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Advances in Potential M-protein Peptide-Based Vaccines for Preventing Rheumatic Fever and Rheumatic Heart Disease

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Abstract

Rheumatic fever (RF) and rheumatic heart disease (RHD) are post-infectious complications of an infection (or repeated infection) with the Gram-positive bacterium, *Streptococcus pyogenes* (also known as group A streptococcus, GAS). RF and RHD are global problems and affect many indigenous populations of developed countries and many developing countries. However, RF and RHD are only part of a larger spectrum of diseases caused by this organism. The development of a vaccine against GAS has primarily targeted the abundant cell-surface protein called the M-protein. This review focuses on different M-protein–based–subunit vaccine approaches and the different delivery technologies used to administer these vaccine candidates in preclinical studies.

Key Words

Peptide
Vaccine
Streptococcus
Lipids
Conjugation
Antimicrobials

Introduction

Vaccination is one of the most successful and cost-effective methods for the control of infectious diseases that have afflicted humankind throughout history. It is currently an exciting era in vaccination, as there has been a significant reduction in major childhood diseases such as measles, diphtheria, and pertussis in most industrialized countries through the successful implementation of routine vaccination programs. In addition, although many developing countries now have ongoing vaccination programs for some of these diseases, further progress is required to achieve the goal of disease eradication in these countries.

One of the key highlights in the history of vaccination was the official announcement of

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the eradication of smallpox in 1980, with the last case of naturally acquired smallpox occurring in Somalia in 1977. This success can be attributed to several favorable key factors including the availability of a heat-stable vaccine, which protected with a single dose. The World Health Organisation (WHO) is now focusing on other diseases such as polio and measles. For example, the WHO Global Polio Eradication Initiative's strategic plan is to certify global eradication of poliomyelitis by the end of 2008 *(1)*. The success of implementing vaccines and possible eradication programs is influenced by many factors such as public education, compliance, vaccine distribution, and training of health care worker. However, the availability of a safe, stable, efficacious vaccine is crucial.

Traditional vaccines have been based on a whole organism, which has been either attenuated (such as the Sabin oral polio and MMR [measles, mumps, and rubella] vaccines) or inactivated (killed) such as the Salk polio and influenza vaccines. The advantages of inactivated vaccines is that the risk of infection is low and the antigens are presented in a near-natural conformation. However, owing to the inability of the organism to replicate, high amounts of the organism and boosters must be administered (for example, the Salk polio vaccine). In contrast attenuated vaccines may be administered at lower doses (for example, Sabin oral polio and MMR vaccine); however, attenuated vaccines may revert (rarely) to their virulent form and cause disease. The rapid advancements in molecular biology, genomics, and proteomics has led to the development of new types of vaccines which overcome the drawbacks of these traditional vaccines.

Recombinant vaccines are those in which genes that encode the desired antigens are expressed either by a vector (e.g., virus) and used as a vaccine, or the protein product is purified and subsequently used as a subunit vaccine. The major advantage of recombinant vaccines is that the specific antigen can be produced in large quantities at low cost. If the desired antigen requires post-translational modification, the choice of expression systems is vital as prokaryotes such as *Escherichia coli* have little ability for post-translational modification of proteins compared to eukaryotes. The only recombinant vaccine currently licensed and administered in humans is for hepatitis B virus. This vaccine consists of a purified hepatitis B surface protein that is expressed in yeast. Expanding on this concept, DNA vaccines are in development where DNA is directly injected into the host and relies on host cells to translate and express the protein of interest to generate an immune response.

Subunit vaccines typically contain a purified antigen(s) derived from the host organism such as *Bordetella pertussis* antigens in the acellular DPT vaccine or the purified surface antigen in hepatitis B vaccine. The advantages of subunit vaccines include safety (as they are non-infectious) and the fact that specific immune responses can be targeted. However, the disadvantages of subunit vaccines are that it may be difficult to express the antigens in their native conformation and they may be poorly immunogenic. Similar to the subunit vaccines are the conjugate vaccines, the production of which involves conjugation of either carbohydrates or peptides to carrier proteins, which enhance the immune response by acting as a source of T cell help. Examples of such vaccines are the pneumococcal conjugate vaccines (PCV), which contains purified capsular polysaccharides of many types of pneumococcal bacteria conjugated to a carrier protein, and the Hib (*Haemophilus influenzae* type b) vaccine, which contains Hib-polysaccharide, conjugated to a protein carrier, such as diphtheria toxoid, tetanus toxoid, or meningococcal outer membrane protein. Alternate methods such as peptide–lipid conjugations

have been used to promote the desired immune responses. Here we review the progress toward the development of peptides and their derivatives as potential subunit vaccine candidates for controlling rheumatic fever and rheumatic heart disease.

Synthetic peptide vaccines have numerous advantages over classical vaccines including safety, relatively economical production, and easy handling and storage. Solid-phase stepwise synthesis of peptides was first described by Merrifield *(2)*. This process involved the stepwise addition of *t*-butoxycarbonyl (*t*-Boc) protected amino acids to the peptide, which is attached to an insoluble polystyrene support. The temporary protective group is acid labile and removed by trifluoroacetic acid to allow the subsequent addition of another amino acid. Since the publication of this original method, optimization of this process has been attempted by introducing alternate protecting chemistries such as 9-fluorenylmethoxycarbonyl (Fmoc) *(3)*. Recent developments in solid-phase peptide synthesis have previously been reviewed *(4)*.

While peptides vaccines are totally synthetic and have many advantages over traditional vaccines, they do not readily stimulate T cells. Because of their small size, they behave like haptens and require coupling to a protein carrier that is recognized by T cells. It is now known that synthetic peptide-based vaccines could be highly immunogenic provided they contain, in addition to the B cell epitope, T cell epitopes recognized by T helper cells. Such a T cell epitope can be provided by carrier protein molecules, foreign antigens, or within the synthetic peptide molecule itself. In recent years, there have been many developments in peptide vaccines and preclinical studies for a number of infectious diseases caused by a variety of pathogens including bacteria and viruses.

