ABSTRACT

J.L. Armacost. CHARACTERISTICS OF THE IMMUNE RESPONSE TO TYPE III PNEUMOCOCCAL POLYSACCHARIDE INDUCED IN BALB/C MICE THROUGH THE USE OF IMMUNOSTIMULATING COMPLEXES. (Under the supervision of Dr. A. Mason Smith) Department of Biology December 1997.

This research was designed to study the immune response in BALB/c mice to a type III pneumoccocal capsular polysaccharide-bovine serum albumin (BSA) conjugate incorporated in immunostimulating complexes (ISCOMs). The study included the distribution of the immunogen via different routes while monitoring both the systemic and mucosal responses.

This work was based on the original hypothesis that a polysaccharide-carrier protein conjugate incorporated into ISCOMs would (1) induce an anamnestic response consisting primarily of IgG antibodies when delivered subcutaneously (s.q.) and intraperitoneally (i.p.), and (2) generate an IgA response when given orally to BALB/c mice. The humoral immune responses were monitored in serum and intestinal gavages by ELISA and PHA. The quantification of splenocytes producing antibody specific for type III polysaccharide was done by ELISPOT. Peyer's patch cells were characterized after staining thin sections with hematoxylin and eosin.

In preliminary studies, type III pneumococcal polysaccharide was conjugated with BSA using two different chemical reagents: 4-(maleimidomethyl) cyclohexane 1-Carboxyl hydrazide (M_2C_2H) and cyanuric chloride ($C_3Cl_3N_3$). The presence of the ISCOMs structure was verified by electron microscopy, and its incorporated conjugate by carbohydrate assay and protein assay.

ISCOMs injected i.p. and s.q. induced both an IgM and IgG systemic response. The IgG response was shown to be continuous over several weeks. Data obtained by ELISPOT indicated that the spleen was a major source of antibodies produced. Therefore, presented evidence supports the induction of an anamnestic response when the type III polysaccharide was given as part of the ISCOM vehicle.

Oral feeding of the polysaccharide in the ISCOM vehicle did not produce secretory IgA in the intestine as measured in this study. However, oral feeding did produce a systemic IgG response and significant changes in Peyer's patch cell populations. These findings are discussed along with the importance of ISCOMs as a means of inducing protective immunity against some common bacterial polysaccharides.

CHARACTERISTICS OF THE IMMUNE RESPONSE TO TYPE III PNEUMOCOCCAL POLYSACCHARIDE INDUCED IN BALB/c MICE THROUGH THE USE OF IMMUNOSTIMULATING COMPLEXES

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CHARACTERISTICS OF THE IMMUNE RESPONSE TO TYPE III PNEUMOCOCCAL POLYSACCHARIDE INDUCED IN BALB/c MICE THROUGH THE USE OF IMMUNOSTIMULATING COMPLEXES

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Dedication

ך The completion of this small work required patience

כ A small step reaching toward the final hour

ק When "I AM" reclaims His kingdom

and His right

Acknowledgements

The last few years have been the type of challenge that I believe will serve me well throughout the remainder of my life. These skills have and will enable me to better fulfill the purpose to which I was created. Many persons have contributed to this success, and certainly no one can achieve goals in life without the hard work and patience of others. The first acknowledgement rightfully belongs to my parents, who quietly supported me throughout the arduous process that is graduate work. Others who contributed mightily to my education are: Lily Fainter, Dan Whitehead and Tim Charles for their many hours engaged in electron microscopy. Also, my gratitude to Dr. Cindy Putnam-Evans and fellow graduate students Susan McKnight and Michele Kosovac for sharing their experiences and friendship. My thanks to Carolyn Jones, who provided technical support, and to my committee members, who provided valuable perspective and technical acumen. Finally, it is fitting that I acknowledge the person who opened his lab and his years of experience to me, Dr. A. Mason Smith. I am the last of a long line of Dr. Smith's graduate students who have benefited from his skill and direction.

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INTRODUCTION

Streptococcus pneumoniae is a common human pathogen that has been widely studied using a variety of animal models, and most extensively in BALB/c mice. There are approximately 90 serotypes of *S. pneumoniae* (1), based on different polysaccharide capsular reactivities. The study of *S. pneumoniae* dates back to the research of Pasteur, Osler and Avery (2). Serotypes of greatest frequency in human disease are types XIV, IV, III and VIII. Their capsular structures confer virulency to the organisms, and result in the different immune responses between serotypes (3).

S. pneumoniae, bearing polysaccharide type III, (SIII) is one of the few streptococcal capsular structures that has been fully characterized. The acidic-charged capsule consists of repeating disaccharide units of D-glucose in a β-1,4 glycosidic linkage with D-glucuronic acid, with a molecular weight approximating 100-200K (3). The enduring importance of type III among streptococcal disease and the extensive knowledge accumulated on this serotype in animals and humans make it ideal for further work in elucidating immune function.

Much of the last two and one-half decades of SIII research has been conducted in BALB/c mice by Drs. P.J. Baker, Barthold and Braley-Mullen. Past research had discovered SIII, like other polysaccharides, to be a type 2, thymus-independent antigen, (TI-2). TI-2 antigens are not processed through the Major Histocompatibility Complex class II (MHC II) and therefore do not enlist aid from the T helper subset (4). This finding is in contrast to T cell-dependent antigens such as proteins, which are processed

by antigen-presenting cells, (APCs) and presented to T cells as peptides to stimulate cytokine secretion. TI-2 antigens cross-link antigen receptors on B cell membranes specific for the polysaccharide epitopes and induce an Ig-mediated membrane signal transduction (5). This transduction may be aided by cytokines secreted by Natural Killer cells, (NK) (6).

The immune response to SIII has been shown to be regulated by competitive interactions between suppressor T cells and amplifier T cells. These subsets are responsible for regulating the magnitude of the immune response against SIII (7). T suppressor lymphocytes, (Ts) have been shown to abrogate plaque-forming cell (PFC) production by preventing B cell differentiation into plasma cells (8). It has been suggested that another subset of T cells, contrasuppressors T cells (Tcs), may function to offset Ts cell activity (9). When injected intraperitoneally, (i.p.) or subcutaneously (s.q.), SIII induces a specific IgM immune response that is characteristic of TI-2 antigens (10). These competitive interactions of SIII regulation account for the short-lived immune response to polysaccharide and the absence of an anamnestic response in BALB/c mice (10), and in infants <24 months of age (11).

An anamnestic response, also known as a secondary response, is important to generate in human and animal populations that normally do not acquire immune memory to pneumococcal microorganisms. Immune memory to microbes is retained by antigen-specific, long-lived, resting B memory cells that are selected to differentiate into memory cells as a result of initial antigen contact. These memory cells generate a rapid, high affinity response and clonal proliferation on subsequent antigen encounter (5). Antigen,

encountered months or years after the initial immune response, is captured by B memory cells at the interface of cortical lymphoid tissue and follicular structures. The B memory cells become the predominant APC to T memory cells in these responses, and the T cell-derived cytokines in turn direct the Ig isotype switching that enable the production of isotypes other than IgM. Additionally, somatic mutation of variable region B cell genes results in the high affinity binding within the Ig hypervariable regions (5). Cytokines also stimulate the rapid clonal proliferation of these B memory cells into large numbers of plasma cells (12).

The generation of an anamnestic response has been achieved through a variety of immunization routes, conjugation methods and adjuvants. This direction of study has been pursued in order to increase immunity to diseases in which traditional vaccination schedules, formulations and routes have induced poor responses or short-lived immunity. Historically, polysaccharide-protein conjugations have yielded some success in converting bacterial polysaccharides to thymus dependent (TD) antigens when conjugates were administered i.p. or s.q. (13, 14, 15). Despite this limited success, the exact mechanism behind polysaccharide-protein interaction with T-helper cells and the subsequent activation of B cells specific for polysaccharide antigen is unknown. One theory states that bystander B cells specific for the hapten (polysaccharide) are stimulated upon release of cytokines emanating from those T and B cells which specifically target the carrier protein. The fact that bacterial polysaccharide is not processed appreciably by eucaryotic cells makes this argument plausible (5).

Different conjugation methods and varied routes of vaccination have offered

insight, but have also produced unanswered questions. Peeters et.al. (16), conjugated pneumococcal polysaccharide type IV (PSIV) to the carrier protein tetanus toxoid (TT), and alternately, PSIV oligosaccharides conjugated to TT via the carbodiimide method. IgG responses toward PSIV were greater after the i.p. injection of the polysaccharide conjugates than mice injected with the oligosaccharide conjugate which consisted of short carbohydrate moieties. Fewer epitopes on the oligosaccharide could have contributed to a diminished immune response, but this theory was not confirmed. Another problem arose from Peeters' study when mice developed immune tolerance after repeated injections with higher doses of the PSIV-TT conjugate (>0.5μg). SIII, in contrast, has been shown to be optimally immunogenic in BALB/c mice at the low dose of 0.5μg, while higher doses evoke immune tolerance and suboptimal doses induce short-term immune paralysis by T-suppressor cells (17). Peeters' study exemplified the precarious balance needed to produce a conjugate that will induce significant immunologic response toward both the polysaccharide and the carrier. Evoking an anti-TT response with higher doses may have been attained at the expense of tolerizing T cells that were involved in the anti-polysaccharide response. Conjugates of components derived from different pathogens is a useful vaccine strategy in the delivery of immunogens, and optimization of this system requires a precise understanding of the immune responses to all components in the vaccine formulation.

Paul et.al., successfully conjugated SIII to bovine gamma globulin (BGG), using the cyanuric chloride method ($C_3Cl_3N_3$) (18). Anamnestic responses to the vaccine

injected intravenously in rabbits were elicited, but only after priming with SIII, followed closely by a s.q. injection of the carrier protein. These data showed the necessity for the carrier effect and further showed that prior exposure to the carrier could enhance subsequent immune response to a conjugate vaccine (18). Although prior exposure to the carrier might be introduced from the environment, such enhancement could also be absent without priming, as proved to be the case in Paul's study.

The importance of protein-carbohydrate crosslinking related to adjuvanticity was shown by Verheul et.al. (13). Verheul conjugated pneumococcal polysaccharide type XIV, (PSXIV) to BSA using the carbodiimide method. BALB/c mice were intracutaneously inoculated with a mixture of conjugate and a plant saponin, Quil A, as an adjuvant. Verheul's group experimented with different polysaccharide-protein ratios and coupling conditions, and their data showed the effects of protein and polysaccharide ratios and the degree to which conjugate crosslinking had on the immune response.

The importance of crosslinkage as seen from previous studies (13, 14), has been optimized in recent years in conjugation methods by tailoring the experimental conditions to accommodate the structure of both conjugate components. Initially, researchers used crosslinker reagents that conjugated antigens without regard to what effect the bond length between components might have on immunogenicity. The cyanuric chloride ($C_3Cl_3N_3$) reagent, as an example, was first employed as a crosslinker in the late 1960's (19), using a triazine bridge to form lattice structures. The $C_3Cl_3N_3$ reagent's short spacer groups produced tight lattices between conjugate components in which epitopes might not be accessible to soluble antibodies for binding (20). A widely applied method

is the amidation of the carbodiimide reagent to the formation of a reactive isourea intermediate. Innovations to this method came with the addition of hydroxysuccinimide (sulfo-NHS), in order to stabilize the isourea intermediate. Stabilization of the intermediate structure was made possible due to the reagent's ability to work within a broader pH range. This enhanced ability increased the selection of proteins to which carbodiimide could react (20). The addition of an homodifunctional spacer, adipic acid dihydrazide (ADH), improved the immunogenicity of conjugates, but in some cases created excessive crossslinking. Treatment of the antigenic components with ADH resulted in excessive intralinkage of reactive groups within the primary antigen to the exclusion of crosslinkage with the reactive groups of a second antigen. The lack of reactive sites available to the second antigen for binding to the first resulted in a less immunogenic conjugate. Strictly controlled experimental conditions designed to accommodate individual antigen chemistry resolved most problems with the carbodiimide method and homodifunctional spacers, as shown in a study by Verheul (13).

Another class of conjugate spacer, heterobifunctional, was created to maximize crosslinkage within the conjugate components and simultaneously introduce a spacer that incorporated several bond lengths between antigens. As used in the current study, the crosslinker, 4-(maleimidomethyl) cyclohexane 1-Carboxyl hydrazide (M_2C_2H), has a maleimide group and a carboxyl group located at opposite ends of the molecule. As a heterobifunctional crosslinker, M_2C_2H reacts with the antigen moieties in a stepwise, controlled manner. This chemical mechanism yields greater coupling distance

leading to improved T cell interaction with peptide epitopes and increased accessibility of soluble antibodies to epitopes (20).

The degree of crosslinkage and coupling distance between conjugate components has been shown to increase conjugate immunogenicity (20). An additional consideration is polysaccharide chain length and the effect of the protein carrier on the immune response. It had been shown with some polysaccharides that chain length influences the IgG subclass (15), and the strength of the immune response (21). It had also been shown that oligosaccharides rather than high molecular weight polysaccharides induced stronger immune responses in conjugates incorporating *Haemophilus influenzae* type B polysaccharide (15, 21). These observations, however, were not supported by studies that examined this factor using other polysaccharides. Pneumoccocal polysaccharide XIV, for example, induced significant IgG titers after conjugation with bovine serum albumin (BSA) (13), and in another study, IgA titers were detected against *Streptococcus mutans* polysaccharide after conjugation to salivary proteins (22). Peeters et.al. noted higher IgG titers against their polysaccharide-TT conjugate over that of the response shown with their oligosaccharide-TT conjugate (16).

Another consideration in the preparation of a conjugate is the incomplete crosslinkage of polysaccharide to protein resulting in the immune recognition of the unconjugated, repeating epitopes of polysaccharide as a TI-2 antigen (20). This TI-2 recognition could nullify the strategy to create a TD response by associating the polysaccharide with the carrier protein. It is imperative that reagents allow for maximal crosslinkage while avoiding the inappropriate crosslinkage present in homodifunctional

reagents.

Equally important to the immune response against the conjugate vaccine is the influence of the protein carrier. Data on protein carriers have shown that a carrier can influence the generation of specific IgG subclasses, as seen in the examples of *Salmonella*-derived flagellar protein and BSA conjugated to PSXIV (13, 14). Flagellin used as a carrier protein resulted in a IgG_3 subclass response, in contrast to the IgG_1 subclass to PSXIV using BSA as carrier. Other reported studies using *Salmonella* flagellin as a carrier found IgG_{2a} and IgG_3 predominant in the response to streptococcal polysaccharide in mice (23). Contrasted with those findings were the studies that showed tetanus toxoid, keyhole limpet hemocyanin and diptheria toxoid carriers elicited IgG_1 against polysaccharide in mice (24, 25).

Modulation of the strength of the immune response and immunoglobulin subclass is also influenced by introducing adjuvants as a component of vaccines. Oil emulsions such as Freund's (CFA), metal salts such as alum or beryllium, or structures mimicking cell membrane composition provide adjuvanticity (26). The primary action of an adjuvant is to stimulate APCs and naive, unstimulated B cells into the expression of costimulators. These costimulators have been shown to be essential to optimal T cell activation in the presence of processed antigen (5). For example, the costimulator molecule B7 (CD80) is a common structure on APCs. B7 binds the T cell receptor CD28 while simultaneously, processed peptides bound by MHC molecules are then bound by the TCR. Gene upregulation for the production of the regulatory cytokine IL-2 and increased stability of IL-2 mRNA occur with the resultant T cell stimulation. The

adjuvant itself is not immunogenic; however, it might modulate antigen presentation via both major histocompatibility classes to select for CD4+ and CD8+ T cell subsets and work to ablate CD8+ T suppressor populations (26). Another characteristic of some adjuvants is their ability to prolong antigen release in tissue leading to the enhancement of the immune response by prolonging antigen removal and increasing inflammation (27).

Saponin, an adjuvant derived from the Quillaja plant, was used in the current study for its ability to impart adjuvanticity to the SIII-BSA conjugate after intraperitoneal, subcutaneous and oral administration. Other studies have shown saponin to enhance mucosal surface permeability to antigen after oral immunization (28, 29), and to stimulate cytotoxic T lymphocytes (CTLs), and natural killer cells (NK) (30). Saponin has unique properties that enable it to spontaneously form micellar complexes in combination with cholesterol and phosphatidylcholine (PC), while simultaneously creating hydrophilic pores throughout the complex. The saponin molecule consists of a triterpene molecule bearing oligosaccharide moieties at carbon-28 and carbon-3 of the molecule totaling eight sugars per molecule. The five-sugar oligosaccharide on carbon-28 of saponin interacts with other saponin molecules to repel the sugar moieties, and this results in the formation of the hydrophilic pores. The pores enable water-soluble molecules to diffuse through and interact with enveloped, protruding antigen epitopes within the micellar complex. The carbon-3 trisaccharide is speculated to provide a hydrophilic "shield" for overlying and underlying hydrophobic groups (31). Saponin's sugar moities serve the dual purpose of conferring stability on the micellar complex and

together with cholesterol and PC generating the unique "honeycomb" geometry, known as immunostimulating complexes (ISCOMs) (32). Concerns about the immunogenicity of the ISCOMs matrix and its sugar moieties have been addressed by a study that has shown that the matrix does not exert mitogenic effects in vitro (33).

Cholesterol and phosphatidylcholine (PC) constitute a large portion of the ISCOMs matrix with these components mimicking cell membrane chemical composition. This mimicry has been shown to allow the ISCOMs structure to directly adhere to cell membranes in order to expedite antigen processing (34). Additionally, the PC component facilitates the incorporation of protein antigen into the ISCOMs structure (35).

The ISCOMs structure is approximately 40nm in diameter. The structure incorporates both the adjuvanticity of saponin, while creating an environment of close proximity of adjuvant to antigen, and producing a surface studded with multiple antigen epitopes (34). A caveat ascribed to the ISCOMs vehicle is the inability to incorporate non-amphipathic molecules. This problem was overcome by the palmitifying or conjugating of cell membrane proteins to hydrophilic proteins. Another approach was to acidify proteins as was done in the current study with the protein carrier BSA. Acidification exposes hydrophobic surfaces on the protein for interaction with the hydrophobic moieties in the ISCOMs structure. However, it was recognized that denaturation of the protein could potentially change the epitope structure (36).

