recent applications of silica and reverse-phase chromatographic techniques have resulted in purified compounds that retain adjuvant activity.

Kensil et al. (67) separated multiple fractions of varying composition from an initial crude extract of saponin. Several of these fractions possessed significant adjuvant activity. One, designated QS 7, was a weak detergent but nevertheless exhibited adjuvant activity equal to those of other strongly hemolytic fractions. Close association between saponin and antigen was necessary for effectiveness since injection at different sites did not result in increased titers. Of interest, no increase in IgE levels, a potential cause of immediate hypersensitivity, was found. A second fraction, QS 21, was shown recently by Newman et al. (109) to induce class I major histocompatibility complex antigen-restricted cytotoxic T lymphocytes with activity against a synthetic ovalbumin peptide. Since viral proteins generally do not give rise to cytotoxic T lymphocytes, the findings of Wu et al. (151) are significant in establishing the efficacy of 10 to 20 μg of the QS 21 fraction in the presence of alum to induce both humoral and cell-mediated immunity to a recombinant human immunodeficiency virus type 1 envelope protein, gp 160.

A thymus-independent antigen, the polysaccharide fraction from the LPS of *E. coli*, also demonstrated enhanced immunogenicity in mice in the presence of QS 21. IgG2a and IgG2b were elevated most strongly (149). Kensil et al. (68) reported that neutralizing antibodies induced by QS 21 as adjuvant to gp70 envelope protein protected against feline leukemia virus infection.

E. COLI LT

Oral adjuvants that increase mucosal immunity have not emerged until recently. The importance of stimulation of this branch of the immune system becomes obvious with the knowledge that it is the major site of entrance of human pathogens and also possesses the largest composite of lymphoid tissue in the body. Emphasis has been given to cholera enterotoxin (CT), which has been shown in a number of studies to elevate antibody titers following oral immunization (45, 95, 145). CT is composed of two subunits: one is responsible for binding (B) and the other, the active principal (A), affects ADP-ribosyltransferase (96). Its prospects for use in human beings, however, are dampened considerably by the toxicity of the A component. Recently, the adjuvant properties of an orally administered *E. coli* heat-labile enterotoxin (LT), which shares partial structural homology with CT, have been reviewed (98). Although LT also expresses toxicity through ADP ribosylation, differences in receptor binding as well as a process of isolation of LT that results in considerably reduced acute toxicity but retention of adjuvanticity have been described (98). In contrast to CT, LT is not secreted by the bacterium; rather, it must be extracted from the cells, cleaved enzymatically, and reduced prior to exerting any toxicity (22, 24). This property, together with the characteristic of LT (again in contrast to CT) binding to galactose as well as to the ganglioside G_{m1} (which disperses the enterotoxin away from the intestinal tract, its site of toxic action), is thought to retard and reduce the evolvement of enterotoxicity. Of considerable practical importance, LT can be given without complex vehicles or conjugates; it is simply coadministered with antigen orally. The importance of increasing mucosal immunity and the exciting potential of LT has led the U.S. Navy and U.S. Army to begin conducting human phase I trials with this adjuvant.

Convincing experimental studies have documented excellent adjuvant action in animals to a wide range of antigens, e.g., ovalbumin, bovine serum albumin, HSV, *Campylobacter coli*, and influenza vaccine (98, 145). Of concern with any orally administered adjuvant is whether tolerance to dietary substances in healthy individuals might be broken, giving rise to allergies. However, LT did not abrogate oral tolerance (23), and CT did not induce antibody to dietary proteins (108).

DHEA

Adjuvants are particularly applicable for immunization of individuals in whom an immunodeficiency is evident (e.g., AIDS, cancer, virus infections, congenital aberrancies, or aging). Toward this end, an endogenous steroid hormone, dehydroepiandrosterone (DHEA), has been studied recently by Daynes and colleagues (4, 27, 28). Earlier, they demonstrated the capacity of DHEA to influence T-cell function by elevating IL-2 and IFN-γ secretion without affecting IL-4 and IL-5 production (3). Consequently, this immune stimulating hormone appears to affect selectively the Th_1 subpopulation. This pattern of cytokine release is associated with the elevation of cell-mediated immunity, the branch essential for resistance against many viruses and intracellular pathogens.

DHEA is produced when its prohormone, DHEA sulfate (DHEAS), is cleaved by a sulfatase enzyme. DHEAS also was found to possess immunomodulatory properties, affecting primarily the nonmucosal lymphoid tissue, which possesses the highest DHEAS sulfatase activity required for conversion to the active DHEA form (28).

Aged mice as well as humans have been shown to exhibit an age- and stress-dependent decline in endogenous DHEA production. The potential of supplementary DHEA as a particularly effective immunomodulator in immunodeficiency states was demonstrated recently in an aged mouse model system (4, 29). A single topical administration of DHEA was effective at reversing the cytokine phenotype of aging mice to that expressed by mature adult mice. Thus, levels of IL-2, IL-3, and granulocyte macrophage colony-stimulating factor were increased by this hormone. Both primary and secondary antibody responses were enhanced. Similarly, when recombinant hepatitis B surface antigen was used for footpad vaccination, DHEAS, when given either before or with the vaccine, increased recombinant hepatitis B surface antigen-specific antibody in aged mice (4).

Thus, incorporation of DHEAS into the diet, or DHEA given subcutaneously or topically, may repair deficiencies in the suboptimal responses associated with certain disease states and aging. Its potential as an adjunct to human vaccines is being explored.

CONCLUSIONS

Within the past decade basic science research has extended greatly our knowledge of how the immune system is regulated. Multiple inter- and intracellular molecular signals capable of elevating or inhibiting both humoral and cell-mediated immunity have been identified. As a result, exogenous manipulation and magnification of such signals now is possible with a variety of recently defined purified and synthetic molecular adjuvants of minimal toxicity, as described herein. Consequently, the future appears bright for increasing the efficacy of human vaccines heretofore expressing only weak antigenicities, as exemplified by certain tumors, viruses, and synthetic ligands.

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