The interaction of the host's immune system with the vaccine and the mounting of an effective immune response is the key to a successful vaccination. This interaction relies on two different types of immune responses, which follow natural infection or vaccination:

1. A cell-mediated response (T cell, type 1 response).
2. Humoral response (B cell, type 2 like response).

The cell-mediated response is believed to control intracellular bacterial and viral pathogens. The primary effector cells of a type 1–like response are CD8+ cytotoxic T cells. In contrast, the humoral immune response preferentially controls circulating pathogens through antibodies that subsequently activate various mechanisms of pathogen clearance. CD4+ T helper cells (Th cells) are involved in both type 1 – and type 2–like responses.

CD8+ cytotoxic T cells can kill cells that express specific antigens. The action of these CD8+ T cells involves the recognition of proteins, particularly intracellular proteins, that have been processed and presented as peptide fragments by class I MHC molecules. In contrast, CD4+ T cell activation requires the recognition of antigenic proteins that are extracellular and have been processed and presented as peptide fragments by class II MHC on antigen-presenting cells. The mechanism of delivery (vector, polypeptide) can greatly influence the type of T cells activated and the subsequent immune response. A number of different strategies have been developed for peptide delivery, such as conjugation to carrier peptides or lipids or the creation of large peptide polymers.

Group A *Streptococcus* (GAS) and Disease Burden

Infection with group A *Streptococcus* (GAS) can result in a number of clinical man-

Table 1. Selected Major GAS-Associated Disease Manifestations

Non-invasive diseases	Invasive diseases	Post-infectious diseases (sequelae)
Pharyngitis	Septicemia	Rheumatic fever (RF)
Pyoderma	Necrotizing fasciitis (NF)	Rheumatic heart disease (RHD)
Impetigo	Meningitis	Post-streptococcal gomerulonephritis (PSGN)
Tonsillitis	Pneumonia	
Scarlet fever	Cellulitis	
Otitis media	Erysipelas	
	Streptococcal toxic shock syndrome (STSS)	

ifestations ranging from the relatively benign and self-limiting pharyngitis to invasive diseases such as necrotizing fasciitis (Table 1). It has been estimated that in the US, acute pharyngitis accounted for 11 million doctor visits in the year 2000, thus imparting a huge burden on the US health care system *(5).* GAS is the most common bacterial cause of acute pharyngitis and is believed to be responsible for approx 15–30% of acute pharyngitis cases in children *(6,7)* and about 10% of cases in adults *(8).* In contrast, the Centers for Disease Control and Prevention (US) estimates that all invasive GAS diseases account for approx 10,000 cases per annum in the US, resulting in approx 1350 deaths. However, the mortality rate resulting from GAS invasive disease varies depending upon the disease manifestation. For example, the mortality rate is approximately 20% for necrotizing fasciitis and approximately 45% for streptococcal toxic shock syndrome *(7).* While these diseases have a huge economic burden for developing and developed nations, of more global concern are the post-infectious diseases, rheumatic fever (RF) and rheumatic heart disease (RHD).

Walter Cheadle published one of the first complete descriptions of RF in 1889 in the *Lancet* describing the "various manifestations of the rheumatic state." Cheadle and other physicians observed an association between tonsillitis and RF; however, it was believed at that time to be a manifestation of RF and not the cause. Interestingly, joint manifestations associated with the disease RF were first described as early as the 1600s. Moreover, the physician Guillaume Baillou is believed to be the first to use the term "rheumatism" in his treatise, which was published in 1642, more than 20 yr after his death *(9).*

Over the following centuries the five major manifestations of RF were described including joint symptoms (polyarthritis), carditis, chorea, erythema marginatum, and subcutaneous nodules (Fig. 1). However, it was not until 1931 that a single etiology was proposed for RF when the American physician Alvin Coburn and the British physician William Collis working independently both postulated that a hemolytic variety of *Streptococcus* caused RF. At approximately the same time Dr. Rebecca Lancefield was working on the serological typing of hemolytic *Streptococcus.*

Fig. 1. Timeline of selected key events in defining group A streptococcus associated diseases including rheumatic fever *(9,30,31,39,77).* Timeline is not to scale.

~ 400 BC
Hippocrates mentions migratory arthritis, possible RF?

Early 1600s AD
Guillaume Baillou uses the term "rheumatism".

1675
Thomas Sydenham distinguishes scarlatina from measles.

1676
Thomas Sydenham describes polyarthritis.

1686
Thomas Sydenham describes St Vitus Dance (Chorea).

1788
David Pitcairn associates heart disease with rheumatism.

1788
Edward Jenner independently associates heart disease with rheumatism.

1805
Haygarth relates rheumatism to sore throat.

1810
Charles Wells notes nodules and rash (erythema marginatum) in connection with cases of acute rheumatism and rheumatism of the heart.

1861
Trousseau describes "rheumatic sore throat".

1874
Billroth describes the bacteria streptococcus.

1879
Pasteur discovers streptococcus in the blood of a patient dying of puerperal sepsis.

1880
J Kingston Fowler notes lag period between tonsillitis and rheumatism

1881
Barlow and Warner document the connection of nodules and erythema marginatum with acute rhe umatism.

1883
Frederick Fehleisen isolates the streptococcus bacteria.

1884
Rosenbach names *Streptococcus pyogenes.*

1888
Walter Cheadle gives a comprehensive description of the 5 clinical manifestations of rheumatism (ie rheumatic fever). Also associates tonsillitis with rheumatism.

1903
Hugo Schottmuller distinguishes haemolytic from non-haemolytic streptococcus on blood agar plates.

1906
Gabritschewsky produces a vaccine from streptococc us isolated from scarlet fever patients. Vaccine administered to children in Europe.

1928
Rebecca Lancefield types haemolytic streptococcus using antisera that recognises the M-protein.

1931
Alvin Coburn defines single etiology for rheumatic fever (*Streptococcus pyogenes*).

1931
William Collis independently defines single etiology for rheumatic fever (*Streptococcus pyogenes*).

1944
T. Duckett Jones defines guidelines (Jones Criteria) for diagnosis of rheumatic fever.

1945
Plumber et al. treat haemolytic streptococcal throat infection with penicillin.

1946
Young et al. administers heat-killed streptococcal vaccine to US Armed Forces personnel (clinical trial).

1960
Willard Smith administers partially purified M-protein vaccine to a limited number of adults and children (clinical trial).