The immunomodulating effects of ISCOMs have been extensively analyzed in studies using a variety of proteins as a conjugate or in the unconjugated state (37, 38, 39,

40). ISCOMS injected i.p. have shown the ability to achieve extensive contact and adhesion to peritoneal cells as a result of saponin's surfactant properties. It is theorized that the rigidity of the ISCOMs structure allows it to be internalized within the cells lining the mucosa such as macrophages and small lymphocytes prior to structure degradation (41). The clearance of ISCOMs from the peritoneum was shown to be rapid but less rapid than that seen with other micellar structures. This increased association with peritoneal APCs prior to relocation throughout the lymphatics may enhance processing and presentation to B cells and T-helper cells in the spleen, thereby modulating the immune response to a significant degree. It is reasonable to conclude, based on Watson's work, that the SIII-BSA ISCOMs would follow the same route after i.p. injection (42). Another intestinal cell type of importance to ISCOMs is the M (microfolding) subset. The M cells are largely devoid of villi or fully functioning lysosomes needed to process and degrade macromolecules. Their high lipid content and low protein content make their contact with and adhesion to lipid molecules favorable (43). Transport of water-soluble molecules such as BSA proceeds at a less efficient pace. It is possible because of the lipid components of ISCOMs and the adhesive qualities of M cells that a favorable interaction between the two does occur.

The stability of ISCOMs exposed to gastric and intestinal enzymes after oral administration might have been a concern, but the ISCOMs structure had been shown to resist the degradative effects of gastric acids and bile salts from the gastrointestinal tract (44). ISCOMS have also been shown to generate an immune response after oral immunization without inducing immune tolerance. This effect was documented as an

unusual finding in the midst of a large body of research showing that ingested antigen, proteins in particular, invariably led to immune tolerance (45, 46).

Oral immunization becomes an ideal immunization route for stimulating both systemic and mucosal immunity as a result of its ease of delivery. This route places the immunogen in direct contact with mucosal cells that transport antigen into microenvironments containing lymphocytes. Among these areas of concentrated lymphoid cells are the Peyer's patches located predominantly throughout the serosal side of the distal colon and rectal mucosa in humans (47), and in mice are concentrated in the ileum (48). The location of the lymphoid tissue along the gut provides for direct stimulation of B cells committed to the production of secretory IgA. Plasmablasts that have been activated in lymphoid tissue germinal centers in lymph nodes and spleen, and those originating in Peyer's patches have the ability to migrate from the site of activation to other sites. Some plasmablasts leave germinal centers and migrate into the bone marrow where they undergo final differentiation into plasma cells and secrete much of the available humoral antibody (49). IgA-secreting plasmablasts follow a different migration route beginning in Peyer's patches with eventual movement into the efferent lymphatics to mesenteric lymph nodes and into the blood. The plasmablasts are then distributed to sites such as the lamina propria of the intestine, lacrimal glands, sweat glands, salivary glands and lactating mammary glands. Secreted IgA found in these localized areas is transported across epithelial cells of mucosal tissue (46).

Introducing antigen via the mucosal route often leads to systemic tolerance (50). The mechanisms generating an immune response against ingested antigen are dependent

on complex mucosal interactions and cell-cell interactions. Antigen processed through the intestinal mucosa for example might be presented to Th2 helper cells, which secrete IL-5 and IL-6 necessary for IgA synthesis. Concurrently, this processed antigen could induce a tolerogenic signal toward serosal Th1 and Th2 cells. Th1 might be induced to secrete IFN-gamma to down-regulate Th2 cells while Th2 cells in turn might secrete IL-10 to down-regulate the Th1 cells. Clonal anergy would result from this Th1/Th2 cyclic feedback. Systemic unresponsiveness could also result from tolergenic signals toward CD8+, cytotoxic T lymphocytes (CTLs). Affected CTLs might secrete excessive levels of TGF-β to ablate B cell differentiation into IgG-secreting plasma cells (50). It could be critical in some disease states to design a vaccine that elicits both an IgA mucosal response and an IgG systemic response; however, a response generating CTLs such as with viral infections possibly would take priority over generating an IgA humoral response. The ISCOMs matrix has been shown to effectively induce CD8+ CTLs , and it could prove protective against viruses in the absence of a significant IgA response (50).

There are many reports in the literature addressing oral tolerance, with most of these discussing the use of soluble antigens (51, 52). Even with this early recognition of the tolerance phenomenon, no consensus has been reached on any one mechanism responsible for oral tolerance (5). Current paradigms include the close relationships between the various subpopulations of T cells and the regulatory functions of cytokines produced by these cells.

Specific IgA regulation by a T cell subset has been shown by the T cell subset Th3. The Th3 subset has been shown to be partly responsible for eliciting a strong

suppressive immune response in the presence of oral protein antigens (53). Th3 cells might ultimately work in conjunction with CD8+ suppressor cells to effect tolerance toward orally ingested antigen. Th3 cells secrete transforming growth factor beta (TGF-β), in quantities theorized to induce tolerance to some antigens while, in contrast, directing IgA class-switching by inducing RNA transcripts from the unrearranged germline. Ultimately, IgA class-switch recombination is induced by other cytokines (54, 55).

A myriad of cells that encounter orally ingested antigen influence the processing of antigen and the subsequent immune response to the potential immunogen. Studies have suggested that antigen processing differences exist within intestinal epithelial cells (IEs) apart from that of B cells and other antigen-presenting cells. It is also speculated that IEs handle antigen in a less degradative process, with the implication that processing of antigen by these cells induces oral tolerance (56). It has also been suggested that aggregated protein is subtly denatured by IEs to reveal hydrophobic epitopes which in turn could bind to CD8+ T suppressor cells. These cells in turn, nonspecifically act to suppress immune responses (57). A study of oral tolerance in rats showed that anti-IA antisera blocked oral tolerance in rats after ingestion of protein. Such research supports the theory that IEs are instrumental in oral tolerance where ingested antigen is a protein (58).

Intra-epithelial cells (IELs), in contrast to IEs are speculated to retain antigen (59). In humans, these cells secrete an adhesion molecule, human mucosal lymphocyte antigen-1 (HML-1), which homes T-helper lymphocytes to antigen retained by IELs (5).

It has also been theorized that lipid interfaces between antigen and intra-epithelial cells could confer a mode of antigen processing sufficient to avoid stimulation of T-suppressor populations (60). IELs are widely dispersed in mucosal tissue throughout the body where antigen-antigen contact is imminent. These cells bear numerous T cell markers which include both αβ and γδ T cell receptors. It has been speculated that γδ-bearing IELs are important to the maintenance and generation of IgA in the mucosa even when systemic unresponsiveness occurs toward an orally administered vaccine (50).

As noted, protein-carbohydrate vaccines have some history of success in animals and humans injected i.p. or intracutaneously with those conjugate formulations, (13, 15, 16). Some degree of mucosal immunity has also been achieved with conjugates and in vaccines of unconjugated protein when used in conjunction with adjuvant vehicles that possess lipid properties (liposomes, ISCOMs), (22, 61, 62). Also, information is now available concerning the use of ISCOMs as vehicles for polysaccharide antigen or conjugates of polysaccharide-protein antigen. One study has used an influenza A nucleoprotein-lipopolysaccharide conjugate in ISCOMs to orally vaccinate C3H/He mice. This vaccine induced high IgA titers in both serum and bronchoalveolar fluids (39). Despite the isolated successes of oral immunization, there still remains much speculation over what causes one antigen to elicit an immune response while another induces oral tolerance.

The current study produced immune responses in BALB/c mice against an SIII-BSA conjugate incorporated within an ISCOMs vehicle. The mice were placed into groups receiving either i.p., s.q. or oral immunizations in order to assess the immune

response via these routes. Preliminary studies evaluated two conjugation methods, 4-(maleimidomethyl) cyclohexane 1-carboxyl hydrazide (M_2C_2H), and the cyanuric chloride method ($C_3Cl_3N_3$) incorporated into ISCOMs and injected i.p. Enzyme-linked immunosorbent assays (ELISAs) and passive hemagglutination assays (PHAs) were used to determine the systemic immune response to each conjugate type. The conjugates were evaluated at each step of their formation and tested for the semi-quantitative protein and carbohydrate content. A modified Lowry (BIO-RAD, Hercules, CA), was used for the protein estimates in the conjugates, while an amino acid analysis (Dr. P. Fletcher, ECU School of Medicine) was done on the ISCOMs preparations. A miniaturized carbohydrate assay modified from Dubois' technique (63), was used for the SIII estimates. Based on the collected data, the M_2C_2H method of conjugation was employed throughout the study. ELISAs were used to detect serum anti-BSA and anti-SIII IgG-specific titers for all immunized groups, and PHAs detected a pan-antibody response specific for SIII in serum. The different methodologies were employed for the detection of the anti-SIII response to obtain specificity and sensitivity. Splenocytes collected from all groups immunized with the conjugates generated by the M_2C_2H method were examined for antibody-secreting cells specific for IgG anti-SIII using the ELISPOT. The ELISPOT provided direct visualization of IgG-secreting spot-forming cells (SFCs) specific for SIII, and was used to detect cells from i.p., s.q. and orally immunized mice.

The purpose of this study was to generate an anamnestic response to the polysaccharide capsule of SIII via i.p., s.q. and oral routes. It has been documented that a humoral response to the polysaccharide capsule as elicited by vaccination does confer

protection to the viable organism in many cases (64, 65, 66). The possibility of eliciting a primary or secondary response in the intestinal mucosa to the conjugate after incorporation in the ISCOMs vehicle was explored. The serum and gavages collected from these orally immunized groups was tested using the ELISA and ELISPOT techniques for antibodies to SIII and BSA of the IgG, IgA, IgM and IgE isotypes. Additionally, Peyer's patches were collected from these oral groups, sectioned and stained with hematoxylin and eosin (HE). Size and relative cell population density can be useful indicators of immune stimulation; therefore, Peyer's patch cells from ISCOMs' groups were examined for morphology and cell density relative to those Peyer's patch cells collected from control mice.

MATERIALS AND METHODS

Animals

All studies were conducted using protocols previously approved by the East Carolina University Animal Care and Use Committee. Male and female BALB/c mice (8-12 weeks old) were obtained from Charles River Breeding Laboratories, Wilmington, MA, or from a breeding stock maintained at the East Carolina University School of Medicine. Animals were maintained in plastic cages, allowed free access to NIH-07 Rodent Diet (Agway, Syracuse, New York) and tap water, and housed in facilities approved by ALAC.

Buffers and Reagents

(See Appendix A)

Pneumococcal Polysaccharide Type III (SIII) Antigen

The SIII used was provided by Dr. P.J. Baker, NIH, Bethesda, Maryland. The method by which the SIII, from *Streptococcus pneumoniae* was prepared has been described previously (10).

Immunization Protocols

BALB/c mice were injected with either ISCOMs preparations in sterile phosphate buffered saline (PBS), or SIII dissolved in sterile PBS at a total volume of 0.2 ml. The

mice were injected either i.p. s.q. or were fed orally. Mice receiving antigens orally were first taken off food for 12 hr. A feeding needle was used to transfer the antigen in sterile PBS at a volume of 0.2 ml directly into the stomach. A normal diet was resumed until 12 hrs prior to a gavage procedure. Protocols specific to each immunization are found throughout the text in appropriate areas.

Sulfhydryl Addition to BSA

Bovine serum albumin (BSA), 67,000 MW grade V (Sigma, St. Louis, MO), was dissolved to a concentration of 60 μM/ml in 50 mM sodium phosphate buffer pH 7.5. N-succinimidyl S-Acetylthioacetate (SATA), (Pierce, Rockford, Ill), was reconstituted to a concentration of 1.3 mg in 0.1 ml of Dimethyl sulfoxide (DMSO). The reconstituted SATA was added at a volume of 0.1 ml for every 60 μM of BSA and incubated at 25°C for 30 minutes. A desalting column (7.0 cm x 0.5 cm) of G-25-150 Sephadex, (Pharmacia, Uppsala, Sweden) with an exclusion limit of 5,000 daltons was equilibrated with ten ml of 50 mM sodium phosphate buffer pH 7.5. The 1.1 ml of BSA-SATA mixture was applied to the column dropwise. The column rate was adjusted to 12 drops/min and 1.0 ml fractions were collected and analysed for spectrophotometric activity at 280 nm using a Beckman model 26 spectrophotometer (Beckman, Irvine, CA). Fraction numbers 3, 4 and 5 were pooled from each column run. Pooled fractions were concentrated in a Centriplus-30 tube (Amicon, Beverly, CA) and centrifuged at 1500 x g for 30 min at 25°C until the concentrate approached one-half its original volume. The

concentrate was returned to its original volume using 50 mM sodium phosphate buffer pH 7.5. The pooled BSA-SH concentrates were stored at 4°C.

Oxidation of Carbohydrate Vicinal Moieties

Vicinal hydroxyl groups on the glucose and glucuronic acid moieties of SIII polysaccharide were modified to aldehyde form in the presence of sodium meta-periodate. Sodium acetate, at a concentration of 0.1 M pH 5.5 was used to dissolve 10 mg/ml of SIII polysaccharide. To each 10 ml of dissolved SIII was added 0.1 ml of 0.1 M sodium meta periodate. All tubes were wrapped in aluminum foil and kept in the dark one hr at 25°C. Glycerol, at 15 mM concentration was added for every 10 mg/ml of oxidized SIII and incubated on ice in darkness for five minutes to stop the reaction. Maintaining total darkness, excess oxidant was removed by filtering the reactants through an Amicon model 52 filtration system. The device was fitted with a 43 mm PM-30 membrane of 30,000 MWCO, (Amicon, Beverly, CA), and its housing covered with foil to eliminate incident light. A 100 ml reservoir contained the reactants with 50 ml of 0.1 M sodium acetate buffer pH 4.5. The system was operated under pressure generated from a nitrogen tank and concentration proceeded until the solution approached its original volume.

Cross-linkage of SIII to M_2C_2H

The cross-linker 4-(N-maleimidomethyl) cyclohexane 1-Carboxyl hydrazide hydrochloride • ½ dioxane (M_2C_2H) (Pierce, Rockford, Ill), was added to the oxidized

SIII moieties at a concentration of 1 mM per ~3.33 mg/ml of SIII. The mixture was agitated on a shaker, (LabIndustries Inc., Berkeley, CA.), for two hrs at 25°C. Excess M_2C_2H was filtered and concentrated as described above. A 100 ml reservoir held the reactants and 50 ml of 0.1 M sodium phosphate buffer pH 7.0, with the filtration performed in normal light. The concentrated solution was removed to 12 x 75 mm glass tubes and divided into aliquots of approximately 3.33 mg SIII/0.5 ml.

Deprotection of BSA-SH

Several hours prior to BSA-SIII polysaccharide conjugation, protected BSA sulfhydryl derivatives were deacetylated to free sulfhydryls for conjugation via maleimide groups bound to SIII polysaccharide. Deacetylation solution, (appendix A), was added at a volume of 0.1 ml to every 60 μM/ml of derivatized BSA. This preparation was covered with parafilm and incubated two hrs at 25°C. The mixture was concentrated using a Centriplus-30 filtration tube (Amicon, Beverly, CA), and brought to a protein concentration of 2.5 mg/0.5 ml with 50 mM sodium phosphate buffer pH 7.5.

Conjugation of BSA to SIII

Deacetylated BSA-SH aliquots at approximately 2.5 mg/0.5 ml were paired with oxidized SIII-M_2C_2H aliquots containing approximately 3.33 mg/0.5 ml. Each tube of BSA-SH and SIII-M_2C_2H contained 1.0 ml. The tubes were gently mixed, covered and incubated for a minimum of two hrs at 25°C. The conjugates were stored at 4°C.

Cyanuric Chloride Conjugations

Paul's method for covalently linking a carbohydrate to a protein was used with minor modifications (18). A concentration of 25.0 mg of SIII was dissolved in 0.67 ml of distilled water and held at 4°C. 2,4,6-trichloro-s-triazine ($C_3Cl_3N_3$) (Sigma, St. Louis, MO), at a concentration of 2.9 mg was dissolved in 0.15 ml of dimethylformamide (DMF), and then added to the SIII maintained at 4°C and vortexed vigorously for 20 min while maintaining a temperature of 4°C. The reactant tubes were then stirred on a magnetic stirring plate while contained in a beaker of ice for an additional 40 minutes. A concentration of 25.0 mg of BSA was dissolved in 0.67 ml of PBS pH 7.4 and was added to the cold SIII-$C_3Cl_3N_3$ mixture while continuing to stir for 1h at ambient temperature. The SIII-BSA-$C_3Cl_3N_3$ mixture was refrigerated at 4°C overnight and dialyzed in four liters of PBS pH 7.4 at 25°C for 24 h.

Lipid Mixture for the Formation of ISCOMs

Decanoyl-N-methylglucamide (MEGA-10) (Sigma, St. Louis, MO), was heated gently at a concentration of 2 g/10 ml in distilled water (20% w/v) until fully dissolved. One ml of phosphatidylcholine, (Sigma, St. Louis, MO) was added to 100 mg of cholesterol in 1.0 ml of chloroform (Sigma, St. Louis, MO), in a glass beaker, and this mixture was gradually added to cooled MEGA-10. This mixture was extensively stirred to yield a final volume of 12 ml containing ~8.33 mg/ml of cholesterol. One ml aliquots were removed to 2.0 ml capacity glass vials and were stored frozen at –70°C.

ISCOMs Formation

SIII-BSA conjugates prepared for ISCOMs incorporation were estimated to contain ~2.5 mg of BSA and 3.33 mg of SIII per aliquot. To each aliquot was added 0.1 ml of 1 M glycine buffer, pH 2.5. A frozen lipid mix aliquot was thawed and gently vortexed. A volume of 0.020 ml of lipid mix was added for every 2.0 mg of protein. A quantity of 1.5 mg of Quillaja saponin, (Sigma, St. Louis, MO), was added for every 2.0 mg of protein. Each aliquot was sonicated for 15 min at 20°C and was removed and set aside undisturbed for one hr at 25°C. Each aliquot was sealed in a dialysis bag of MWCO 12,000-14,000 (SpectraPor, Houston, TX), and dialyzed against five liters of 0.1 M glycine buffer pH 2.5 for 24 hrs at 25°C. Additional dialysis was done for 48 hrs at 25°C against five liters of PBS pH 7.4 with buffer changes every 12 hrs. The dialysis bags were removed at 72 hrs and aliquots were stored at 4°C.