1980s
Resurgence in GAS virulence and associated diseases in developed countries

1988
Bessen and Fischetti investigate conserved region peptides from the M-protein as potential vaccine candidates.

1997
Minimal B-cell epitope of the conserved region of the M-protein is defined by Hayman et al.

2001
Ferretti et al. publishes M1 GAS genome.

2004
Recombinant subunit vaccine of multiple amino-terminal epitopes of the M-protein used in a phase I clinical trial.

Extensive research has been conducted in determining the mechanism by which GAS causes RF/RHD. It is now accepted that RF/RHD is an autoimmune disease in which T cells and possibly antibodies induced by GAS are thought to play a role in disease pathogenesis by inducing immune responses that cross-react with human tissues *(10).* In order to mitigate against possible autoimmune complications, subunit vaccines, as opposed to whole organism approaches, have been the focus of GAS vaccine development.

RF/RHD are still a major problem in many developing nations and many indigenous populations of developed nations *(11).* For example, Indigenous Australians in the Northern Territory of Australia have one of the highest documented incidence rates (651/100,000) of RF worldwide *(12).* In the World Health Organization (WHO) World Health Report 2000, it was estimated that global RHD mortality in 1999 was approx 376,000 deaths with the majority of these in the WHO regions of South-East Asia and the Western Pacific *(13).*

A recent review of the global burden of streptococcal diseases *(14,15)* estimated that currently there are approx 15.6 million existing cases of RHD plus 460,000 new RHD cases and 349,000 RHD-related deaths each year. Moreover, it was also estimated that there are approx 663,000 new cases of invasive GAS disease each year resulting in 163,000 deaths. The majority of these RHD and invasive GAS disease cases occur in less developed countries. The global burden of non-invasive GAS-associated diseases such as pyoderma and pharyngitis was also estimated at 111 million current cases of pyoderma and 616 million new cases of pharyngitis each year *(14,15).* These data taken together with the dynamic epidemiology of GAS in combination with episodic resurgence of GAS virulence highlight the need for a prophylactic vaccine.

Human clinical trials of vaccines to prevent RF were conducted even before a single etiology *(Streptococcus pyogenes)* was clearly defined for the disease in 1931. For example, in 1906, a vaccine was produced from streptococcal bacteria isolated from scarlet fever patients. This vaccine was subsequently administered to children in Europe as discussed in refs. *9* and *16.* However, once *S. pyogenes* was identified as the organism responsible for RF and RHD, vaccine research focused on the M-protein. During the 1960s and 1970s multiple studies were conducted using purified M-protein or M-protein derivates in adults *(17,18)* and children *(19,20)* with mixed results. However, several studies reported adverse side effects *(21,22).* In one study, in a high-risk population, 2 of 21 subjects developed RF following vaccination with M-protein *(23);* however, it could not be determined whether this was due to the subjects previous exposures to GAS or the vaccine. Moreover, the purity of the vaccine preparation used in this study has been questioned *(24).* In contrast, between 1964 and 1978 Fox et al. administered purified M-protein to a total of 144 individuals and during these clinical trials no cases of RF were reported (reviewed in refs. *9* and *24*).

Vaccine Strategies to Prevent Group A Streptococcal Infection

Several streptococcal target antigens have been investigated as potential vaccines to prevent GAS infection and its associated diseases. Antigens of interest include SCPA *(25),* MtsA/GRAB *(26),* and the cell-wall carbohydrate *(27).* While these antigens hold promise as potential vaccine candidates, the focus of this review will be on the M-protein *(28)*–derived peptide vaccine strategies.

Two regions of the abundant cell-surface virulence factor called the M-protein (Fig. 2)

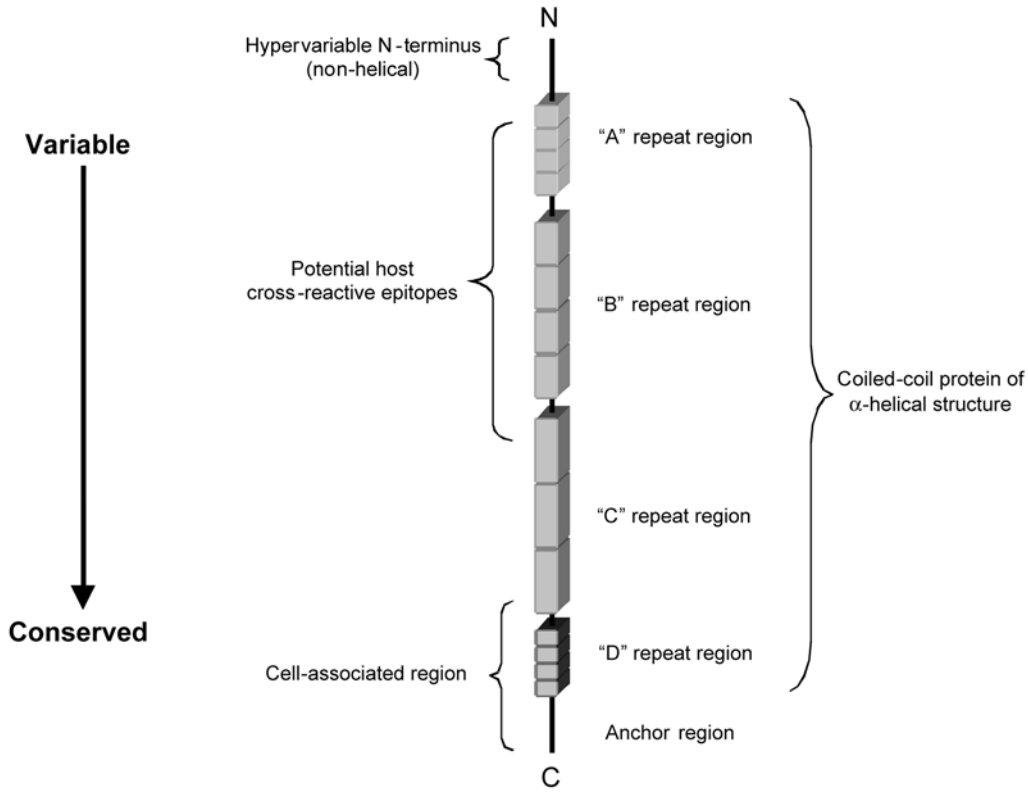

Fig. 2. Diagrammatic representation of the M-protein, which is major cell surface protein of GAS and has been the focus of vaccine development.