Sucrose Gradient of ISCOMs

Two solutions of 40% and 10% sucrose (w/v) respectively were prepared in distilled water. A volume of 4.2 ml of the 40% sucrose solution was pipetted into a 14 x 95 mm polyallomer centrifuge tube (Nalge, Rochester, NY), with care taken to avoid contacting the tube walls. Atop this was laid the the same volume of a 10% sucrose solution. A volume of 2.5 ml of ISCOMs dialysate was laid atop the 10% sucrose solution so that the total volume of the three solutions sat within 0.5 cm of the tube lip, but not less than 1.0 cm below the tube lip. ISCOMs aliquots were pooled to achieve a

ratio of 5 parts 40% sucrose/5 parts 10% sucrose/3 parts ISCOMs. Tubes were centrifuged at 60,000 x g for 18 hrs at 20°C in a 40Twi rotor using a Beckman model L5-50 ultracentrifuge (Beckman, Irvine, CA). One ml fractions from the gradient were siphoned off using a pasteur pipette and removed to separate glass 12 x 75 mm test tubes. A discrete, narrow white band approximating 0.5 ml was visible in each tube and tentatively identified as ISCOMs. All fractions were removed to separate dialysis bags throughout preliminary studies and extensively dialysed against five liters PBS pH 7.4 for 48 to 72 hrs at 25°C with frequent buffer changes. All fractions were tested for spectrophotometric activity at 280 nm and analysed for carbohydrate content. In gradient preparations from later studies, only those bands demonstrably discrete and white were pooled, dialyzed and tested for spectrophotometric activity and carbohydrate activity. Dialyzed fractions were centrifuged at 500 x g for one minute at 25°C. One ml of supernatant was removed and tested for carbohydrate content. Successive centrifugations were performed until the upper one ml supernatant contained neglible carbohydrate content. The supernatant was discarded at this point and the ISCOMs preparation stored at 4°C.

Carbohydrate Analysis of SIII

A phenol-sulfuric acid method adapted to microtiter plates was used for semi-quantitative detection of reducing substances (63). Estimates of type III pneumococcal polysaccharide were obtained throughout various stages of ISCOM preparation. Known quantities of purified pneumococcal polysaccharide type III were used to prepare seven

standards ranging from 0.5 mg/ml to 3.0 mg/ml. All samples were tested in triplicate in a 96-well microtiter plate (Costar, Cambridge, MA). Each sample, at a concentration of 0.025 ml was dispensed into the microtiter wells and the plate was placed on ice and transferred to a fume hood. Phenol, 5% (v/v), was pipetted at a volume of 0.025 ml into each well using a multi-channel pipette (Costar, Cambridge, MA) to both dispense and mix sample and phenol. Concentrated sulfuric acid, at a volume of 0.125 ml , was pipetted into each well using the multi-channel pipette as before while keeping the microtiter plate on ice. The plate was removed to an 80°C water bath, and after a 30 min incubation was placed on ice until the temperature approximated 25°C. The reaction wells were read on an Anthos 2001 spectrophotometer microtiter plate reader, (Anthos Labtec Instruments, Salzburg, Austria), at a wavelength of 492 nm.

Protein Analysis of SIII-BSA Conjugates

Eight standards derived from BSA (Sigma, St. Louis, MO), ranged in concentration from 0.5 mg to 4.0 mg/ml. These were prepared from two stocks of BSA: 1.0 mg/ml and 5.0 mg/ml prepared in phosphate-buffered saline, (PBS), pH 7.4. SIII-BSA conjugates and BSA-SH preparations derived from the SATA protocol and standards were tested in duplicate. Each standard was pipetted at a volume of 0.1 ml into a 12 x 75 mm test tube, (Fisher Scientifics, Fair Lawn, NJ). Samples were dispensed at 0.05 ml per tube. To each tube was pipetted 0.5 ml of an alkaline copper tartrate solution, reagent A (BIO-RAD, Hercules, CA) and 4.0 ml of Folin solution, reagent B (BIO-RAD, Hercules, CA). Each tube was covered, vortexed and incubated at 25°C for 15

min. Tubes were vortexed and 0.1 ml of each sample pipetted into a 96-well flat-bottomed microtiter plate (Costar, Cambridge, MA). Absorbances were read from the Anthos 2001 spectrophotometric plate reader, at a wavelength of 690 nm.

High Performance Liquid Chromatography (HPLC) of ISCOMs

High performance liquid chromatography was done using samples of BSA ISCOMs and SIII-BSA ISCOMs (courtesy of Dr. P. Fletcher, ECU School of Medicine). The samples were vortexed gently, placed in acid-washed test tubes and diluted 1:2 with a mixture of 12N HCl and 0.5% phenol. The tubes were gently vortexed, evacuated of all air and sealed. The samples were placed into a heating block for 20 hrs at 110°C for hydrolysis. After hydrolysis, the tubes were opened and dried down in a Savant Speed-Vac Concentrator (Farmingdale, NY). Amino acid analysis was performed on a 20 µl of sample using a Dionex amino acid system (Dionex Corporation, Sunnyvale, CA), using a cation exchange column (St. John Assoc. Beltsville, MD) and a sodium citrate buffer system over a period of 65 minutes. Detection was by ninhydrin post-column reaction read at 570nm. The amino acid data was analyzed using the Dionex Data AI-450 acquisition software (Dionex Corporation, Sunnyvale, CA) and the EXCEL® program (Microsoft Corp., Redmond, WA).

Transmission Electron Microscopy of ISCOMs

A 3% ammonium molybdate (w/v) negative stain was dissolved in distilled water

and filtered via a 0.22 μM syringe-adapter (Millipore, Bedford, MA). Carbon-coated copper-grids (E. Fullam, Latham, NY), were loaded with 5 μl of the pooled ISCOMs for five minutes under a petri dish lid. ISCOMs excess was wicked off of the grid using the edge of a filter paper sheet (Whatman International Inc., Maidstone, England). Immediately after wicking off the ISCOMs preparation, 5 μl of 3% ammonium molybdate was applied to one side of the grid while simultaneously, a sheet of filter paper was used to wick off the negative stain from the opposite grid side. Dried grids were loaded into a transmission electron microscope (JEOL 1200EX, Peabody, MA), and examined at 40K magnification for hexagonal-shaped spheres approximately 40 nm in diameter. Appearance of these objects confirmed the formation of ISCOMs.

SIII binding to nitrocellulose membranes

The fixing of pneumococcal polysaccharide (SIII) to nitrocellulose membranes (Costar, Cambridge, MA), for use in ELISPOT assays, required conjugating SIII to poly-L-lysine hydrochloride, (MW 30,000-70,000) (Sigma, St. Louis, MO), following a protocol described by Gray, with minor modifications (68). SIII was dissolved at 1.0 mg/ml in distilled water for use as a stock solution. Ten 12 x 75 mm test tubes labelled "A" contained 0.5 ml of 0.01N NaOH pH 11.0. SIII stock, at a concentration of 0.1 ml was added to the "A" tubes and was gently mixed for ten seconds. Cyanuric chloride crystals, (Sigma, St. Louis, MO) were added to each tube at a concentration of 0.5 mg/tube. The tubes were covered and periodically rolled between the hands for 30 min.

The pH of the tubes was closely monitored after 30 minutes until a pH range of 8.2-8.4 was reached. Immediately upon reaching this pH range, 0.1 ml of 1% poly-L-lysine dissolved in 0.05 M TRIS, pH 8.1 (69), was dispensed into each tube. The contents were gently swirled, covered and incubated overnight 4°C. The tubes were centrifuged at 500 x g for one min and the supernatant recovered. Care was taken to avoid contaminating the supernatant with undissolved cyanuric chloride crystals. The SIII-poly-L-lysine conjugates were diluted in PBS pH 7.4 to a concentration of 100 ng/0.1 ml per ELISPOT well. The strips were incubated in a sealed humidifying chamber overnight at 25°C. The strips were then stored at 4°C for up to two weeks prior to using.

ELISPOT for the Detection of anti-SIII IgG SFCs

Pneumococcal polysaccharide type III conjugated with poly-L-lysine was incubated in eight-well nitrocellulose strips (Costar, Cambridge, MA), at a concentration of 100 ng/0.1 ml in PBS pH 7.4 per well overnight at 25°C. The strips were washed twice with PBS/Tween by flooding the plates and flicking out the contents. One percent skim milk/ PBS/Tween was dispensed at a volume of 0.3 ml into each well for 30 min at 37°C. The blocking agent was decanted and the strips were washed once with PBS/Tween as described. Harvested spleen cells previously washed and counted were pipetted at 10^5 cells/0.1 ml per well in sterile Hank's balanced salt solution (Appendix A). Each sample was run in quadruplicate. Control spleens were also harvested and treated identically to those of test spleens, with some ELISPOT wells acting as reagent controls. Strips were inserted into a humidifying chamber, covered loosely with parafilm and incubated

overnight at 37°C in 5% CO_2. Each strip in its holding plate was flooded with PBS/Tween and the contents flicked out for a total of two brief washes. Five additional washes were done by flooding the strips with PBS/Tween and leaving the plate undisturbed for 5 min followed by flicking out the contents. Goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma, St. Louis, Mo) and diluted 1:1000 in PBS pH 7.4 was pipetted into each well at a volume of 0.1 ml followed by incubation overnight at 4°C in a humidified chamber tightly sealed with parafilm. Strips were flooded with PBS/Tween and washed four times for 5 min each as previously described. Alkaline phosphatase substrate, prepared as described in appendix A, was pipetted into each well at a volume of 0.05 ml and incubated overnight in a humidifying chamber at 25°C. Wells were examined using a model Stemi DRC dissecting scope (Carl Zeiss Inc., Thornwood, NY), at 20-25x magnification. Those spots exhibiting symmetry and bluish halos were counted as spot-forming cells (SFCs).

ELISAs for the Detection of anti-SIII and anti-BSA Antibodies

The ELISA (enzyme-linked immunosorbent assay) was performed using 96-well plates (Costar, Cambridge, MA), and to each well was dispensed 0.001 mg/0.1 ml of pneumococcal polysaccharide SIII in PBS pH 7.4 and incubated overnight 25°C. Plates were washed once by flooding with PBS followed by flicking out the contents. The plates were blocked with 0.3 ml of 1% skim milk in PBS, pH 7.4 (w/v), (Difco Labs, Detroit, MI) for 1 h at 37°C. Plates were then washed once with PBS/Tween as above. The primary antibody consisted of mouse sera collected on days 5, 10 and 21. Sera were

diluted serially starting at a dilution of 1:100 in PBS/Tween. The first vertical row on each plate was designated as a reagent blank with each plate having a row devoted to pooled control serum run along with the test sera. Test serum was incubated in the SIII-coated plates for 2 h at 37°C or was covered and incubated overnight at 4°C. Plates were then washed for 10 min in PBS/Tween in a shaking bath (American Optical, Buffalo, NY)). Biotin conjugated to goat anti-mouse IgG (Zymed, San Francisco, CA), diluted 1:1000 in PBS/Tween was dispensed at 0.1 ml/well and incubated for 1h at 37°C. Plates were washed 10 min in PBS/Tween, again using a shaking bath. Strep-avidin conjugated alkaline phosphatase (Zymed, San Francisco, CA), diluted 1:1000 in PBS/Tween was dispensed at 0.1 ml/well and incubated for 30 min at 37°C. Plates were washed three times 5 min each with PBS/Tween in the shaking bath. Alkaline phosphatase substrate (Sigma, St. Louis, MO), dissolved in a glycine buffer, pH 10.4 (Appendix A) was dispensed at 0.1 ml/well and incubated for 30 min at 25°C. Plates were read on an Anthos 2001 spectrophotometric plate reader, at a wavelength of 405 nm approximately 30 min post-substrate. The highest titer was taken as an absorbance one-third above that of the reagent blank. The same protocol was used to determine anti-BSA IgG titers in mouse sera, except that 250 ng/well of BSA was used as the coating antigen in carbonate buffer pH 9.6 (Appendix A). The plates were incubated at 4°C overnight and alkaline phosphatase was used as the developing enzyme. ELISA for IgA, IgE and subclasses of IgG were performed identically to the protocol described for detecting IgG antibodies against SIII, except that biotin conjugated antibodies specific for murine IgA, IgE, IgG_1, IgG_{2a}, IgG_{2b} or IgG_3, were used.

Harvesting Spleens for the ELISPOT

Previously immunized mice were anesthesized with ethyl ether (Fisher Scientific, Pittsburgh, PA) and sacrificed after cardiac puncture, followed by cervical dislocation. Approximately 1.0 ml of blood was aspirated from the heart and immediately refrigerated for later centrifugation at 1000 x g 10 min to separate the clot. Serum was stored at 4°C for later analysis by ELISA.

Sacrificed mice were secured to a dissection board and cleaned with 70% ethanol. Spleens were removed and transferred immediately to 5.0 cm x 5.0 cm plastic petri dishes containing 0.5 ml of cold, sterile Hank's balanced salt solution (HBSS) (Appendix A), pH 7.0. Spleens were teased through stainless steel screens to prepare single-cell suspensions. The harvested cells were removed to 15 ml sterile centrifuge tubes (Corning, Cambridge, MA) and splenocytes were washed once with 0.85% sterile, cold saline and centrifuged at 1000 x g for 5 min at ambient temperature. Following one more wash with HBSS, the cells were suspended in a volume of 5.0 ml with HBSS and placed on ice. A 1:10 dilution of cells in saline was prepared, followed by a 1:2 dilution with trypan blue (Sigma, St. Louis, MO). Cells were counted for each individual spleen. Tubes containing splenocytes were covered and refrigerated at 4°C and tested within 24 hrs using the ELISPOT assay.

Serum Absorption for the PHA

Mouse sera was heated in a 56°C water bath for 30 min to remove complement activity, followed by cooling to ambient temperature. A volume of 2.0 ml of sheep

erythrocytes (Remel, Lenexa, Kansas) was washed four times with sterile 0.85% saline at 1000 x g 25°C for 5 min. All supernatant was removed at the last wash, leaving 1.0 ml of packed erythrocytes. Packed washed sheep erythrocytes were added in equal volume to each heated individual mouse serum and incubated for 1 h at 25°C. Incubation was then continued for 1 h at 4°C. The reactant tubes were centrifuged at 1000 x g for 5 min at ambient temperature and the serum was removed to 10 x 75 mm test tubes and stored at 4°C until used.

Passive Hemagglutination Assay (PHA)

SIII stock solution was prepared at 1 mg/ml in 0.85% saline (w/v). A volume of 0.15 ml of the SIII stock was pipetted into another tube containing 1.0 ml of 0.85% saline. The final 1.15 ml solution was transferred to a 15 ml centrifuge tube containing 0.5 ml of packed sheep erythrocytes washed four times with 0.85% saline. A 1.0% (w/v) solution of chromic chloride ($CrCl_3$) was prepared in 1.0 ml of 0.85% saline. A volume of 0.1 ml was removed from the $CrCl_3$ stock to another tube containing 0.9 ml of 0.85% saline to make a 0.1% $CrCl_3$ solution. The 15 ml tube containing the SIII and erythrocyte solution was vortexed gently followed by adding the 0.1% $CrCl_3$ solution. The mixture was allowed to sit for 5 min at ambient temperature, and was immediately followed by washing the cells in 0.85% saline for a total of two washes for 5 min each at 1000 x g. Two more washes in Hank's balanced salt solution (HBSS) were done at 1000 x g. The last supernatant was discarded, leaving 0.5 ml of SIII-coupled erythrocytes. Cells were diluted to a concentration of 0.7% using HBSS and pipetted into a dispensing boat. A

volume of 0.025 ml of modified barbital buffer (MBB) containing 0.1% fetal calf serum (Hyclone, Logan, Utah) was pipetted into each well of a 96-well V-bottom microtiter plate (Costar, Cambridge, MA). Calibrated 25 µl loops for each serum sample (Dynatech Labs Inc., Alexandria, VA), were used to dispense absorbed serum. Each sample was dispensed to the first well in its respective row of wells and serially diluted starting from a 1:2 dilution and ending at 1:4096. Horse anti-SIII serum, a gift from Dr. P.J. Baker, served as positive control, and pooled normal mouse serum was the negative control. A volume of 0.025 ml of 0.7% SIII-coated sheep erythrocytes was pipetted into each well. The plates were covered with a plastic lid, gently swirled and incubated overnight at ambient temperature. The plates were read on a mirrored plate reader (Cooke Lab Products, Alexandria, VA), with the titer endpoint taken as the last well demonstrating agglutination.

Preparation of Peyer's Patches for Microscopic Examination

Groups of mice fed ISCOMs and control groups of mice given water only, were selected for Peyer's patch studies. Mice were anesthesized using ethyl ether (Fisher Scientific, Pittsburgh, PA) and sacrificed after cardiac puncture. The mice were then secured to a dissection board. After making a ventral incision, the proximal section of the small intestine was located and excised to an approximate length of 6 cm. The excised intestine was transferred to a dissecting microscope (Bausch and Lomb, Rochester, NY), and examined under 20X magnification for protruding, well-circumscribed white masses identified as Peyer's patches. All Peyer's patches were snipped off flush with the

intestine surface and immediately transferred to vials containing Telly's fixative (Appendix A). The Peyer's patches remained in fixative for 24 h and thereafter were transferred to a solution of 70% ethanol. The Peyer's patch tissues were prepared as 7 μm sections after paraffin embedment and stained with hematoxylin and eosin (prepared by Dr. H. Burden, ECU School of Medicine). Slides were examined under 100X and 400X magnification for between-cell distance, and nucleus diameter. These parameters were used to determine cell density and cell maturity within Peyer's patch sections for comparison to that of control groups (under the supervision of Dr. J. Christie, ECU School of Medicine).

Gastric lavage (gavage) method for the collection of intestinal secretions

Food was withheld from BALB/c mice overnight with the mice allowed access to water *ad libitum* prior to administering the gavage. Gavage solution (Appendix A) was allowed to warm to ambient temperature (70). Animals were held vertically, without anesthesia, and fed 0.5 ml of gavage fluid through a stainless steel feeding tube (Popper & sons, Inc. New Hyde Park, N.Y.). Mice were fed gavage fluid every fifteen minutes for a total of four times. Thirty minutes after the last feeding, mice were intraperitoneally injected with 0.2 ml of a 0.1 mg solution of pilocarpine (Sigma, St. Louis, MO) prepared in 0.2 ml of PBS. Mice were placed atop a 12 cm x 12 cm wire mesh consisting of 12.5 mm dimension squares. This mesh was placed within a 100 mm x 15 mm petri dish. Trypsin inhibitor (Sigma, St. Louis, MO) was prepared as 0.1 mg/ml in phosphate buffered saline (PBS) (Appendix A) with 50 mM of ethylenediaminetetraacetic acid

(EDTA). A volume of 3.0 ml of the trypsin inhibitor was added to the petri dish. The trypsin inhibitor prevented ezymatic degradation of proteins, including immunoglobulins in the intestinal secretions. A plastic beaker with holes punched in its top for air was placed over the petri dish to enclose the animal. Approximately thirty minutes after administering the pilocarpine, most intestinal secretions had fallen to the petri dish base below the wire mesh. The animals were returned to their cages where solid food and a pedialyte solution (Abbott Labs, Chicago, IL) were available to them. A volume of 3.0 ml of the secretions and trypsin inhibitor was transferred to a 15.0 ml centrifuge tube and brought to a final volume of 6.0 ml with PBS. Using a glass rod, the mucus was broken up and followed by vigorous vortexing. The tubes were centrifuged at 650 x g for ten minutes. A volume of 3.0 mls of the supernatant were transferred to a polycarbonate centrifuge tube. A volume of 30 µl of 100 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO) in 95% ethanol was added to the supernatant as an additional trypsin inhibitor. The mixture was centrifuged at 27,000 x g for twenty minutes at 4°C. One ml aliquots of the clarified supernatant were removed to two freezer tubes of 1.8 ml capacity. Volumes of 10 µl of PMSF and 10 µl of 1% sodium azide (Sigma, St. Louis, MO), were added to each aliquot and allowed to stand for fifteen minutes at ambient temperature. A volume of 50 µl of calf serum (Hyclone, Logan, UT)) was added to each aliquot to serve as a substrate for any residual protease activity. Vials were stored at -70°C until further use. Aliquots were tested for IgG, IgE and IgA by ELISA as described.

RESULTS

Conjugation Methods and Their Effects on the Immune Response to SIII

Preliminary studies were done to compare the effects that two different methods for conjugating SIII to the carrier protein bovine serum albumin (BSA) would have on the immune response to SIII in BALB/c mice. Two groups of mice were injected i.p. with either the antigen complex of SIII-BSA conjugated by the $C_3Cl_3N_3$ method or the M_2C_2H method delivered in ISCOMs. Within each group, mice were given either 25 μg or 50 μg of antigen on days 0 and 16. All mice were bled on days 5, 10 and 21 and the IgG titers were determined by ELISA.

Day 21 serum IgG titers were examined for statistical differences within each group as well as between each group. As shown in fig. 1, there was no statistical difference in $\log_2$ IgG serum titers from mice receiving 25 μg of the antigen conjugation produced by M_2C_2H or $C_3Cl_3N_3$ methods. Those mice receiving 50 μg of antigen conjugated by the M_2C_2H method showed a significant difference over those receiving 50 μg of $C_3Cl_3N_3$ conjugated antigen ($p<0.05$).

Sera were also subjected to a passive hemagglutination assay (PHA) in which SIII was conjugated to sheep erythrocytes. This assay provided a comparison with titers determined by ELISA and reflected all immunoglobulin isotypes produced in response to SIII including the predominant IgM class. Three experimental groups of mice were

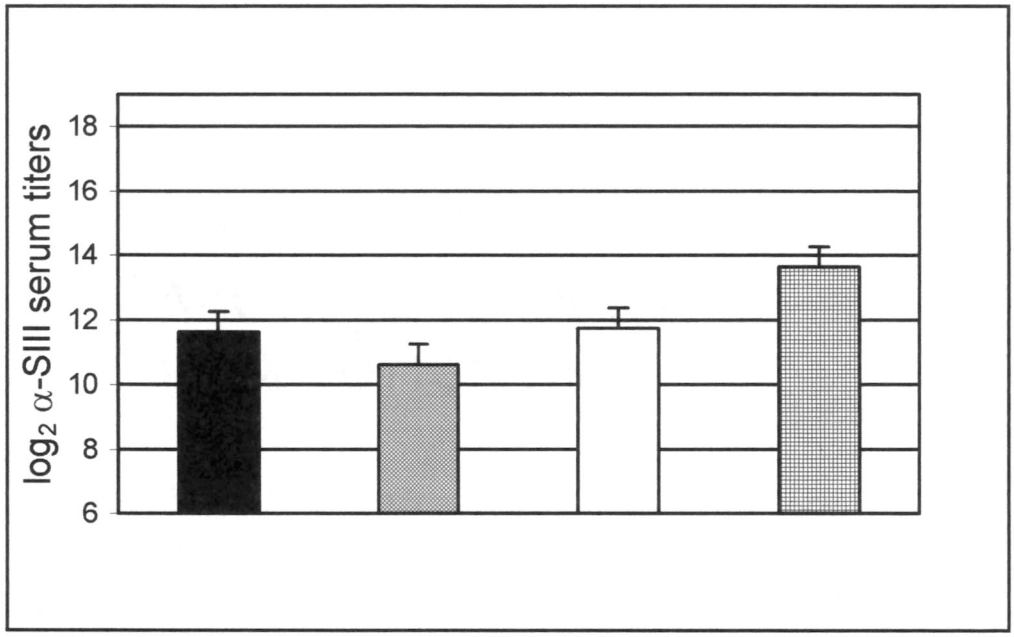


Fig. 1 Four groups of BALB/c mice were injected i.p. on days 0 and 16 with ISCOMs prepared using two different conjugation protocols as follows: 25 μg M_2C_2H ▨ n= 5 , 25 μg $C_3Cl_3N_3$ ■ n= 3. 50 μg M_2C_2H ▩ n= 8 , and 50μg $C_3Cl_3N_3$ □ n= 9 respectively. Bars represent the mean log_2 anti-SIII IgG serum titers ±SEM from day 21. The symbol "α" is used throughout the figures as a designation for "anti".

studied. The first two groups were injected with SIII-BSA ISCOMs derived from conjugates prepared using the cyanuric chloride reagent. The data from these two groups were combined for the purpose of analysis and comparison to a third group of mice injected with ISCOMs derived from a conjugate prepared from the M_2C_2H reagent. The first group was given 25 μg of SIII-ISCOMs i.p. on days 0 and 16 while the second group was given 50 μg i.p. of the same preparation on the same days. Antibody titers measured on days 5, 10 and 21 were not significantly different between the two dosage groups of the same conjugate ($p>0.05$). The antibody titers reached 1:2000 on days 5 and 10 with a subsequent drop to 1:128 by day 21. For a comparison of conjugation methods, a third group of mice was included in the experimental design (fig. 2). These mice received 25 μg i.p. of SIII-BSA ISCOMs prepared by the M_2C_2H method. This group received the immunogen on days 0 and 16 and was bled on days 5, 10 and 21. The titers produced by this group on days 5 and 10 were not significantly different ($p<0.05$) from those of the $C_3Cl_3N_3$ groups except for day 21 where the titer reached 1:400. These data showed that the PHA could be used to measure anti-SIII levels among the experimental groups and that there was a higher titer reached following a second injection of immunogen on day 16 in the group receiving ISCOMs prepared with M_2C_2H conjugated reagents. The data suggested that the M_2C_2H conjugated preparation possibly remained immunogenic longer in vivo or presented more epitopes than in those ISCOMs prepared using the $C_3Cl_3N_3$ conjugation method. From these data, it was decided to use the M_2C_2H

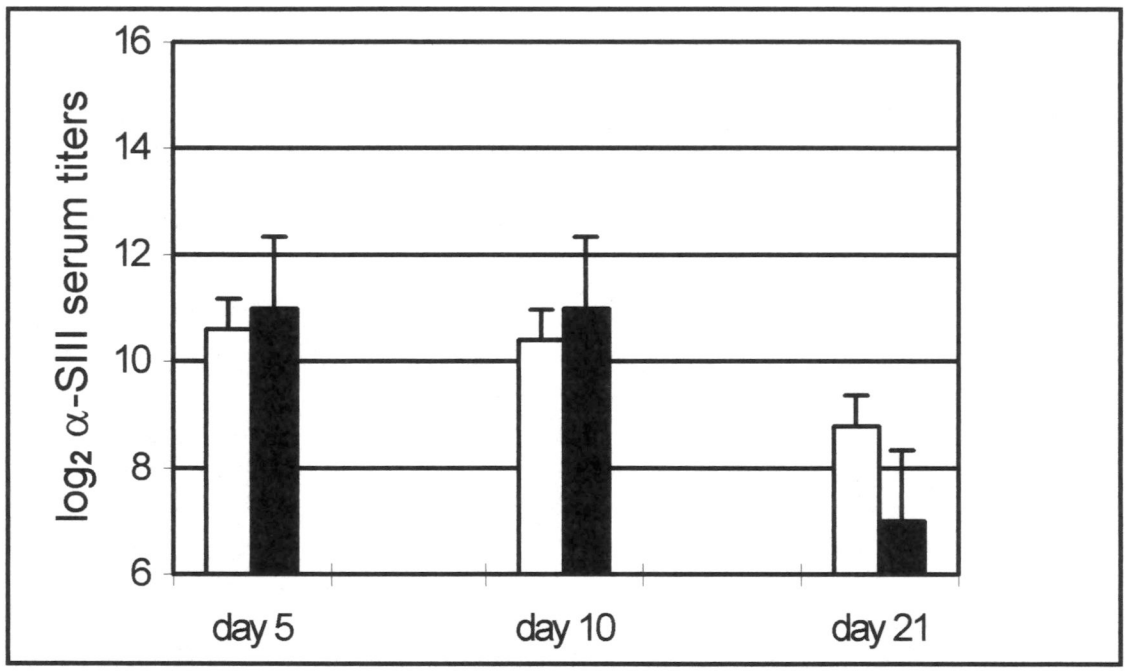


Fig. 2 Serum titers obtained by PHA following i.p. injection on days 0 and 16 with ISCOMs prepared by the $C_3Cl_3N_3$ and M_2C_2H conjugation methods. The mean log_2 anti-SIII titers ±SEM are shown for days 5, 10 and 21. Day 21 serum titers showed a significant difference between the mean titer of the mice receiving the M_2C_2H conjugate □ n= 23, and the titer from the $C_3Cl_3N_3$ conjugate group ■ n= 8 ($p<0.05$).

conjugation method and to use 25 μg of SIII as the estimated quantity of ISCOMs, with this value determining what would constitute a single dose per mouse for the remainder of the study.

Immune Response to BSA as the Carrier Protein

It was important to determine the extent of the immune response to the protein BSA which could indicate the efficiency of the protein as a carrier molecule. The experimental design included two groups of mice given 50 μg of BSA, with one group receiving the protein as a free antigen in sterile saline and the other as a part of the ISCOMs vehicle. The immunogen was given i.p. in both groups on days 0 and 10. Blood was collected on day 19 and the IgG levels determined by ELISA. There was a significant difference in the titers produced by the group injected with BSA and those given BSA-ISCOMs ($p<0.05$). The ISCOMs group produced IgG reciprocal titers averaging 1:4000 on day 19 while those given BSA alone averaged 1:800.

It was of interest to determine what, if any, effects the method of conjugation had on the carrier protein. This determination was made after reviewing serum anti-BSA IgG titers, as tested by the ELISA. One group of mice was injected i.p. with ISCOMs generated from M_2C_2H conjugated SIII-BSA, and a second group was injected i.p. with ISCOMs containing SIII-BSA produced via the $C_3Cl_3N_3$ method. The experiment was biased toward the $C_3Cl_3N_3$ group which received 50 μg of ISCOMs in comparison to the M_2C_2H group, which received 25 μg. Both groups received the immunogens on days 0

and 16 with blood being drawn on day 21. The IgG titers showed no significant difference ($p>0.05$) with the $C_3Cl_3N_3$ conjugation group producing average reciprocal titers of 1:100000 and the M_2C_2H conjugation group with average titers of 1:80000.

These data suggested that the BSA was active as a carrier and that the ISCOMs carrying the SIII-BSA conjugate produced by the M_2C_2H method could be used as an immunogen at the dosage of 25 μg to produce titers that were readily assayed by ELISA. It was decided to use the ISCOMs prepared from the SIII-BSA conjugated using the M_2C_2H method after the data collected indicated that this method produced an immunogen that more effectively generated an IgG response against SIII.

Results of Conjugation Preparations and ISCOMs Formation

The formation of polysaccharide-protein conjugates was undertaken using the M_2C_2H reagent and the $C_3Cl_3N_3$ reagents respectively, as described in the Materials and Methods section. The cyanuric chloride method employed a substitution reaction (19), and the effect of this reaction was to form an extensive lattice structure of protein and polysaccharide. In the present study, a significant loss of conjugate occurred during the formation of the SIII-BSA conjugate due to the formation of an insoluble gelatinous precipitate. A biological effect in mice injected with the cyanuric chloride formed conjugate was fewer numbers of Peyer's patches as compared with uninjected mice.

ISCOM formation was found to be highly pH-dependent. A pH of approximately 2.5 during the formation of SIII-BSA ISCOMs yielded the most abundant quantity of ISCOMs, and those ISCOMs were more consistent in size and shape in contrast to those

ISCOMs formed at higher pH levels as seen by electron microscopy (fig. 3). ISCOMs formation required extensive dialysis at pH 2.5 for 24 h followed by 48 h of dialysis in PBS pH 7.4. During the initial hours of dialysis, the dialysate appeared aggregated but was dissipated after physically disrupting the contents of the the dialysis tubing. Following this technique, the dialysate appeared homogeneous for the duration of the dialysis.

Results of BSA and SIII semi-quantitative assays

Semi-quantitative carbohydrate and protein analysis was performed after conjugating the SIII moiety to the BSA carrier and again, after ISCOMs formation to determine the degree of component incorporation at each step. BSA was incorporated as an SIII-BSA conjugate at ~25% with the use of the M_2C_2H reagent, while SIII was incorporated at ~20% using the same conjugating reagent. Therefore, both components were incorporated at an approximately equal ratio (Table 1). When the $C_3Cl_3N_3$ reagent was used, BSA incorporation was a low 8%.

After ISCOMs formation, sucrose gradient purification and dialysis were performed and the SIII content was determined using the phenol-sulfuric acid carbohydrate assay. The results of testing numerous ISCOMs preparations indicated that the SIII was incorporated at ~11.9% in those preparations where the M_2C_2H reagent was used. In contrast, ~22% of the SIII was incorporated when cyanuric chloride was used. From these data, it can be concluded that the M_2C_2H reagent is more efficient at

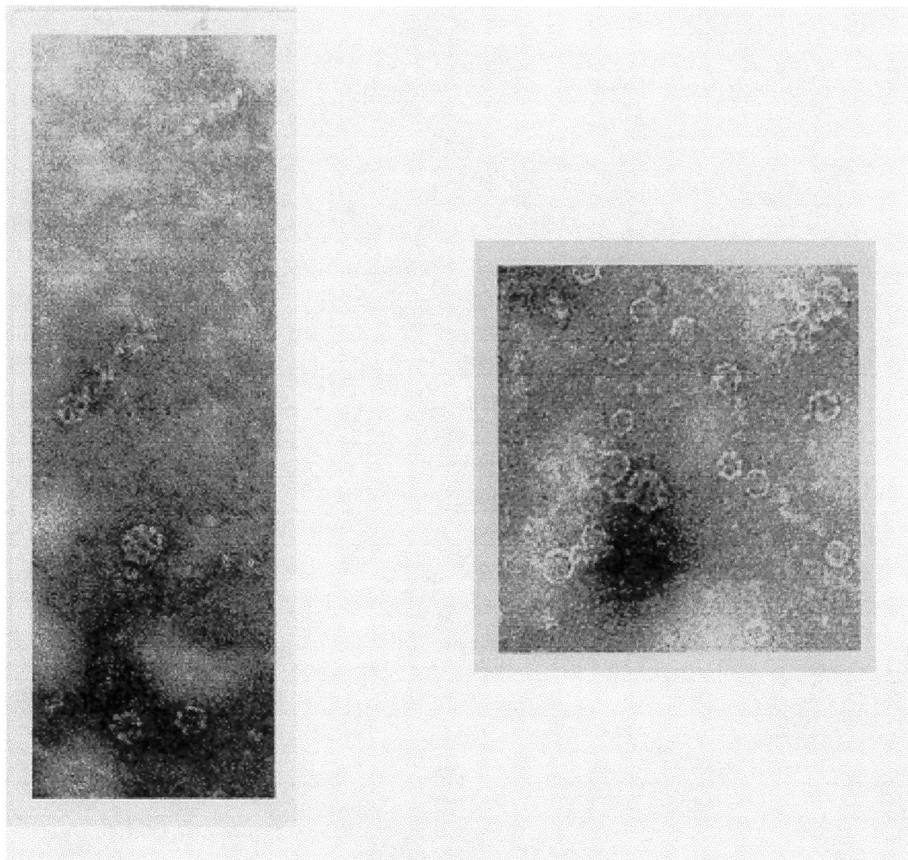


Fig. 3 Photomicrographs of an ISCOMs preparation showing the consistent, uniform size of individual ISCOMs having an approximate diameter of 40 nm.Taken with a JEOL transmission electron microscope at 40K magnification with 3% ammonium molybdate as the negative stain.

conjugating the BSA carrier to SIII while the $C_3Cl_3N_3$ was more efficient at incorporating SIII into its conjugates.

The presence of protein was not detected using the standard modified Lowry protein assay, nor was there evidence of protein after subjecting the ISCOMs to a trichloroacetic acid precipitation prior to performing the protein assay. In lieu of the Lowry method, an amino acid analysis was performed on both SIII-BSA ISCOMs and BSA ISCOMs preparations to yield an estimate of BSA protein (71). The amino acid analysis (courtesy of Dr. P. Fletcher, ECU School of Medicine), yielded an estimated 95 μg/ml of BSA in the SIII-BSA ISCOMs, and 76 μg/ml in the BSA ISCOMs. Based on the average SIII concentration from SIII-BSA ISCOMs of 1.66 mg/ml, an approximate ratio of 17:1 of SIII to BSA was found in these conjugate ISCOMs (table 1). The SIII-BSA ISCOMs dose contained 1.5 μg of BSA for approximately every 25 μg of SIII per dose.