have been the target of vaccine development, the hypervariable N-terminal region *(29,30)* and the conserved C-terminal region *(31)*. Vaccines based on both of these regions have demonstrated protective potential in animal models. Expanding on this, peptide vaccines based on the M-protein are a practical approach in developing a GAS vaccine because potential host-cross reactive epitopes within the M-protein that may be responsible for the clinical conditions RF and RHD can be eliminated from a peptide vaccine as opposed to the whole cell or whole protein based vaccines.

The hypervariable N-terminal region of the M-protein has been shown to be highly immunogenic and induces a strain specific immunity. Therefore, several diferent approaches have been used to combine different N-terminal epitopes together as one construct (Table 2). Substantial progress has been made with a recombinant multivalent protein that consists of multiple N-terminal epitopes of the M-protein from different GAS strains (emm types). Initial studies utilized a hexavalent construct *(30);* however, more recently the number of epitopes has been expanded to increase the vaccine coverage for predominant GAS strains found in the USA *(29)*. In a recent study, this 26-valent vaccine candidate was found to induce antibodies that could opsonize not only the specific M-types represented in the vaccine but also subtype variants which can have small differences in amino acid identity

Table 2. Summary of Selected Preclinical Studies of M-Protein–Derived Peptide Vaccine Candidates for GAS

Study/vaccine candidate/strategy	Vaccine type	Preclinical data/route/mechanism	Reference(s)
N-terminal based vaccine strategies			
Hexavalent or 26-valent recombinant	Multivalent recombinant protein vaccine of N-terminal regions	Intramuscular in humans/via broadly protective opsonic antibodies	*30,32*
N-terminal peptides linked to tetanus toxoid (TT)	Peptide-protein Conjugate	Subcutaneous in mice/via opsonic antibodies	*46*
N-terminal peptides linked via lipid core peptide (LCP)	Lipopeptides IgG and opsonization	Subcutaneous in mice/via peptide specific serum	*72*
Heteropolymer (seven N-terminal and one C-terminal peptides)	Peptide polymer	Subcutaneous in mice/via systemic IgG and opsonic antibodies	*48*
C-terminal (conserved region) based vaccine strategies			
Conserved region peptides linked to CTB	Peptide–Protein conjugate	Intranasal in mice/via blocking attachment of bacteria to the mucosal epithelium via secretory IgA	*31*
Conserved region peptides linked to KLH	Peptide–protein conjugate	Intranasal in mice/via serum IgG	*36*
Conserved region peptide-specific mixed with GAS	Passive transfer (peptide-specific secretory IgA)	Intranasal in mice/via inhibiting binding of bacteria to mucosal epithelium	*34*
J8-DT	Peptide–protein conjugate	Subcutaneous in mice/via serum IgG and opsonic antibodies	*42*
J8-LCP	Lipopeptides	Subcutaneous in mice/via peptide specific serum IgG	*71*
J14/proteosomes	Peptide–protein vesicle	Intranasal/via serum IgG and mucosal IgA	*43*
Conserved region proteins expressed in *Lactococcus lactis*	Recombinant protein via live vector vaccine	Intranasal in mice/mucosal IgA and serum IgG-IgA in controlling the infection at the point of infection	*76*

indicating that the 26-valent vaccine could opsonize subtype variants that may arise in a highly immunized population *(32)*. These studies also demonstrated that the 26-valent vaccine candidate was well tolerated when administered in phase I and phase II human clinical trials *(33)*.

In contrast to the hypervariable N-terminal region of the M-protein, the C-terminal portion is highly conserved between GAS strains and therefore a possible vaccine candidate (Table 2) that may protect against multiple GAS strains. Bessen and Fischetti previously demonstrated that antisera against three peptides representing the conserved region of the M-protein derived from an M6 GAS isolate could passively protect when mixed with GAS and subsequently administered intranasally to mice *(34)*. A primary route of GAS infection in humans is via colonization of the mucosal epithelium of the pharynx. Colonization followed by tissue invasion can lead to local suppurative complications or systemic infections *(10)*. On mucosal surfaces such as the mucosal epithelium, immunoglobulin A (IgA) is one of the primary defense mechanisms of the host to prevent bacterial infection and inhibit bacteria binding to these cells *(35)*. Expanding on this concept, the three conserved region peptides were conjugated to cholera toxin B subunit (CTB) and administered intranasally in mice. These vaccinated mice had significantly reduced pharyngeal colonization following intranasal GAS challenge compared to the control mice *(31)*. In addition, studies by Bronze et al. utilized two synthetic peptides from the conserved region of the M-protein (M type 5) for intranasal immunization of mice. Approximately 65% of immunized mice were protected from death following mucosal GAS challenge. Protection was also afforded against heterologous serotypes of GAS *(36)*.

We have previously defined a peptide corresponding to the conserved C-repeat region of M-protein (peptide p145) that elicits an opsonic *(37,38)* and protective antibody response. In order to identify the minimal protective B cell epitope and eliminate potentially host cross-reactive T cell epitopes from peptide 145, a series of overlapping 12mer peptides were made. However, these short 12mer peptides were not recognized by p145 antisera possibly due to a loss of their conformation *(39)*. In order to maintain an alpha-helical coiled-coil structure similar to that of the whole M-protein, coil–coil promoting moieties from the yeast GCN4 DNA-binding protein were added to both sides of the 12mer insert (Fig. 3). Three of these chimeric peptides (J7, J8, J9) were recognized by p145 antisera *(39)*. Moreover, antisera raised to these peptides were capable of in vitro opsonization of GAS *(39)*. We also defined a slightly larger chimeric peptide representing J7, J8, and J9 [referred to as J14 (Fig. 3)]. This peptide epitope was also conformationally constrained and did not contain any potentially deleterious T cell epitopes from the M-protein *(38–40)*. More recently, we have demonstrated that J14 and J8 can induce a protective immune response in mice against both intraperitoneal and intranasal GAS challenge *(41–43)*.