Immune Response to SIII Without Adjuvant Stimulation

Previous reports had shown that SIII injected in sterile saline without adjuvant or conjugation to a carrier produced essentially an IgM response (67). The optimal dose of SIII had been shown to be 0.5 μg in mice. It was necessary for comparative purposes to determine serum levels of IgG specific for SIII as a result of injection of 0.5 μg of the antigen intraperitoneally. The mice were bled on days 5 and 10 and sera tested using both ELISA and PHA assays (fig. 4). Results of the ELISA studies showed that compared to

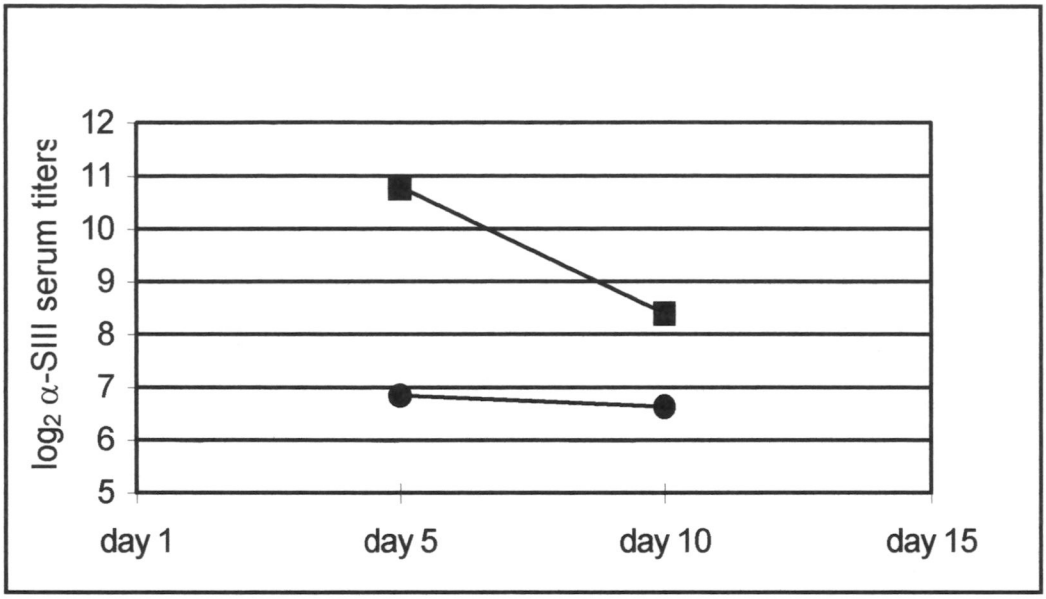


Fig. 4 The murine immune response to soluble SIII. A groupof BALB/c mice (n= 5), was injected i.p. on days 0 and16 with 0.5 μg of soluble SIII in saline. Mean log_2 titers were obtained by ELISA ● and PHA ■ on days 5 and 10. No significant difference was found between the IgG titers detected by ELISA on days 5 and 10 ($p>0.05$) . A significant difference in the pan-antibody responses between the means for sera tested by PHA was found ($p<0.05$).

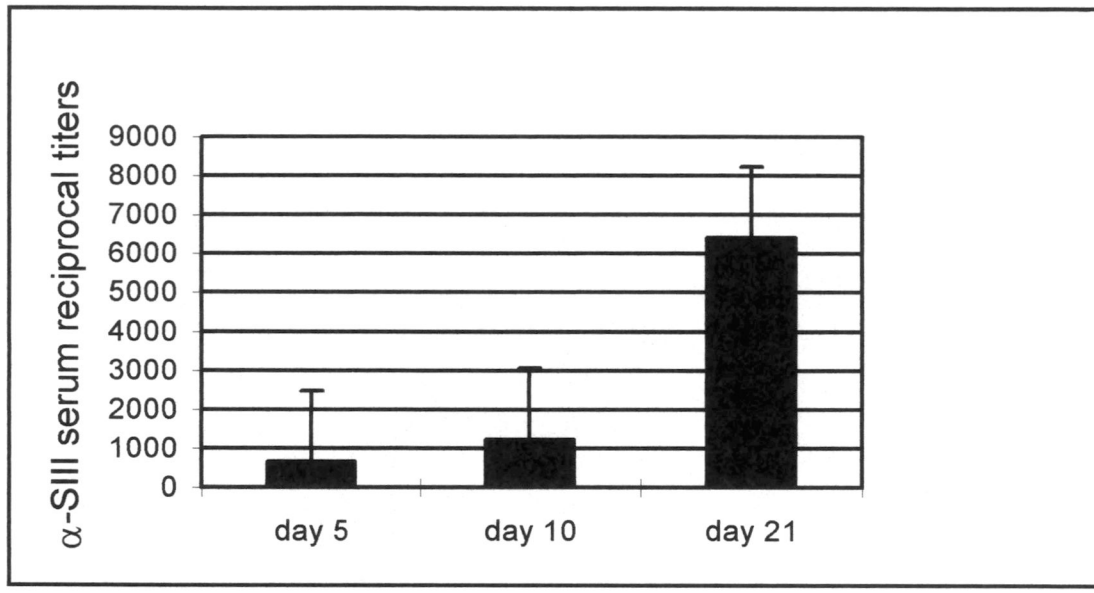


Fig. 5 Serum anti-SIII IgG titers of mice (n= 31) injected i.p with 25 μg of SIII-BSA ISCOMs on days 0 & 16. Bars express the mean reciprocal IgG titers on days 5, 10 and 21 ±SEM. Significance is seen between the means fordays 10 and 21($p<0.05$).

pre-immunization levels of IgG, there was no significant difference on the days tested ($p>0.05$). In contrast, the PHA data revealed titers on days 5 and 10 of 1:2000 with a subsequent decrease to 1:400 by day 21. These results are consistent with an IgM response to a polysaccharide such as SIII given without conjugation to a carrier protein and without adjuvant stimulation.

The Generation of an Anamnestic Response to SIII

Experiments were designed to detect the presence of IgG specific for SIII in immune serum from a large population of mice (n=31). The mice were given a primary i.p. injection of 25 μg of ISCOMs on day 0 followed by a second injection at the same dose on day 16. ELISA data shown in fig. 5 indicated that by day 5 the IgG titer had reached a titer of 1:600 with an increase by day 10 to 1:1200. Following a second injection on day 16, the titer had risen to 1:6000. Statistical significance was found between the means of day 5 and day 10 $\log_2$ titers ($p<0.05$), and for days 5 and 10 versus day 21 $\log_2$ titers ($p<0.05$).

PHA data from this same group using days 5, 10 and 21 sera showed no significant difference between the means of day 5 and 10 reciprocal titers of 1:1000. The PHA measured predominantly the IgM isotype response, and as found previously when SIII was injected alone, the titer had dropped to 1:400 by day 21 even after receiving the second injection of antigen on day 16. These data offer evidence that IgG class antibodies

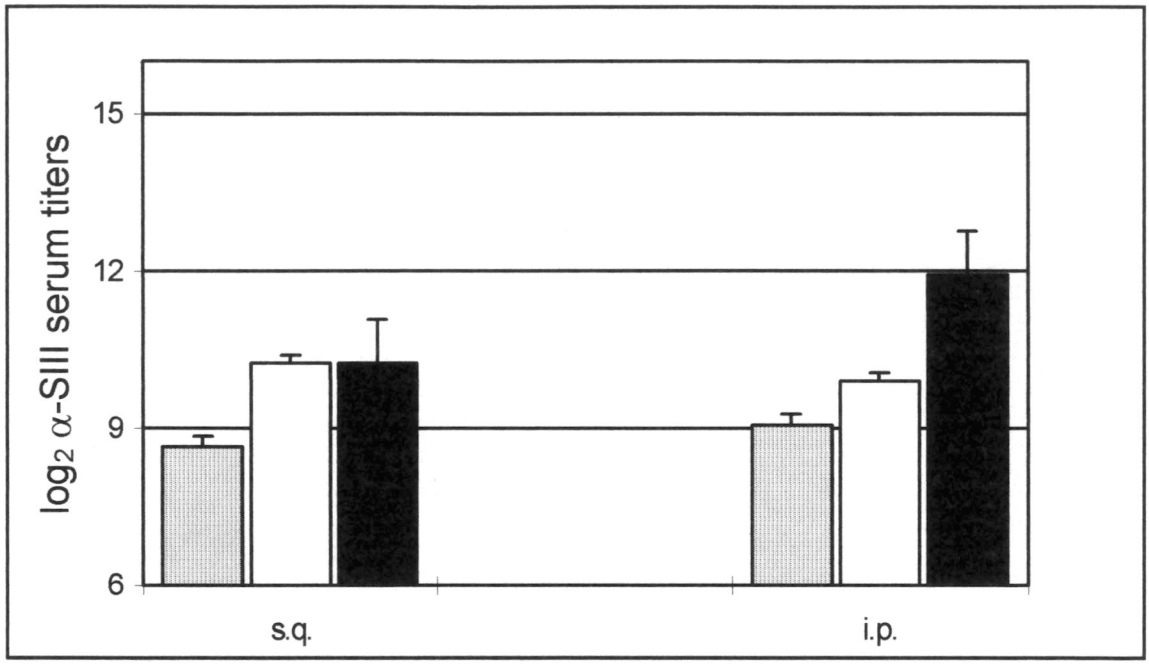


Fig. 6 A comparison of the total IgG response to 25 μg of SIII-BSA ISCOMs prepared by the M_2C_2H conjugation protocol delivered either i.p. (n=31) or s.q. (n=5) on days 0 and 16. Bars represent the mean $\log_2$ anti-SIII titers ±SEM on day 5 ▨, day 10 □, and day 21 ■. A significant difference between day 21 titers produced by s.q. and i.p. injection was found ($p<0.05$).

can be induced with SIII conjugated to a protein carrier such as BSA and that an anamnestic response can be elicited.

A Comparison of the Subcutaneous Route of Injection with the Intraperitoneal Route of Injection

Experiments were designed to compare titers of serum anti-SIII titers of a group of mice injected subcutaneously with a group injected intraperitoneally. The two groups of mice were injected with 25 μg of SIII-BSA ISCOMs on days 0 and 16 and were bled on days 5, 10 and 21. As seen in Fig. 6, both routes of injection induced titers that were not significantly different ($p>0.05$) on days 5 and 10. By day 21, the titers in the group injected s.q. had reached 1:1000 as determined by ELISA. The titer of 1:6000 generated by day 21 in the i.p. group was significantly higher ($p<0.05$). Thus the more direct route to the spleen and mesenteric lymph nodes was found to produce the highest titers.

IgG Subclass Distribution Following ISCOMs Immunizations

Previous experiments in this study were performed to show the production of all subclasses of IgG immunoglobulin molecule in the BALB/c mouse. It was of interest to determine which subclass was dominant in response to the ISCOMs model established in these studies. It had been previously reported that the major subclass of IgG was IgG_3 in response to polysaccharides (72). Experimentally, two groups of mice were given 25 μg of SIII-BSA ISCOMs with one group receiving the immunogen i.p. while the other

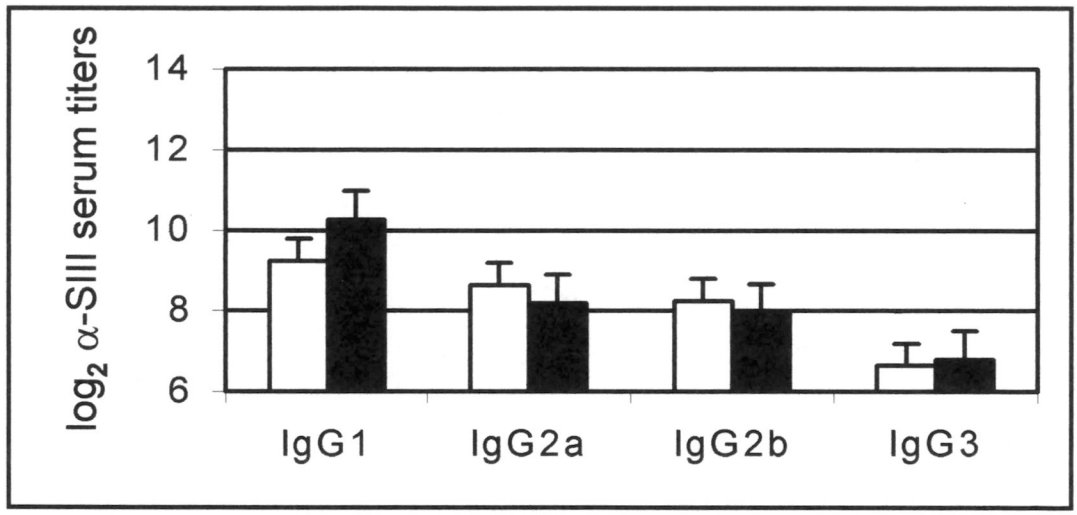
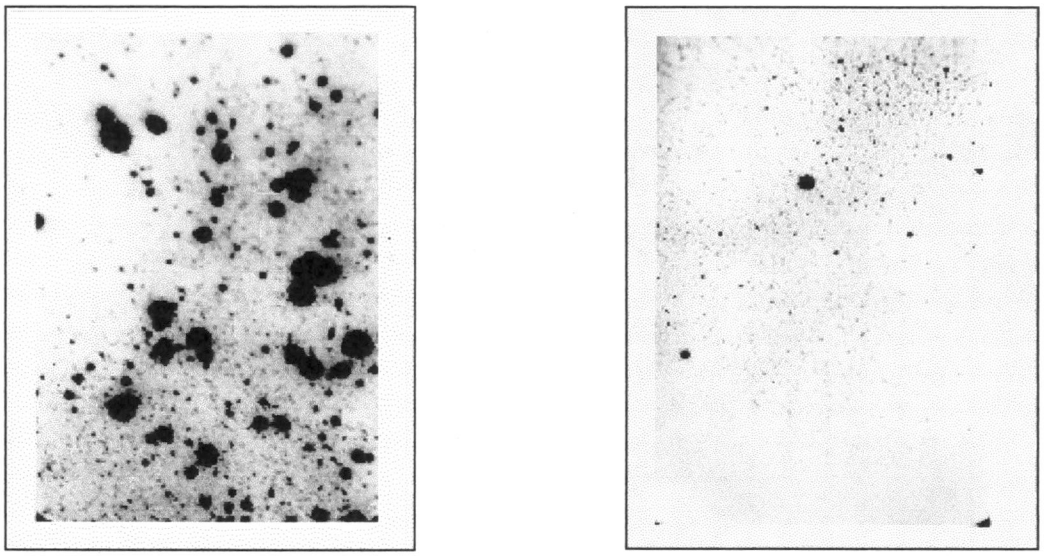


Fig. 7 IgG subclass expression in mice were injected with 25 μg of SIII-BSA ISCOMs either s.q. ■ n= 5, or i.p. □ n= 31, on days 0 and 16. Bars represent the mean serum IgG subclass anti-SIII $\log_2$ titers ±SEM from day 21. A significant difference was found between the IgG_1 subclass compared to all other subclass means regardless of injection route. No demonstrable difference in subclass distribution or titer was seen between routes of injection.

group was injected s.q. Using the ELISA to detect subclass antibodies specific to SIII, it was found that the IgG_1 subclass was significantly higher ($p<0.05$) in both groups than any other subclass (fig. 7). Both groups produced titers in the following order of predominance: $IgG_1>IgG_{2a}>IgG_{2b}>IgG_3$. In contrast to other groups, the IgG_3 subclass titer was not significantly higher than pre-bleed control mice in both groups. Again, the s.q. route of injection did not produce IgG_1 levels comparable to those titers seen in mice injected i.p. (1:600 and 1:1300, respectively). The results of these experiments strongly support the hypothesis that the use of ISCOMs as a means of inducing an IgG response in BALB/c mice is valid. The data also have shown the specific response to be dominated by the IgG_1 subclass.

The Effects of SIII ISCOMs on the Humoral IgM Response

The passive hemagglutination assay (PHA) was used in conjunction with the ELISA to determine isotypes involved in the humoral response to SIII when the antigen was included in ISCOMs. It was assumed that the PHA was measuring the IgM response during the early bleeds and could possibly show an increase following a booster injection on day 16 following the primary injection. A comparison was made between two groups of mice injected with either 25 μg or 50 μg of ISCOMs prepared with the $C_3Cl_3N_3$ SIII-BSA conjugates. It was found that there was no significant difference in serum titers from sera taken on days 5, 10 or day 21 between the groups. Serum titers on day 5 and day 10 reached 1:2000 and had dropped to 1:100 by day 21. As shown in fig. 2, a third



A. B

Fig. 8 The detection of spleen cells producing IgG antibodies specific for SIII by ELISPOT. **A.** The results from a test well of an ELISPOT assay photographed at a 20X magnification with a Zeiss dissecting microscope. Mice (n=13) were injected with 25 μg of SIII ISCOMs i.p. on days 0 and 16 and spleens extracted on day 21. **B.** ELISPOT results photographed at 20X from a group of mice injected on the same days using the same route of injection with 0.5 μg of soluble SIII.

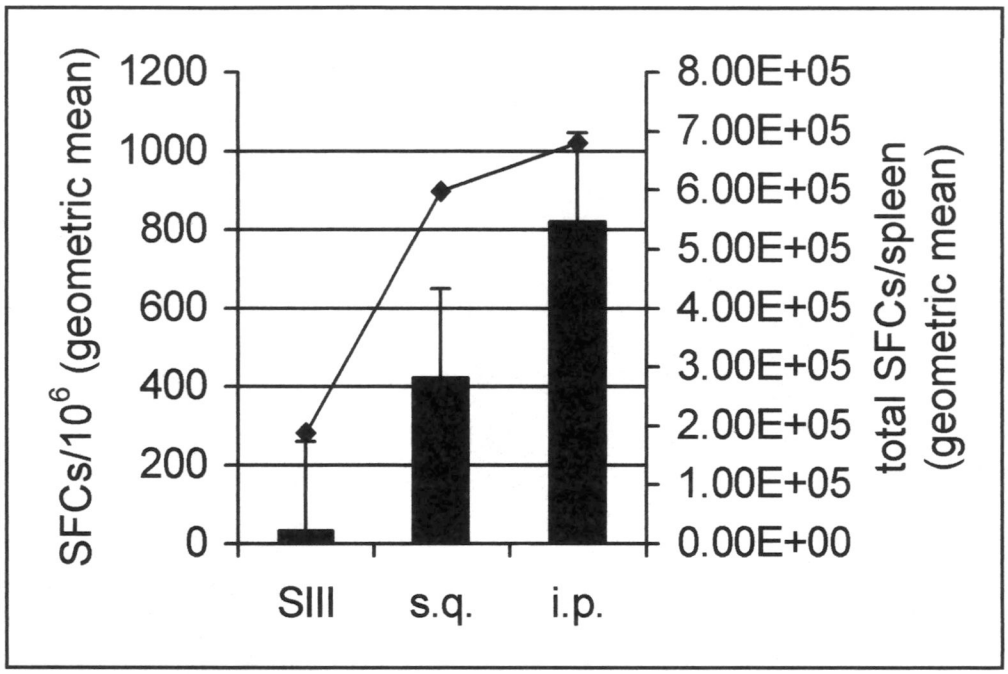


Fig. 9 Quantification by the ELISPOT assay of spleen cells secreting anti-SIII. The SIII group was injected i.p. with 0.5 μg of soluble SIII in sterile saline on days 0 & 16. Two other groups received either s.q. and i.p. ISCOMs at a dosage of 25 μg on days 0 & 16. Spleen cells were harvested on day 21 and quantified as spot-forming cells (SFCs) /10^6 splenocytes and as total SFCs/spleen. Background SFCs from control mice given water *ad libitum* only were subtracted from total SFCs detected in the experimental groups. The results as SFCs ±SEM are expressed in the bar graph while the line graph indicates the SFCs ±SEM/10^6 spleen cells. All data are expressed as the geometric mean of three separate experiments.

group of mice, after receiving a 25 μg dose of ISCOMs produced via the M_2C_2H method on days 0 and 16, produced a reciprocal titer of 1:1000 on days 5 and 10. On day 21, the titer had reached 1:400, which was significantly higher ($p<0.05$) than the titers reached by the group immunized with ISCOMs prepared using the $C_3Cl_3N_3$ reagent.

Anti-SIII Spot-Forming Cells Identified in the Spleens of Immune Mice

The use of the ELISPOT assay yielded additional data concomitant with serum assays by directly detecting cells producing IgG antibodies specific for SIII, as seen in fig. 8. Comparisons were drawn from mice receiving either SIII in saline, or SIII-BSA ISCOMs injected s.q. or i.p.. Data were obtained from spleen cells harvested on day 21 from the three groups after subtracting background counts taken from the unimmunized mice (fig. 9). There was a significant difference between the geometric means of the IgG-producing counts of 281 and 1021 SFCs/10^6 for the group injected i.p. with soluble SIII and the i.p. ISCOMs group, respectively. There was no significant difference between the s.q. count of 896 compared to the count obtained from the i.p. group. Spleens harvested from mice orally fed ISCOMs had their splenocytes tested by ELISPOT and were found to have no significant numbers of IgG-secreting cells specific against SIII.

IgA Response to SIII-BSA ISCOMs

It was of interest to search for isotypes in serum other than IgG and IgM. Day 21 sera were collected from mice injected i.p. with ISCOMs and the ELISA system was used to detect the presence of IgA antibodies specific for BSA. It was found that anti-

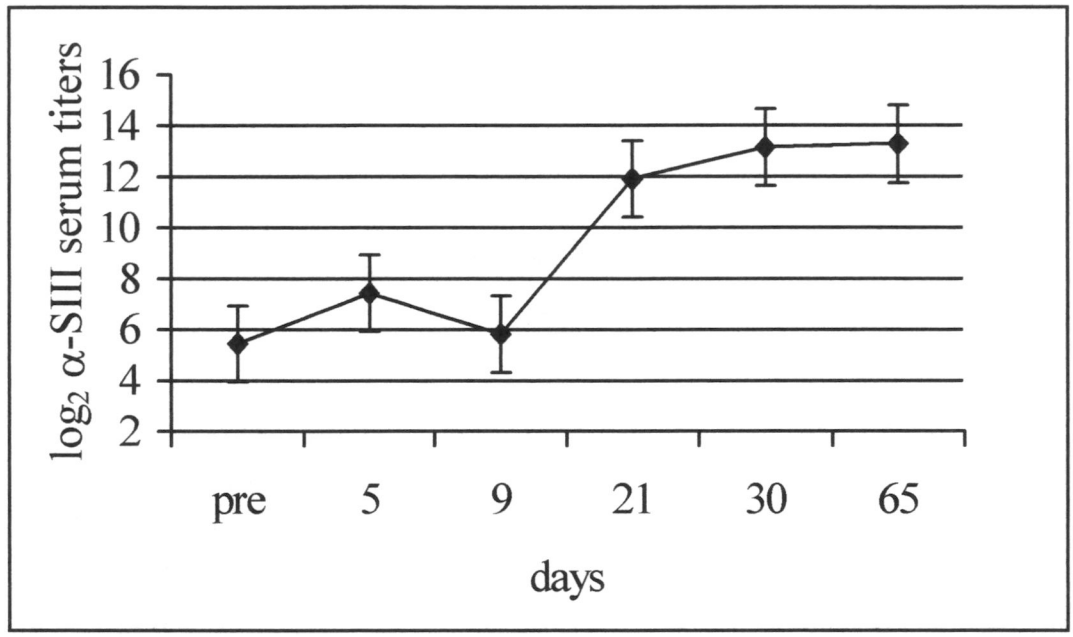


Fig. 10 Kinetics of the anti-SIII IgG antibody response in mice injected i.p. with 25 μg of SIII-BSA ISCOMs on days 0, 21 and 60. Points represent mean $\log_2$ titers ±SEM for days 5, 9, 30 and 65. Day 21 represents the $\log_2$ mean of another ISCOMs group injected with 25 μg i.p. on days 0 & 16 that was included to demonstrate the trend of the antibody response. Significance established by two-tailed t-tests was seen between pre-treatment and day 5, days 5 and 9, days 9 and 30 and days 21 and 30 (for all: $p<0.01$).

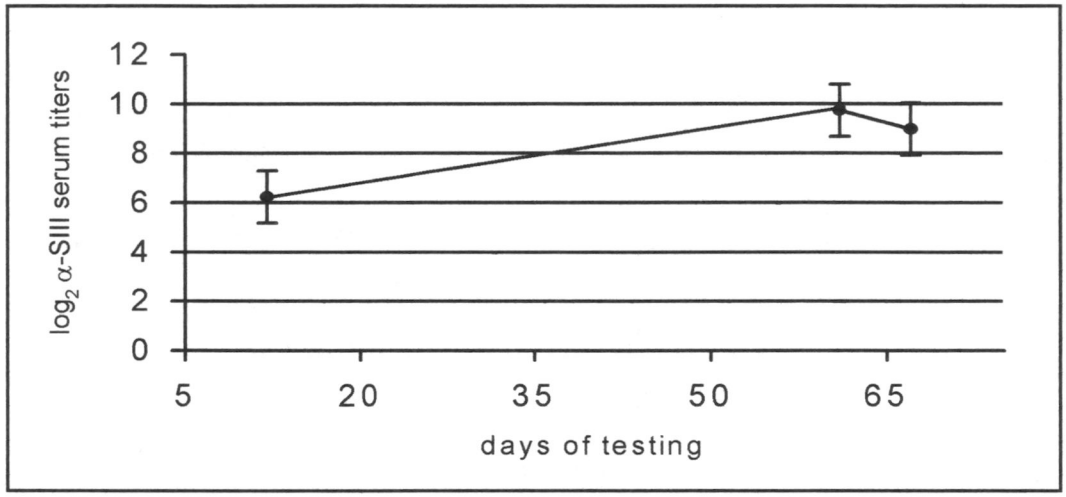


Fig. 11 The systemic IgG response to SIII following oral immunization with ISCOMs. A group of mice (n= 4), was fed 100 μg of ISCOMs on days 0, 6, 7, 54 & 56. Subsequent to the feedings, the mice were bled on days 12, 61 and 67, and the sera tested by ELISA to determine the IgG-specific response. Points represent $\log_2$ titers ±SEM.

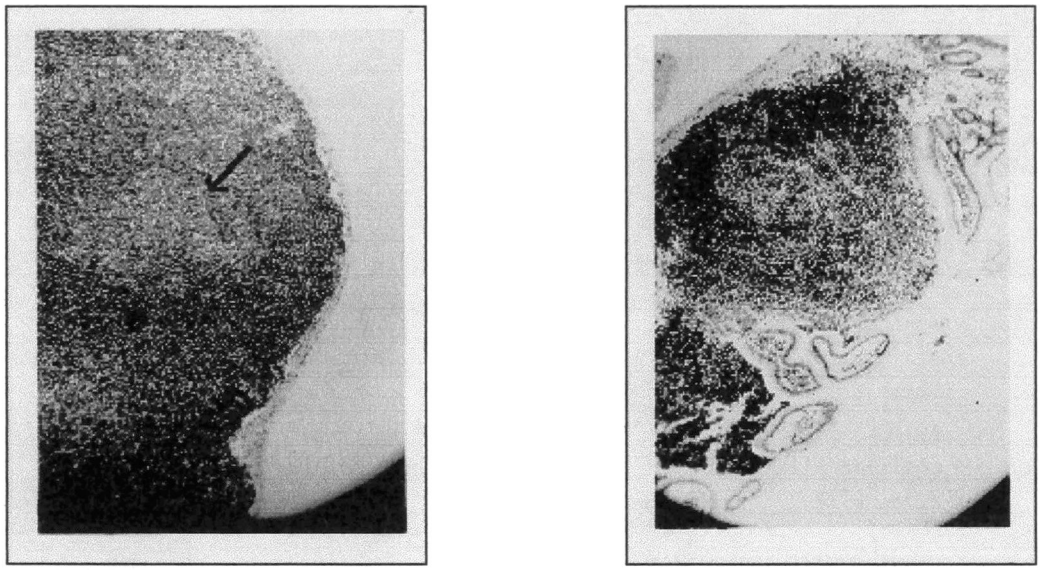
BSA specific titers averaged 1:2000 while control titers reached only 1:100. No anti-SIII IgA serum titers were detected in any group regardless of immunization route.

Long-Term Anti-SIII IgG Immune Response in Mice

Additional experiments were done to assess whether a long-term sustainable anti-SIII IgG titer was possible and to compare these titers to those titers of the primary response. A group of BALB/c mice (n= 8) was injected i.p. with 25 μg on days 0, 21 and 60 as shown in figure 10. A second group of mice (n=24), was previously injected i.p. with 25 μg of ISCOMs on days 0 and 16. This second group's day 21 data was added to figure 10 for comparison to the long-term group (n=8). Mean reciprocal IgG anti-SIII titers of 225, 65, 6400, 9375 and 10,000 were obtained on days 5, 9, 21, 30 and 65 respectively. Student's t-tests showed significance between the $\log_2$ means of pre-treatment serum and day 5 serum titers ($p<0.05$). Also, there was significance between means on days 5 and 9 and between days 9 and 30 ($p<0.05$). No significance between the means of $\log_2$ titers was seen in comparisons between days 30 and 65.

Oral Immunization With SIII-BSA ISCOMs

The oral route of immunization was compared to i.p. and s.q. immunization routes by testing sera and gavage fluid for IgG and IgA specific for SIII and BSA. Two groups of mice were given oral doses of SIII-BSA ISCOMs using the M_2C_2H conjugation method. Group I was administered 100 μg of ISCOMs on days 0, 6, 7, 54 and



A. B.

Fig. 12 Photomicrographs of Peyer's patches. A group of mice was fed 100 μg of SIII-BSA ISCOMs as described under RESULTS. All photographs were taken at 100X using a Zeiss microscope. **A.** A 7 μm section through a Peyer's patch taken on day 21 from an orally fed mouse. The arrow indicates a germinal center containing B cells at various stages of maturation surrounded by an area containing T cells and follicular cells. **B.** A thin section through a Peyer's patch from a control mouse that received water only.

56. Group II had 300 μg of ISCOMs administered on days 0, 2, 28 and 30. Group I anti-SIII serum titers from days 12, 61 and 67 showed mean IgG reciprocal titers of 157, 1657 and 891 respectively (fig. 11). Mean reciprocal anti-SII IgG serum titers for Group II on days 14 and 35 were 35 and 702 respectively. Sera tested for anti-BSA IgG showed that group I (day 30) titers averaged 1:8000, while group II (day 14) titers averaged 1:700. Therefore, oral immunization was found to produce a systemic IgG response at both dose levels against SIII and BSA. In addition to the protocols used for groups I and II, mouse groups Ib and IIb were established, and both groups were given the same 100 μg per mouse dose of ISCOMs but on different days than the groups I and II. To determine if multiple doses over a short period of time would produce antibodies specific for SIII in orally fed mice, the group Ib mice were administered ISCOMs on days 0, 1, 14 and 15, while group IIb received the the same antigen on days 0, 1, 2, 3, 14, 15, 16 and 17. Gavage fluid from groups I and II and groups Ib and IIb were tested for the presence of IgA, IgG and IgE specific for either BSA or SIII and they were found to be negative for antibodies against both antigens.

Effect of SIII-BSA ISCOMs on Murine Peyer's Patch Cells

Oral immunizations were done to determine the effect of antigen on immune cells present within the Peyer's patches. Peyer's patches were prepared as thin sections from one control group and from two test groups to compare the degree to which cells had undergone proliferation and stimulation. Germinal centers within the discrete lymphoid

tissue consist of large, differentiated cells seen as lighter stained areas, surrounded by smaller cells probably of T-cell lineage and interspersed with macrophages that stained darker, as seen in fig. 12. Distance between cells served as a parameter of density that was useful as an indirect measurement of cell proliferation. The diameter of the cell nucleus was another parameter for indicating differences between stimulated and unstimulated cell populations. Group 1 was fed 100 μg of SIII-BSA ISCOMs on days 0, 1, 14 and 15. Group 2 was fed 100 μg of SIII-BSA ISCOMs on days 0, 1, 2, 3, 14, 15, 16 and 17. The Peyer's patches from these groups were removed between days 21 and 35, with measurements and statistical summary of these data shown in Tables 2 and 3. Peyer's patches from mice fed saline were compared with those from the experimental groups. Measurements of the nucleus diameter and distance between cells from slides prepared from fixed and hematoxylin and eosin stained 7 μm sections showed that there was no significant difference in nucleus diameter or distance between cells in either test group fed ISCOMs ($p>0.05$). There was, however, a significant difference in means between the control group when compared to either ISCOMs groups ($p<0.05$).

PROTEIN DATA					
		(Lowry method)			
BSA-SIII conjugates (M_2C_2H rgt.)			**BSA-SIII conjugates ($C_3Cl_3N_3$ rgt.)**		
start	end	recovery	start	end	recovery
10mg	2.8mg	28%	25mg	2mg	8%
32mg	7.1mg	22%			
		(amino acid analysis)			
SIII-BSA ISCOMs			**BSA ISCOMs**		
0.095mg/ml			0.076mg/ml		
CARBOHYDRATE ASSAY FOR SIII					
SIII-BSA Conjugates (M_2C_2H)					
start	end	recovery			
10mg	2.3mg	23%			
10mg	1.7mg	17%			
SIII-BSA ISCOMs (M_2C_2H rgt.)			**SIII-BSA ISCOMs ($C_3Cl_3N_3$ rgt.)**		
start	end	recovery	start	end	recovery
20mg	2.8mg	11.4%	25mg	5.4mg	22%
40mg	5.23mg	13%			
40mg	3.38mg	8.45%			
19.5mg	2.9mg	14.9%			

Table 1 Summary of protein analyses and phenol-sulfuric acid carbohydrate analyses on representative samples of SIII-BSA conjugations by the cyanuric chloride method and the M_2C_2H methods, and after incorporation of the respective conjugates in ISCOMs. Starting and ending quantities of carrier and immunogen are given, as determined from the quantity of components from start of conjugation process through ISCOMs formation and sucrose gradient purification. The incorporation rate of components is represented as the recovery percentage.

Peyer's patch sections (between-cell distance in microns)				
		control	**grp. Ib**	**grp. IIb**
mean		2.67	1.21	1.27
# observations		n= 45	n= 45	n= 30
# mice		n= 3	n= 16	n= 2
t-tests				
control vs. grp. Ib		**P(T<=t) two-tail**	**9.13E-12**	sig.
grp. Ib vs. grp. IIb		**P(T<=t) two-tail**	**0.697**	not sig.

Table 2 Mice orally fed 100μg of ISCOMs on days 0, 1, 14 & 15 (group Ib, n=16), and days 0, 1, 2, 3, 14, 15, 16 and 17 (group IIb, n=2). Summary of between-cell distance data of Peyer's patch thin sections (7 μM), stained with hemoxylin and eosin for comparison .

Peyer's patch sections (nucleus diameter in microns)				
		control	**grp. Ib**	**grp. IIb**
mean		4.9	6.32	6
# observations		n= 45	n= 45	n= 30
t-tests				
control vs. grp. Ib		**P(T<=t) two-tail**	**3.1E-08**	sig.
grp. Ib vs. grp. IIb		**P(T<=t) two-tail**	**0.391**	not sig.

Table 3 Mice from groups Ib (n=16) and IIb (n=2) orally fed 100μg of ISCOMs from table II. Peyer's patch sections (7 μM) stained with hemoxylin and eosin. Sections were used to compare nucleus diameter in test groups to control groups which received water or saline. Student's t-tests between groups Ib & IIb show no significance ($p>0.05$), with significance between the control group and group Ib ($p<0.05$).

DISCUSSION

Although the results of extensive research on the response to pneumococcal polysaccharide type III made by BALB/c mice have been reported, little data was available pertaining to the development of techniques for generating isotype shifts and the subsequent anamnestic response. One problem faced in the development of efficient vaccines in humans for protection against Streptococci is related to the polysaccharide antigens present in the gram positive organisms. The purpose of the work reported herein was to study the use of a relatively new approach for delivering purified type III pneumococcal polysaccharide to BALB/c mice in order to generate a lasting IgG response. The approach to the problem was to conjugate the polysaccharide to a protein carrier and include both in an adjuvant-containing structure that could safely be delivered via different routes. The delivery system of choice was immunostimulating complexes (ISCOMs) which was structurally large enough to contain all the necessary components for the induction of an immune response.