Human salivary IgA specific for the parent conserved region peptide, p145, is able to opsonize heterologous strains of GAS *(44)*. In early human clinical trials using the M-protein *(45)*, patients who were immunized intranasally with M-protein had both reduced throat colonization and clinical illness compared with patients vaccinated systemically indicating the importance of a local immune response in protection. Taken together, these data strongly suggest that one of the preferred routes of immunization to prevent GAS infection is intranasal administration; however, a

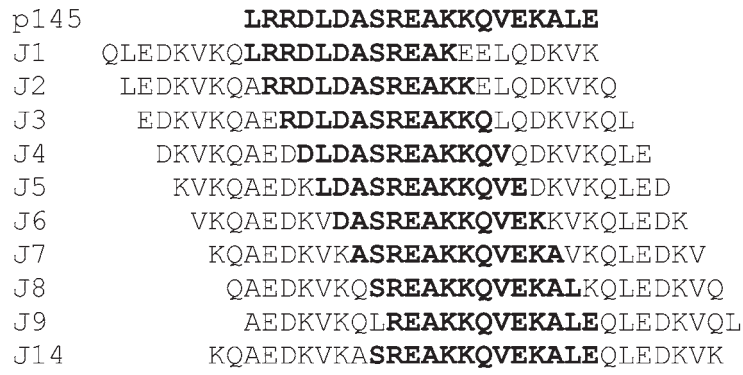

Fig. 3. Overlapping peptides (including GCN4 flanking sequence) used to map the minimal B cell epitope within peptide p145, which was derived from the conserved region of the M-protein. Amino acids from the M-protein are shown in bold. Non-bold amino acids are flanking region sequence derived from the DNA binding protein, GCN4, designed to promote the correct helical folding.

lack of suitable human mucosal adjuvants limits the potential of this approach.

Conjugates

Peptide–protein conjugates have been extensively used to enhance the immune responses of peptide haptens in outbred populations. As previously discussed three conserved region peptides from the M-protein were conjugated to CTB before being administered intranasally to mice *(31)*. Expanding on this concept peptides that represent the N-terminal hypervariable region and sub-N-terminal regions of the M-protein from numerous clinical isolates of GAS from the Northern Territory of Australia have been synthesized *(46)*. These peptides ranging in size from 19 to 33 amino acids have been conjugated to either tetanus toxoid (TT) or diphtheria toxoid (DT) and formulated with complete Fruend's adjuvant (CFA) and administered subcutaneously to mice. Peptide-specific antisera were capable of opsonizing the homologous GAS strain in vitro *(46)*. However of more interest was the ability of selected peptide-antisera to cross-opsonize heterologous GAS strains, indicating that some N-terminal peptides and in particular selected sub-N-terminal epitopes may protect against more than a single GAS serotype.

The minimal B cell peptide epitopes (Fig. 3) from the conserved region of the M-protein (J8 and J14) were immunologically non-responsive in most outbred mice but were found to be responsive in inbred mice of the H-2^k haplotype *(39)*. To overcome this immunological non-responsiveness the peptides were conjugated to DT. These peptide–DT constructs when administered with CFA were found to be highly immunogenic in both inbred and outbred mice *(42)*. Moreover, peptide antisera were capable of in vitro opsonization of multiple GAS strains representing different M-types. Intranasal immunization of B10.BR mice with this conjugated peptide (J8/J14-DT) using CTB as a mucosal adjuvant has also been shown to reduce throat colonization following intranasal GAS challenge *(47)*.

In the search for a human compatible formulation, the conserved region peptide conjugated to DT was formulated with alum and subcutaneously administered to mice *(42)*. Peptide-specific antibodies were induced and bound the surface of a panel of GAS isolates. These antisera also opsonized multiple GAS

strains in vitro, similar to that observed when the peptide conjugate was administered with CFA. In addition, the peptide conjugate also induced significant protection in both inbred and outbred mice following intraperitoneal GAS challenge with both a reference GAS isolate, M6, and a non-typable clinical isolate from the Northern Territory of Australia (88/30) *(42)*.

Polymers (Heteropolymers)

A novel approach to combining multiple peptides into a single immunogen was the polymerization of multiple peptides to form an immunogen larger than 400 kDa in size. These polymers, termed heteropolymers, consisted of seven different peptides derived from the N-terminus of the M-protein of different *S. pyogenes* strains and a single conserved region peptide from the M-protein *(48)*. The seven N-terminal peptides were selected for inclusion into the heteropolymer because these peptides were derived from the M-protein of the seven most prevalent clinical isolates of GAS circulating in the Northern Territory of Australia *(48)*. The conserved region peptide was included due to its broad specificity and its ability to protect against multiple GAS strains in the murine model *(39)*.

To create the polymer, individual peptides representing portions of the M-protein were assembled using fluorenylmethoxycarbonyl chemistry, and a residue of Lys (4-methyltrityl) was inserted at the C-terminus *(49)*. After removal of the N-terminal fluorenylmethoxycarbonyl group, the exposed N-terminal amino group was acetylated and the 4-methyltrityl group of the C-terminal lysine was then removed. The amino group exposed at the C-terminal lysine was then derivitized with acryloyl chloride prior to the polymerization of individual peptides.

When administered subcutaneously with CFA, these large polymers induced strong systemic serum IgG immune responses in both inbred B10.BR (H-2^k) and outbred Quackenbush mice. Sera collected from these mice were capable of in vitro opsonization of homologous and heterologous GAS strains, i.e., GAS strains represented on the heteropolymer by an N-terminal peptide epitope and those represented by only the conserved region peptide, respectively *(48)*. Mice immunized with the heteropolymer had a significantly larger number of survivors following lethal systemic GAS challenge compared with control groups administered with PBS in adjuvant. Protection was not only afforded to GAS strains represented by the N-terminal peptide in the polymer (homologous strains) but also against heterologous strains where protection was attributed to the conserved region peptide *(48)*.

Lipopeptides (Including Pam2Cys and LCPs)

Bacterial lipoproteins contain the unusual amino acid S-glycerylcysteine, which is acylated by three fatty acids *(50)*. These lipoproteins are found in the outer membrane of Gram-negative and selected Gram-positive bacteria. Through genomic sequencing the number of putative lipoproteins identified vary between bacterial species. It is predicted that *Borrelia burgdorferi* contains 105 putative lipoproteins or more than 8% of its genome, while only 20 lipoproteins were identified from the *Helicobacter pylori* genome. The outer membrane lipoprotein OspA from *B. burgdorferi* has induced protective immunity in mice following oral administration *(51)*. Furthermore, this lipoprotein has also been used in human vaccine trials *(52)*. Further investigation demonstrated that the lipoproteins (from different organisms) were capable of stimulating murine B cell growth *(53),* activating the nuclear factor kappa-B *(54)* and stimulating cytokine production *(55)*.

In the original work, a series of N-terminal–derived synthetic analogs of lipoproteins were synthesized and found to be highly immunogenic *(56).* The N-terminal lipid region of the lipoproteins was then combined with peptides to form lipopeptides. Lipopeptides have been administered successfully both subcutaneously and intranasally and were capable of inducing both arms of the immune system, i.e., both cellular and antibody responses. The synthetic lipopeptides were shown to be as active as native lipoproteins in terms of activation of B cells *(56),* monocytes *(57),* and other immune cells *(58,59).* The adjuvant-like properties of lipopeptides such as Pam3Cys, which is based on the lipid palmitoyl, have extensively been studied. Vaccination of both guinea pigs and cattle with B and T cell peptide epitopes from the foot and mouth disease virus has induced long lasting protection *(60).*

While lipopolysaccharide (LPS) activates dendritic cells (DC) through TLR4, it has been demonstrated that Gram-positive cell-wall components (such as peptidoglycan and lipoteichoic acid) and mycobacterial cell-wall components (such as lipoarabinomannan and mycolylarabinogalactan) activate cells by a different receptor (TLR2). It has recently been reported that TLR4 and TLR2 activate human DC through different mechanisms with TLR4 inducing a Th1-type response in contrast to TLR2, which induced Th2-type responses *(61).* The immunostimulatory properties of bacterial lipoproteins for TLR2 have been attributed to the presence of a lipoylated N-terminus therefore suggesting that N-terminal derivatives of the lipoproteins (such as Pam3Cys or Pam2Cys) may interact with TLR2. Moreover, recent studies have demonstrated that infection with either Gram-positive bacteria or fungi induced Toll-dependent expression of the antimicrobial peptide, drosomycin, highlighting the importance of peptides in the innate immune system *(62).*

Recent work has demonstrated that Pam2Cys does interact with TLR2 *(63).* This synthetic N-terminal derivative has been synthesized with a number of different B cell and T cell epitopes such as luteinizing hormone–releasing hormone *(64)* and has potential as both carrier and adjuvant for a peptide vaccine against GAS.

Expanding on this observation and utilizing multiple antigenic peptides (MAPs) technology to build branched peptide structures *(65)* Toth et al. developed a new chemistry that allowed the development of lipid core peptides (LCP), which combine MAPs physically linked to lipid core structures of different compositions *(66–69).* The LCPs are designed to have both carrier and adjuvant properties for the incorporated antigen. Utilizing this technology Hayman et al. *(70)* developed LCP constructs with the p145 peptide from the conserved region of the M-protein. These LCP–p145 constructs consisted of an oligomeric polylysine core with multiple copies of the peptide p145 conjugated to a series of lipoamino acids, which act as an anchor for the antigen.

Seven different LCP constructs based on the p145 peptide sequence were synthesized and the immunogenicity of the compounds was examined *(70).* It was demonstrated that the number of lipoamino acids and the spacing between the alkyl side chains in the constructs affected the immunogenicity. Moreover, the most immunogenic constructs contained the longest alkyl side chains *(70).* Selected constructs also induced antibodies that had the same fine specificity as those found in endemic human serum. These antibodies were capable of in vitro opsonization of GAS therefore demonstrating the potential of LCPs as a vaccine delivery technology.

Olive et al. further demonstrated the potential of this technology by using the J8 con-

served region peptide epitope alone *(71)* or in combination with up to three different peptides derived from the N-terminus of the M-protein *(72)* in immunogenicity and protection studies. The immunogenicity of these LCP constructs varied depending on their peptide composition but constructs were capable of inducing antigen-specific serum IgG and broadly protective opsonic antibodies, when administered subcutaneously with and without additional adjuvant to inbred B10.BR (H-2^k) mice. LCP constructs were also immunogenic and induced antigen-specific serum IgG in outbred Quackenbush mice (unpublished data). Furthermore, parenteral immunization of mice with LCP constructs was shown to induce protection from lethal systemic GAS infection *(73)*. Together, these data highlight the potential of the LCPs for use in the development of a GAS vaccine.

Protein Vesicles ("Proteosomes")

The J14 peptide has also been administered in conjunction with protein vesicles termed proteosomes *(43)*. These proteosomes can be made from the outer membrane proteins (OMP) of a variety of bacterial species including meningococci and other *Neisseria* species *(74)*. To promote non-covalent complexing of J14 to proteosomes a hydrophobic anchor consisting of a fatty acid lauroyl chain was added to the epsilon-amino moiety of the lysine near the J14 carboxyl terminus. Intranasal administration of the J14/proteosome formulation without additional adjuvant to outbred mice led to high titers of J14-specific serum IgG and mucosal IgA antibodies. Following intranasal GAS challenge, these immunized mice demonstrated increased survival and reduced GAS colonization of the throat compared to mice administered proteosomes alone *(43)*.