Extensive research has been published on the immune response and regulation of pneumococcal polysaccharide type III in BALB/c mice. SIII is a thymus-independent type 2 antigen (TI-2) (67); therefore the outcome of the anti-SIII immune response is partly dependent on T-cell help. This is in contrast to thymus-independent antigens classified as type 1 (TI-1) antigens which can directly activate B-cells (73).

The IgM-dominant immune response to SIII in BALB/c mice has been well documented (74), and the studies reported here have demonstrated the typical IgM

response devoid of isotype switching to IgG (fig. 4). This study used SIII as a soluble antigen for intraperitoneal (i.p.) immunization to ensure both the immunogenicity of the stock SIII and to establish a baseline for comparison with other mice injected with the conjugated antigen incorporated within ISCOMs. These data supported conclusions made by other investigators as to the murine immune response against the SIII antigen when given without a carrier or adjuvant.

The conjugation of SIII in this study was performed using two different methods in the preliminary stages of the study. SIII was conjugated to bovine serum albumin (BSA) using a 4-(N-maleimidomethyl) cyclohexane 1-Carboxyl hydrazide, (M_2C_2H) reagent. SIII and BSA were linked via the M_2C_2H molecule to create a stable covalent bond. As a comparison, the cyanuric chloride method ($C_3Cl_3N_3$), was employed to conjugate SIII to BSA. The two conjugate products, prepared by the two methods were separately incorporated into the cage-like structure called immunostimulating complexes (ISCOMs). A comparison of the M_2C_2H and $C_3Cl_3N_3$ conjugation methods showed that the cyanuric chloride method's single triazine structure linked the carrier protein to the polysaccharide (19). The M_2C_2H method, in contrast, employed a heterobifunctional cross-linker consisting of an electrophilic maleimide bound to the protein's thiol group, with linkage to a nucleophilic hydrazide covalently bound to the polysaccharide's reactive aldehyde (75). The M_2C_2H molecule acted as a spacer, which decreased steric hindrance between the protein carrier and the polysaccharide. Diminished steric hindrance within conjugates has been shown to increase immunogenicity as a direct result of greater T cell interaction between helper T cells and the carrier protein (13, 76). Additionally, the

formation and recovery of the conjugate using the $C_3Cl_3N_3$ method was hindered when the majority of the conjugate aggregated into an insoluble gelatinous mass during its formation. This caveat is ascribed to the short linkage existing between the carrier and polysaccharide after attempts are made to form large matrixes within a lattice structure (20).

The use of the M_2C_2H reagent for conjugation of protein and carbohydrate is a recent methodology and some documentation on its use was found in a formula of similar chemical reactivity, 4-(4-N-maleimidophenyl) butyric acid hydrazide, (MPBH) (75). The M_2C_2H conjugation method provided the opportunity to study the affects it might have on the ability of the immune system to respond against a large polysaccharide structure such as SIII when covalently bound to the moderately-sized protein BSA.

Preliminary data comparing the $C_3Cl_3N_3$ and M_2C_2H methods showed conclusively that comparable IgG titers were produced by both methods, (fig. 1). The data showing IgG titers specific for SIII and BSA, along with hemagglutination assays specific for SIII, confirmed what other investigators had observed. Protein-polysaccharide conjugates induce the immune system to respond in a T-cell dependent manner toward the conjugate with concomitant appearance of multiple isotypes after antigen exposure (77). The present data showed, however, that the conjugates formed from the M_2C_2H reagent produced a more sustained titer in the PHA assay (fig. 2). The Student's t-tests of serum titers from all days studied showed a difference in means between both methods at a 99% significance level. It is speculated that the nature of the bond created by the $C_3Cl_3N_3$ method is degraded in a way that renders a subtle difference

in antigen processing and presentation by antigen-presenting cells. Also, as previously noted, the decreased distance between conjugate components resulting from the use of the cyanuric chloride method could lead to fewer T cell interactions with the protein carrier resulting in a less sustained immune response to the conjugate.

Additional rationale for using the M_2C_2H method was found after examining the rates of polysaccharide and protein incorporation as conjugates both before and after ISCOMs incorporation. The rates of protein and carbohydrate recovery after conjugating components showed that polysaccharide incorporation in conjugates and in ISCOMs was high using the cyanuric chloride method. However, protein content was approximately half that in those conjugates formed using the M_2C_2H reagent (table 1). Conjugates prepared using the M_2C_2H reagent yielded an approximately equal ratio of protein to polysaccharide. The M_2C_2H method was used solely in these current studies after reviewing preliminary data and comparing the efficacy of conjugation methods in immune response induction. The paucity of literature on the cyanuric chloride method, problems encountered in conjugate formation, shorter molecular distance between conjugate components, and the method's absence among present day conjugations for vaccine lent little justification for further use.

The formation of the vaccine currently under study also enlisted the aid of the ISCOMs adjuvant. Adjuvants such as Quil A (saponin), flagella protein and MPL (monophosphoryl lipid A) have previously been added to conjugate formulations, in an attempt to both direct the immune response toward a specific isotype and enhance conjugate immunogenicity (13, 14). The ISCOMs structure's unique adjuvant properties

are attributed to saponin (32). Saponin is a triterpene derived from the Quillaja plant having hemolytic properties when injected i.v., but has little toxicity when administered orally (44). Saponin has long been used as a veterinary vaccine adjuvant (78), and it has been shown to permeate mucosal surfaces and increase antigen uptake(28, 29). Saponin's mitogenic influence toward cytotoxic lymphocytes and natural killer cells has been previously reported (30). Revelant also was the finding that saponin enhanced the primary immune response to thymus-independent antigens, such as SIII. This enhancement is possible either by inducing antigen-specific B cells to differentiate into memory cells, or by making B cells more receptive to T cell signals (79).

A carrier protein's influence on the immune response against the bound polysaccharide was also an important consideration in the design of the vaccine formulation used in the current study. The utility of enlisting BSA as a protein carrier was decided after measuring the response to BSA in mice. The low anti-BSA average reciprocal titer of 800 reflects the relatively poor immunogenicity of BSA after it was injected as an unconjugated antigen. This finding was in contrast to the significant anti-BSA IgG titer seen after immunization with BSA-ISCOMs. This same enhancement of IgG response was shown repeatedly in studies as a result of saponin's presence in the ISCOMs structure, (32, 33), as well as in studies where saponin and antigen associated freely in a vaccine emulsion (13).

Further ELISA analysis to detect anti-BSA IgG titers was conducted on sera from mice injected i.p. with SIII-BSA ISCOMs. Anti-BSA IgG titers were not significantly different between the two conjugation groups; however, the increase of anti-BSA IgG

titer in those mice injected with SIII-BSA ISCOMs over that of BSA-ISCOMs was striking. This stronger immune response illustrates the adjuvanticity of saponin, and is attributed also to the sizable SIII molecule in covalent linkage with BSA. A previous study examined anti-BSA IgG titers in mice after immunizing with several conjugates of BSA to different-sized molecules (80). The study theorized that a higher molecular weight in the polysaccharide (dextran), induced substantial anti-BSA titers by exposing higher numbers of BSA epitopes on the large polysaccharide surface for longer periods of time in vivo. The longer exposure period would provide continuous T-cell stimulation and recruitment over that seen after injection with BSA alone (80). The current data support this previous study's demonstration of the substantial enhancement of anti-BSA IgG titers as directly attributed to BSA's association with the polysaccharide.

The generation and enhancement of anti-SIII IgG titers as a result of polysaccharide association with BSA possibly provide a reciprocating relationship. Conjugation of the two molecules resulted in higher immune responses to both components but for different reasons. Polysaccharide bulk, increased BSA epitope exposure and possibly small differences in antigen presentation have enhanced anti-BSA IgG titers. The T-dependent characteristics of proteins and their subsequent processing within APCs, along with the isotype switch to IgG that is characteristic of protein antigen processing are imparted to SIII by virtue of the polysaccharide-protein conjugation.

The effects of isotype switching and isotype subclass predominance toward pneumococcal polysaccharide after conjugation to a protein was further illustrated in a study linking pneumococcal polysaccharide (type XIV) to BSA (14). This

carbodiimide/sulfo-NHS-generated conjugate was injected i.p. into mice and resulted in isotype switching predominantly to the IgG_1 subtype. In the study with type XIV polysaccharide, it was theorized that the carrier protein was the predominant influence on the IgG subtype; however, some influence was seen on isotype ratios when the conjugate was injected in conjunction with various adjuvants. Previous studies of conjugates with polysaccharides had typically induced an immune response which was predominantly of the murine IgG_3 subclass (81). It was also previously reported that IgG_3 was most protective towards pneumococcal infections, but different studies have shown that other IgG isotypes were protective (72, 82). IgG subclasses have been reported to differ in opsonization strength, but in vitro assays to measure opsonic activity have not shown a correlation between opsonic activity and protection against pneumoccocal infections regardless of IgG subclass (1). It is reasonable not to discount the potential protective benefits of the predominant IgG_1 before further studies are done. The current study examined IgG subclasses specific for SIII in mice injected i.p. or s.q. with SIII-BSA ISCOMs, and the data confirmed the predominance of the IgG_1 isotype, (fig. 7). This finding supports the previously published data.

An anamnestic response upon re-exposure to the same antigen is of prime consideration to the efficacy of a vaccine. The ability to generate an anamnestic response will determine the host's success in mounting an effective immune defense towards an infective agent months or years after an initial immunization. Exposure to the SIII antigen typically does not induce an anamnestic response, but such a response is possible after prolonged intervals between immunizing doses with SIII, although the secondary

response is short-lived (83). Experiments with rabbits have shown that isotype switching to IgG, with the generation of a lasting anamnestic response, was possible following conjugation of the SIII polysaccharide to bovine gamma globulin (BGG) using a cyanuric chloride conjugation technique (18). This protocol induced an anamnestic response, but only after first priming with SIII and its protein carrier separately, weeks before administration of the SIII-BGG conjugate (18).

The current study examined IgG titers specific for SIII in BALB/c mice which were injected with SIII-BSA ISCOMs over a period of 65 days (figs. 10 and 5). IgG subclasses produced by day 65 showed the same IgG_1 predominance found in day 21 sera. A concomittant sharp decline in IgG_{2a} and IgG_{2b} subclasses was seen by 65. These data provide direct evidence supporting the existence of an anamnestic response after immunizing with SIII-BSA ISCOMs as characterized by a substantial enhancement of the IgG titer. The possibility of lasting immune protection is provided by these studies, although it is well documented that high serum antibody titers generated by vaccine do not always translate into protection against the infection (40). It is reasonable to suggest that challenging BALB/c mice with viable Streptococcus pneumoniae of the type III capsular polysaccharide would be a logical continuation of the current research.

Preliminary trials were conducted to determine the optimal dose and the degree of vaccine immunogenicity after the incorporation of SIII-BSA into ISCOMs. These criteria relate directly to immune tolerance and the most efficient route of immunization. The data from the current studies showed an increase in the immune response to SIII after i.p. injection over s.q. injection on day 21 (fig. 6). The estimated dose of 25μg, based on SIII

content, did not induce tolerance after i.p. and s.q. injection, nor was there evidence of tolerance at the higher dose of 50μg. These findings are in contrast to the experiments of Peeters' group, in which doses higher than 0.5μg of a pneumococcal polysaccharide type IV/tetanus toxoid conjugate induced tolerance in mice (16). There are several possibilities as to why immune tolerance was not noted in the present study. The ISCOMs structure may have masked some of the conjugates' potentially tolerogenic epitopes from having the ability to induce immune tolerance. Another possibility is that removal of unconjugated residue from the conjugate/ISCOMs preparation via the sucrose gradient method precluded the introduction of any unconjugated polysaccharide into the vaccine formulation. Peeter's group purified their conjugate via dialysis, high-speed centrifugation and gel column chromatography. Their protocol yielded ~10% unbound polysaccharide as determined by rocket immunoelectrophoresis. Despite this finding, Peeters was able to eliminate the polysaccharide residue as the cause of T suppressor cell stimulation in the PS4 conjugate. The quantity of unconjugated polysaccharide in the original vaccine was determined to be insufficient to cause the tolerance noted in their initial vaccination trial. No explanation was given for the retention of TI-2 characteristics in the PS4 conjugate and the attendant suppression (16). Peeters did not eliminate the possibility of a problem with the carrier protein. Proteins commonly found in the environment or in other vaccine formulations, and which are repeatedly encountered by the immune system may induce tolerance to the hapten or polysaccharide moiety. This problem has been reported with the use of tetanus toxoid (83). Additionally, there are

structural differences in pneumococcal polysaccharides, between conjugation methods, and in carrier proteins that could influence suppressor populations. Further work would be necessary before drawing firm conclusions.

Recent success has been reported in generating a significant immune response while avoiding tolerance after administering ovalbumin orally in conjunction with the adjuvant ISCOMs (85). Speculation about this success centers on the membrane interaction between intestinal cells having immune function potential and the lipid properties of ISCOMs (85). Two cell types figure prominantly in any initial encounter with a luminal antigen. Intra-epithelial cells of the CD8+ lymphocyte subset line the intestinal lumen alongside the intestinal epithelial cells (IEs). Evidence suggests that IEs indirectly initialize the act of immune suppression to oral immunogens (57). Another cellular subset, the M (microfold) cell, is found overlaying the Peyer's patches in the murine intestinal lumen. The lower protein content of M cells functions to slow the endocytosis of water-soluble molecules, such as albumins (43). This lowered protein content is in contrast to the lipid/protein ratios found in other intestinal cells. It has been speculated that the lipid matrix of a vaccine vehicle such as the ISCOMs could enhance its uptake into intestinal cells (85). Although M cells are not mentioned as specific attractants of ISCOMs, the altered ratio of lipid/protein in M cells makes the idea of a heightened association between the two entities a logical hypothesis. The importance of Peyer's patch (PP) cells in mucosal immunity might be appreciated when it is recognized that the majority of isotype switching to IgA occurs in the PP cells, and that IgA is the major protective isotype found in mucosal tissue (54). Application of the ISCOMs as an

oral adjuvant and vaccine vehicle is appropriate as evidenced by the body of research previously reported. Studies attest to the ISCOMs structures' extended time interaction with peritoneal cells (41, 44).

The current study examined the effects of SIII-BSA antigen after oral administration of ISCOMs. Intestinal gavages and sera were tested by ELISA to detect IgG and IgA. Additionally, the gavages were tested for IgE, in the event that a Type I, hypersensitivity reaction was induced. Gavages showed no titers above those of mice fed ISCOMs alone. Importantly, significant titers of IgG against SIII and BSA were detected in sera by ELISA in orally-fed mice (fig. 11). Peyer's patch cells in ISCOMs-fed mice also showed quantitative differences (fig. 12, tables 2 and 3) over those of control mice fed the SIII-BSA conjugate or saline alone. In contrast, the ELISPOT protocol used to quantitate antibody-secreting plasma cells specific for SIII found no significant numbers of IgG and IgA-secreting cells in the spleens of these orally immunized animals above background. Significance of these findings is unclear since no titers of any isotype class from gavages were detected in any group. The presence of a systemic response but concurrent absence of a mucosal response after oral feeding is unusual, since the opposite occurrence is more commonly reported (50). The evidence for an IgG systemic response in the absence of a detectable mucosal IgA response against SIII and BSA suggests that a cytokine suppressive action against the production of IgA is at work. Extensive research has documented that TGF-β is partially responsible for directing the isotype switch of B cells to IgA, (55), followed by Th_2-derived cytokines Il-5 and IL-6. These latter cytokines are responsible for influencing the terminal differentiation of IgA-

committed B cells to antibody-secreting plasma cells (86). A major problem in generating an IgA response comes from the regulation of these cytokines. TGF-β concentrations operate within a narrow range to either enhance IgA direction or, conversely, to inhibit isotype switching to IgA (54). Recently, a lymphocyte subset known as Th3, which is presumed to be located in the mucosa, has been implicated as an important cell for the production of TGF-β (53). It is reasonable to speculate that this cell type could be involved in the absence of IgA in the gavages and sera of the mice immunized with ISCOMS in this study, yet allow a systemic IgG response to occur. This can not be the entire explanation for the absence of IgA directed against SIII and BSA, since other ISCOMs preparations have successfully elicited IgA responses either locally or systemically with varying degrees of success (37, 60, 87). Recent studies have used purified fractions of saponin such as QS-21, as this fraction was found to be less toxic than the crude extract (88, 89). What specific properties isolated saponin fractions might have apart from the crude saponin used in the current study, and their effects on cytokine ratios will require more research to determine the reasons behind the phenomenon described here.

Studies were necessary to further understand how the conjugate-ISCOMs were handled in vivo following oral and i.p. administrations. Quantification of the splenic B cell response towards the T-cell dependent antigen became imperative (fig. 8). First, it was necessary to show a transfer of antigen to the spleen, and second, to show a specific IgG response to the SIII as part of the conjugated antigen. The ELISPOT assay was

performed using the spleen cells of i.p. injected mice (fig. 9). The four-fold increase in spot-forming cells (SPC), found in ISCOMs-injected mice over those of control mice and in SIII-injected mice indicated relocation of antigen to the spleen via the lymphatics, with some antigen possibly carried to the spleen within peritoneal APCs (41).

The sharp contrast of cell-antigen contact between oral and i.p. routes shows the advantage of the i.p. route's lower risk of inducing immune tolerance, followed by the high serum IgG titers in evidence (fig. 5). The oral route may induce high levels of IgA and IgG systemically and in localized regions if tolerance can be avoided. Future studies in oral feedings using more varied dosages and immunization time-tables should include assays to directly sample cytokines within the mucosa. Immunohistological techniques for Peyer's patch cells could be used to distinguish cell populations that produce various cytokines as well as antibody isotypes.