The hydrophobic nature of the proteosome OMP is believed to help in antigen uptake by antigen-presenting cells and macrophages. While OMPs are known to be B cell mitogens and polyclonal activators in mice and humans, only recently has the major component of proteosomes, neisserial porins, been shown to be critical to TLR2 and MyD88 activation *(75)*. Therefore, proteosome OMPs can fulfill the roles of both carrier protein and adjuvant for a range of haptens *(74)* including the J14 peptide, which was previously shown to be immunologically non-responsive in most outbred mice.

Expression of Vaccine Candidates by Live Commensal Organisms

Expanding on the principle of a vaccine based on the conserved region of the M-protein, several studies have investigated the potential of live commensal organisms as vectors to mucosally deliver recombinant protein vaccines. Mannam et al. have recently developed a genetically modified non-pathogenic *Lactococcus lactis* strain that expresses a protein derived from the conserved region of M-protein on its surface *(76)*. Intranasally vaccinated mice developed both mucosal IgA and serum IgG specific for the conserved region antigen, whereas mice vaccinated subcutaneously only developed conserved region-specific serum IgG *(76)*. In addition, intranasally vaccinated mice were protected from intranasal GAS challenge as opposed to mice vaccinated subcutaneously, which were not protected, suggesting the importance of IgA in controlling GAS infection at the point of infection. Mannam et al. also found that cohorts of mice that did not produce conserved region-specific IgG but had significant levels of mucosal IgA, survived following intranasal GAS challenge suggesting that antigen-specific IgA contributed to survival even in the absence of significant antigen-specific serum IgG *(76)*.

Conclusions

Several different approaches have been used in the attempts to develop a vaccine that will prevent GAS infection and its associated diseases including RF and RHD. A subunit peptide vaccine is a logical approach in developing a GAS vaccine based on the M-protein because this abundant cell-surface protein contains host cross-reactive epitopes that may be responsible for initiating RF and RHD. A subunit vaccine that utilizes defined regions of the M-protein that does not contain potentially detrimental epitopes is a preferred vaccine for this organism.

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References

1. WHO: Global Polio Eradication Initiative : Strategic Plan 2004–2008. World Health Organization, Geneva, 2003.
2. Merrifield RB: Solid phase peptide synthesis I: The synthesis of a tetrapeptide. J Am Chem Soc 1963;85: 2149–2153.
3. Carpino LA, Han GY: The 9-fluorenylmethoxycarbonyl amino-protecting group. J Org Chem 1972;37:3404–3409.
4. Fields GB, Nobel RL: Solid phase peptide synthesis utilizing 9- fluorenylmethoxycarbonyl amino acids. Intern J Pep Prot Res 1990;35:161–214.
5. Cherry DK, Woodwell DA: National Ambulatory Medical Care Survey: 2000 summary. Adv Data 2002;1–32.
6. Bisno AL: Acute pharyngitis. N Engl J Med 2001; 344:205–211.
7. Bisno AL, Rubin FA, Cleary PP, Dale JB: Prospects for a group A streptococcal vaccine: rationale, feasibility, and obstacles—report of a National Institute of Allergy and Infectious Diseases Workshop. Clin Infect Dis 2005;41:1150–1156.
8. Komaroff AL, Pass TM, Aronson MD, et al: The prediction of streptococcal pharyngitis in adults. J Gen Intern Med 1986;1:1–7.
9. Massell BF: Rheumatic fever and streptococal infection. Harvard University Press, Boston, 1997.
10. Cunningham MW: Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 2000;13:470–511.
11. Steer AC, Carapetis JR, Nolan TM, Shann F: Systematic review of rheumatic heart disease prevalence in children in developing countries: the role of environmental factors. J Paediatr Child Health 2002;38:229–234.
12. Carapetis JR, Wolff DR, Currie BJ: Acute rheumatic fever and rheumatic heart disease in the top end of Australia's Northern Territory. Med J Aust 1996;164: 146–149.
13. World Health Organization: World Health Report. Office of Publications, World Health Organization, Geneva, 2000, pp. 164–169.
14. Carapetis J: A review of WHO activities in, the burden of, and the evidence for strategies to control group A streptococcal diseases: part 3—the current evidence for the burden of group A streptococcal diseases. University of Melbourne, Melbourne, 2004, pp. 1–49.
15. Carapetis JR, Steer AC, Mulholland EK, Weber M: The global burden of group A streptococcal diseases. Lancet Infect Dis 2005;5:685–694.
16. Thomson D, Thomson R: The role of the *Streptococci* in scarlet fever. Annals of the Pickett-Thomson Research Laboratory 1930;4:244–252.
17. Beachey EH, Stollerman GH, Johnson RH, Ofek I, Bisno AL: Human immune response to immunization with a structurally defined polypeptide fragment of streptococcal M protein. J Exp Med 1979;150: 862–877.
18. Fox EN, Wittner MK, Dorfman A: Antigenicity of the M proteins of group A hemolytic streptococci. 3. Antibody responses and cutaneous hypersensitivity in humans. J Exp Med 1966;124:1135–1151.
19. Massell BF, Michael JG, Amezcua J, Siner M: Secondary and apparent primary antibody responses after group A streptococcal vaccination of 21 children. Appl Microbiol 1968;16:509–518.