The data demonstrated that an isotype switch from the predominant IgM non-anamnestic response toward the TI-2 antigen SIII became one that was predominantly IgG_1 after i.p. immunization. The response was sustained from 21 days to 65 days against SIII and the carrier BSA. It has been suggested that the conversion of the SIII antigen to a T-cell dependent antigen rests largely in its covalent linkage to BSA. The enhancement of the immune response to BSA was contingent on the increased presentation of epitopes as evidenced by the high titer of BSA after conjugation to SIII. The enhancement of the immune response against SIII was evident in the IgG titer seen by day 21 and in all mice, IgG-specific titers to SIII of >1:10,000 by day 65. This response can be attributed to the conversion of SIII to a TD antigen and perhaps to the ISCOMs structure in some yet

unknown way. This contribution of the ISCOMs structure is of particular relevance where higher dosages of conjugate-ISCOMs are injected without inducing tolerance.

Oral immunization using the SIII-BSA ISCOMs did induce significant IgG sera titers against IgG in BALB/c mice. In contrast, the data showed no IgA production in gavages and sera. These data, along with the lack of SIII-specific SFC from the spleen are an enigmatic finding when contrasted to the positive quantitative data derived from the Peyer's patches. A cytokine ratio not conducive to the generation of an immune response, an overabundance of TGF-β or some other suppressive cytokine may be responsible. The strong evidence of a systemic IgG response to both SIII and BSA suggests that antibody-secreting cells are circulating throughout mesenteric nodes, for example. This may account for the negative ELISPOT data.

Conjugates of protein and SIII polysaccharide incorporated into ISCOMs and the effects of such a formulation have not previously been reported. The findings herein demonstrate the merits of further studies with such a formulation relating to the effects on mucosal immunity, particularly in light of the positive systemic immune response to this vaccine. Experimental design focusing on cytokine production and localized immune reactions should further the understanding of the mucosal immune mechanism and its response to polysaccharide antigens.

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APPENDIX A

Buffers and Reagents

Phenol C_6H_5OH - 5% distilled water (Fisher Scientific, Pittsburgh, PA)

50 mM Sodium phosphate - 45 mM Na_2HSO_4, 5 mM NaH_2PO_4, 1 mM EDTA, pH 7.5

100 mM Sodium phosphate - 95 mM Na_2HPO_4, 5 mM NaH_2PO_4, 50 mM NaCl, pH 7.0

4-(N-maleimidomethyl) cyclohexane 1-carboxyl hydrazide hydrochloride · ½ dioxane M_2C_2H) 3 mM in DMSO (Pierce, Inc. Rockford, Ill)

100 mM Sodium meta periodate - $NaIO_4$ in distilled water, (Fisher Scientific, Pittsburg, PA)

15 mM glycerol - $C_3H_8O_3$

N,N-Dimethylformamide (DMF) - C_3H_7NO, (Sigma, St. Louis, MO)

5-Bromo-4-chloro-3-indolyl phosphate-toulidine salt (BCIP)

$C_8H_6BrClNO_4P \cdot C_7H_9N$ (BIO-RAD, Hercules, CA)

Acetate - 100 mM $CH_3COONa \cdot 3H_2O$, 100 mM NaCl, pH 4.5

"Labelling" solution - 100 mM $CH_3COONa \cdot 3H_2O$, pH 5.5

Poly-L-Lysine · hydrochloride (PLL)-30,000-70,000 MW 1% PLL in 50mM TRIZMA-base pH 8.1, (Sigma, St. Louis, MO)

Trypan Blue - $C_{34}H_{24}N_6O_{14}S_4NO_4$	0.5% in PBS with 0.05% NaN,dye content 50% (Direct blue #14), (Sigma, St. Louis, MO)
Chromic chloride -	$CrCl_3 \cdot 6H_2O$ 1% in 0.85% saline, (Fisher Scientific, Pittsburgh, PA)
ELISA substrate buffer -	5x stock, 2 mM $ZnCl_2$ 1 mM $MgCl_2$,100 mM glycine to 100 ml distilled water, pH 10.4
Modified barbitol buffer (MBB) -	15.68 mM $C_8H_{12}N_2O_3$, 9.08 mM $C_8H_{11}N_2Na$, 0.75 mM Ca_2Cl, 757.0 mM NaCl
Hank's balanced salt solution (HBSS) -	1.3 mM $CaCl_2$ 0.5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$ 138.0 mM NaCl, 3 mM $NaHCO_3$ 0.3 mM $NaHPO_4 \cdot 7H_2O$
Phosphate-buffered saline (PBS) -	150 mM NaCl, 7 mM Na_2HPO_4, 3 mM KCl, 1.0 mM KH_2PO_4, pH 7.4
PBS Tween -	0.05% Tween 20 (Sigma, St. Louis, MO) in PBS
Blocking buffer -	1% skim milk (Difco Labs, Detroit, MI) in PBS/Tween 20
Coating buffer -	15 mM Na_2HCO_3, 0.05% NaN_3. pH 9.6
Nitro blue tetrazolium (NBT) -	$C_{40}H_{30}Cl_2N_{10}O_6$, (Sigma, MO)

ELISPOT alkaline phosphatase substrate -	1.5 mg BCIP to 0.1ml DMF, 3.0 mg NBT to 0.1 ml DMF, 10 ml of ELISPOT substrate buffer pH 9.8
ELISPOT substrate buffer -	100 mM $NaHCO_3$, 1.0 mM $MgCl_2$,pH 9.8
Ethyl ether - $C_2H_5OC_2H_5$	(Fisher Scientific, Pittsburgh, PA)
TRIZMA-base - $C_4H_{11}NO_3$	20.59 g/l (Sigma, St. Louis, MO)
ACT buffer -	9 volumes of 0.83% NH_4Cl, 1 volume TRIZMA-base, pH 7.2
Deacetylation solution -	50 mM Na_2HPO_4, 500 mM NH_2OH · HCl (hydroxylamine hydrochloride), (Pierce, Inc.Rockford, Ill), 25 mM EDTA, PH 7.5
Telly's Fixative -	1476 ml 95% ethanol 524 ml distilled water 200 ml formaldehyde 100 ml glacial acetic acid
Gavage solution -	25 mM NaCl, 40 mM $NaSO_4$, 10 mM KCl, 20 mM $NaHCO_3$, 145g/L Polyethylene glycol (~33,500g/mole) pH 7.8, 530 mOSM

APPENDIX B

Summary Of Statistical Data

Treatment: 0.5 μg soluble SIII i.p. on days 0 & 16 p. 44

		day 5	day 10	day 21		
ELISA data α-SIII IgG titers					**P(T<=t) two-tail**	**0.373 d 5 & d 10**
	mean	6.84	6.64	7.24	**P(T<=t) two-tail**	**0.177 d 5 & d 21**
n= 5	**std dev**	0.44	0	0.89	**P(T<=t) two-tail**	**0.208 d 10 & d 21**
	sem	0.2	0	0.4		
PHA data α-SIII titers						
	mean	10.8	8.4			
n= 5	**stdev**	0.45	0.89			
	sem	0.2	0.4			

Treatment: SIII-BSA ISCOMs 25 μg i.p. pp. 40, 41

anti-BSA IgG titers			
		day 21	
Conjugation method		**log_2**	
$C_3Cl_3N_3$	**mean**	16.64	
n=4	**stdev**	0	
M_2C_2H	**mean**	16.26	
n=8	**sd**	0.51	
	sem	0.18	

P(T<=t) two-tail 0.0796

P value shows no significant difference between the means of these two groups

ELISA anti-BSA IgG log_2 titers pp. 40, 41

	mouse#	day 7	day 19
Treatment			
	1	9.32	12.32
BSA	2	10.32	12.32
ISCOMs	3	9.32	11.32
	4	10.32	11.32
mean		9.82	11.8
stdev		0.58	0.58
sem		0.29	0.29
	1	nd	8.32
BSA	2	nd	10.32
	3	nd	8.32
	4	nd	10.32
mean			9.32
stdev			1.15
sem			0.58

Student's t-test	
P(T<=t) two-tail	**0.008237**

P value shows a significant difference between the means of these two groups.

Comparison of PHA log_2 titers over time between an ISCOMs group and an SIII group p. 47

Treat-ment		Log_2 anti-SIII titers	day 5	day 10	day 21
		mean	10.8	8.4	nd
SIII i.p.	**n**= 5	**stdev**	0.45	0.89	nd
0.5 μg		**sem**	0.2	0.4	nd
		mean	10.6	10.4	8.78
ISCOMs i.p.		**stdev**	0.59	0.72	1.53
25 μg	**n**= 23	**sem**	0.42	0.15	0.41

Student's t-tests	PHA data		Log_2 values		
comparison within groups					
days 5 & 10 within the ISCOMs group			**P(T<=t) two-tail**	**0.2570**	not sig.
days 5 & 10 within the SIII grp.			**P(T<=t) two-tail**	**0.0038**	sig.
comparison between groups					
day 5 for SIII & ISCOMs groups			**P(T<=t) two-tail**	**0.4406**	not sig.
day 10 for SIII & ISCOMs groups			**P(T<=t) two-tail**	**0.0043**	sig.

Treatment: SIII-BSA ISCOMs i.p p. 36

Dose	Conjug. method		ELISA anti-SIII IgG titers		
				log_2 values	
Grp1: 25 μg	**$C_3Cl_3N_3$**		**mean**	11.64	
	n= 3		**stdev**	0	
			sem	0	
Grp2: 50 μg	**$C_3Cl_3N_3$**		**mean**	11.75	
	n= 9		**stdfev**	1.69	
			sem	0.56	
Grp3: 25 μg	**M_2C_2H**		**mean**	10.62	
	n= 5		**stdev**	0.67	
			sem	0.3	
Grp4: 50 μg	**M_2C_2H**		**mean**	13.64	
	n= 8		**stdev**	1.29	
			sem	0.45	

Student's t-tests					
Comparisons between conjugation groups					
25 μg groups	M_2C_2H & $C_3Cl_3N_3$		**P(T<=t) two-tail**	**0.0341**	significant
50 μg groups	M_2C_2H & $C_3Cl_3N_3$		**P(T<=t) two-tail**	**0.0203**	significant
25 μg & 50 μg M_2C_2H groups			**P(T<=t) two-tail**	**0.020**	significant
25 μg & 50 μg $C_3Cl_3N_3$ groups			**P(T<=t) two-tail**	**0.0002**	significant

Treatment: SIII-BSA ISCOMs injected i.p pp. 38, 47, 51

PHA Data			**Anti-SIII log_2 titers**		
			day 5	**day 10**	**day 21**
M_2C_2H		**mean**	10.6	10.4	8.78
n=23		**sd**	0.59	0.72	0.72
		sem	0.42	0.15	0.41
$C_3Cl_3N_3$		**mean**	11	11	7
n=8		**sd**	0	0	0
		sem	0	0	0

			PHA Student's t-tests				
day 5 log_2 titers			**day 10 log_2 titers**			**day 21 log_2 titers**	
P(T<=t) two-tail	**0.0040**		**P(T<=t) two-tail**	**0.0005**		**P(T<=t) two-tail**	**0.0007**
	sig.			sig.			sig.

Treatment: **SIII-BSA ISCOMs injected either i.p. or s.q. on days 0 & 16 p. 49**

ELISA Data				
		Anti-SIII IgG $\log_2$ titers		
		day 5	**day 10**	**day 21**
n= 5	**s.q.**	8.64	10.24	10.24
n= 31	**i.p.**	9.05	9.9	11.92

Student's t-test					
Comparison of p values within the s.q. group					
days 5 & 10		**P(T<=t) two-tail**	**0.0349**	significant	
days 5 & 21		**P(T<=t) two-tail**	**0.0349**	significant	
days 10 & 21		the means are identical			
Comparison of p values within the i.p. group					
days 5 & 10		**P(T<=t) two-tail**	**0.0001**	sig.	
days 5 & 21		**P(T<=t) two-tail**	**2.6E-10**	sig.	
days 10 & 21		**P(T<=t) two-tail**	**9.3E-07**	sig.	
Comparison of p values between i.p. & s.q. groups					
day 5		**P(T<=t) two-tail**	**0.4938**	not sig.	
day 10		**P(T<=t) two-tail**	**0.0374**	sig.	
day 21		**P(T<=t) two-tail**	**0.0003**	sig.	

Treatment: SIII-BSA ISCOMs injected either i.p. or s.q. on days 0 & 16 p. 51

ELISA Data		Anti-SIII IgG subclasses (log_2 titers)			
Route of injection		IgG_1	IgG_{2a}	IgG_{2b}	IgG_3
	mean	9.24	8.64	8.24	6.64
s.q.	stdev	1.14	0.7	1.14	0
n= 5	sem	0.51	0.31	0.51	0
	mean	10.26	8.18	7.95	7.95
i.p.	stdev	0.65	1.56	1.6	0.55
n= 13	sem	0.18	0.43	0.44	0.15

Student's t-test				
Comparison within the i.p. group				
IgG_1 & IgG_{2a}		P(T<=t) two-tail	0.0008	significant
IgG_1 & IgG_{2b}		P(T<=t) two-tail	0.0005	significant
IgG_1 & IgG_3		P(T<=t) two-tail	1.8E-09	significant
Comparison within the s.q. group				
IgG_1 & IgG_{2a}		P(T<=t) two-tail	0.3045	sig.
IgG_1 & IgG_{2b}		P(T<=t) two-tail	0.2662	sig.
IgG_1 & IgG_3		P(T<=t) two-tail	0.00699	sig.

Treatment: **ISCOMs injected i.p. or s.q. compared to control and soluble SIII groups. p. 54**

ELISPOT Data		Anti-SIII	IgG	
	GEOMETRIC MEANS for all groups			
		SFCs/10^6	**Total # SFCs/spleen**	
control	**n**=4	**441**		**3.11E+04**
SIII 0.5 μg	**n**= 4	**281**		**2.19E+04**
ISCOMs s.q.	**n**= 5	**896**		**2.81E+05**
ISCOMs i.p.	**n**= 13	**1021**		**5.46E+05**
	Log_{10} MEANS for all groups			
		SFCs/10^6	**Total # SFC/spleen**	
control		**2.64+0.04**		**4.49+0.23**
SIII 0.5 μg		**2.45+0.13**		**4.34+1.9**
ISCOMs s.q.		**2.95+0.54**		**5.45+0.33**
ISCOMs i.p.		**3.01+0.06**		**5.74+0.23**

		Student's t-test			
	calculated from SFCs/10^6				
sol. SIII & ISCOMs i.p.			**P(T<=t) two-tail**	**0.0033**	significant
ISCOMs i.p. & ISCOMs s.q.			**P(T<=t) two-tail**	**0.8386**	not sig.
	calculated from SFCs/spleen				
sol. SIII & control group			**P(T<=t) two-tail**	**0.2953**	not sig.
sol. SIII & ISCOMs i.p.			**P(T<=t) two-tail**	**2.04E-05**	significant
ISCOMs i.p. & ISCOMs s.q.			**P(T<=t) two-tail**	**0.1226**	not sig.

Treatment: **SIII-BSA ISCOMs 25 μg injected i.p. on days 0, 21 & 60. Sera tested from days 0, 5, 9, 30 & 65. ** Another group injected on days 0 & 16, with sera tested on day 21 p. 57**

ELISA Data		Anti-SIII	IgG			
n=8		**log_2 serum titers**				
	Pre-	**day**	**day**	**day**	**day**	**day**
	treatment	**5**	**9**	**21**	**30**	**65**
mean	5.44	7.44	5.82	11.93	13.15	13.28
stdev	0.64	1.12	0.76	1.43	0.35	0
sem	1.9	0.39	0.27	0.27	0.29	0

Student's t-test					
Pre-treatment & day 5 titers			**P(T<=t) two-tail**	**0.0011**	significant
day 5 & day 9			**P(T<=t) two-tail**	**0.0004**	significant
****day 21 & 30**			**P(T<=t) two-tail**	**0.0005**	significant
day 9 & day 30			**P(T<=t) two-tail**	**1.94E-08**	significant
day 30 & day 65			**P(T<=t) two-tail**	**0.3506**	not sig.

* This table and the one below it are from the identical group. IgG subclass distribution is discussed on p. 57

ELISA Data		**log_2 titers**	Anti-SIII	
	IgG_1	**IgG_{2a}**	**IgG_{2b}**	**IgG_3**
day 30	14.61	8.64	8.64	0
day 65	14.61	9.64	9.64	0
n= 8	pooled sera			

Treatment: SIII-BSA ISCOMs administered orally to Group I (100 μg) on days 0, 6, 7, 54 & 56. Group II was fed 300 μg on days 0, 2, 28 & 30. Discussed on pp. 57, 59.

ELISA anti-SIII IgG			**Log_2**	
n= 4	**Group I**	**mean**	**stdev**	**sem**
	day 12	6.24	1.87	0.94
	day 61	9.73	2.2	1.1
	day 67	8.98	2.14	1.07
			Log_2	
n= 5	**Group II**	**mean**	**stdev**	**sem**
	day 14	5	0.71	0.32
	day 35	9.18	1.07	0.48
	(no t-tests)			

Treatment: Groups I and II as described on p. 59

ELISA anti-BSA IgG titers			log_2	
n=4	Group I	mean	stdev	sem
	day 30	12.96	0	0
n=2	Group II	mean	stdev	sem
	day 14	9.46	0.707	0.5
student's t-test		**P(T<=t) two-tail**	**0.0903**	not sig.

Treatment: **Oral immunization groups of controls (saline or no treatment), Grp. Ib (100 μg ISCOMs on days 0, 1, 14 & 15), and Grp. IIb (100 μg ISCOMs on days 0, 1, 2, 3, 14, 15, 16 & 17).**

Peyer's patch sections (between-cell distance in microns)				
		control	**grp. Ib**	**grp. IIb**
mean		2.67	1.21	1.27
# observations		n= 45	n= 45	n= 30
# mice		n= 3	n= 16	n= 2
t-tests				
control vs. grp. Ib		**P(T<=t) two-tail**	**9.13E-12**	sig.
grp. Ib vs. grp. IIb		**P(T<=t) two-tail**	**0.697**	not sig.

Peyer's patch sections (nucleus diameter in microns)					
		control	**grp. Ib**	**grp. IIb**	
mean		4.9	6.32	6	
# observations		n= 45	n= 45	n= 30	
t-tests					
control vs. grp. Ib		**P(T<=t) two-tail**	**3.1E-08**	sig.	
grp. Ib vs. grp. IIb		**P(T<=t) two-tail**	**0.391**	not sig.	

These oral groups with Peyer's patch data are discussed on pp. 59, 60