20. Fox EN, Pachman LM, Wittner MK, Dorfman A: Primary immunization of infants and children with group A streptococcal M protein. J Infect Dis 1969;120: 598–604.
21. Lyampert IM, Danilova TA, Borodyuk NA, Beletskaya LV: Mechanism of formation of antibodies to heart tissue in immunization with group A streptococci. Folia Biol (Praha) 1966;12:108–115.
22. Beachey EH, Stollerman GH: The common antigen(s) of streptococcal M protein vaccines causing hyperimmune reactions in man. Trans Assoc Am Physicians 1972; 85:212–221.
23. Massell BF, Honikman LH, Amezcua J: Rheumatic fever following streptococcal vaccination. Report of three cases. JAMA 1969;207:1115–1119.
24. Fox EN: M proteins of group A streptococci. Bacteriol Rev 1974;38:57–86.
25. Park HS, Cleary PP: Active and passive intranasal immunizations with streptococcal surface protein c5a peptidase prevent infection of murine nasal mucosa-associated lymphoid tissue, a functional homologue of human tonsils. Infect Immun 2005;73:7878–7886.
26. McMillan DJ, Batzloff MR, Browning CL, et al: Identification and assessment of new vaccine candidates for group A streptococcal infections. Vaccine 2004;22: 2783–2790.
27. Zabriskie JB, Poon-King T, Blake MS, Michon F, Yoshinaga M: Phagocytic, serological, and protective properties of streptococcal group A carbohydrate antibodies. Adv Exp Med Biol 1997;418:917–919.
28. Fischetti VA, Jones KF, Hollingshead SK, Scott JR: Structure, function, and genetics of streptococcal M protein. Rev Infect Dis 1988;10(Suppl 2):S356–359.
29. Hu MC, Walls MA, Stroop SD, Reddish MA, Beall B, Dale JB: Immunogenicity of a 26-valent group A streptococcal vaccine. Infect Immun 2002;70:2171–2177.
30. Kotloff KL, Corretti M, Palmer K, et al: Safety and immunogenicity of a recombinant multivalent group a streptococcal vaccine in healthy adults: phase 1 trial. JAMA 2004;292:709–715.
31. Bessen D, Fischetti VA: Influence of intranasal immunization with synthetic peptides corresponding to conserved epitopes of M protein on mucosal colonization by group A streptococci. Infect Immun 1988;56:2666–2672.
32. Dale JB, Penfound T, Chiang EY, Long V, Shulman ST, Beall B: Multivalent group A streptococcal vaccine elicits bactericidal antibodies against variant M subtypes. Clin Diagn Lab Immunol 2005;12:833–836.
33. McNeil SA, Halperin SA, Langley JM, et al: Safety and immunogenicity of 26-valent group a streptococcus vaccine in healthy adult volunteers. Clin Infect Dis 2005;41:1114–1122.
34. Bessen D, Fischetti VA: Passive acquired mucosal immunity to group A streptococci by secretory immunoglobulin A. J Exp Med 1988;167:1945–1950.
35. Brandtzaeg P: Role of secretory antibodies in the defence against infections. Int J Med Microbiol 2003;293:3–15.
36. Bronze MS, Courtney HS, Dale JB: Epitopes of group-a streptococcal M-protein that evoke cross-protective local immune-responses. J Immunol 1992;148:888–893.
37. Pruksakorn S, Currie B, Brandt E, et al: Towards a vaccine for rheumatic fever: identification of a conserved target epitope on M protein of group A streptococci. Lancet 1994;344:639–642.
38. Pruksakorn S, Galbraith A, Houghten RA, Good MF: Conserved T and B cell epitopes on the M protein of group A streptococci. Induction of bactericidal antibodies. J Immunol 1992;149:2729–2735.
39. Hayman WA, Brandt ER, Relf WA, Cooper J, Saul A, Good MF: Mapping the minimal murine T cell and B cell epitopes within a peptide vaccine candidate from the conserved region of the M protein of group A streptococcus. Int Immunol 1997;9:1723–1733.
40. Brandt ER, Hayman WA, Currie B, et al: Opsonic human antibodies from an endemic population specific for a conserved epitope on the M protein of group A streptococci. Immunology 1996;89:331–337.
41. Batzloff M, Yan H, Davies M, Hartas J, Good M: Preclinical evaluation of a vaccine based on conserved region of M protein that prevents group A streptococcal infection. Indian J Med Res 2004;119(Suppl):104–107.
42. Batzloff MR, Hayman WA, Davies MR, et al: Protection against group A streptococcus by immunization with J8-diphtheria toxoid: contribution of J8- and diphtheria toxoid-specific antibodies to protection. J Infect Dis 2003;187:1598–1608.
43. Batzloff MR, Yan H, Davies MR, et al: Toward the development of an antidisease, transmission-blocking intranasal vaccine for group a streptococcus. J Infect Dis 2005;192:1450–1455.
44. Brandt ER, Hayman WA, Currie B, et al: Functional analysis of IgA antibodies specific for a conserved epitope within the M protein of group A streptococci from Australian Aboriginal endemic communities. Int Immunol 1999;11:569–576.
45. D'Alessandri R, Plotkin G, Kluge RM, et al: Protective studies with group A streptococcal M protein vaccine. III. Challenge of volunteers after systemic or intranasal immunization with Type 3 or Type 12 group A Streptococcus. J Infect Dis 1978;138:712–718.
46. Brandt ER, Teh T, Relf WA, Hobb RI, Good MF: Protective and nonprotective epitopes from amino termini of M proteins from Australian aboriginal isolates and reference strains of group A streptococci. Infect Immun 2000;68:6587–6594.
47. Olive C, Clair T, Yarwood P, Good MF: Protection of mice from group A streptococcal infection by intranasal immunization with a peptide vaccine that contains a conserved M protein B cell epitope and lacks a T cell autoepitope. Vaccine 2002;20:2816–2825.
48. Bran5dt ER, Sriprakash KS, Hobb RI, et al: New multideterminant strategy for a group A streptococcal vaccine designed for the Australian Aboriginal population. Nat Med 2000;6:455–459.
49. Jackson DC, O'Brien-Simpson N, Ede NJ, Brown LE: Free radical induced polymerization of synthetic peptides into polymeric immunogens. Vaccine 1997; 15:1697–1705.
50. Hantke K, Braun V: Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal

end of the murein- lipoprotein of the Escherichia coli outer membrane. Eur J Biochem 1973;34:284–296.
51. Luke CJ, Huebner RC, Kasmiersky V, Barbour AG: Oral delivery of purified lipoprotein OspA protects mice from systemic infection with Borrelia burgdorferi. Vaccine 1997;15:739–746.
52. Keller D, Koster FT, Marks DH, Hosbach P, Erdile LF, Mays JP: Safety and immunogenicity of a recombinant outer surface protein A Lyme vaccine. JAMA 1994; 271:1764–1768.
53. Melchers F, Braun V, Galanos C: The lipoprotein of the outer membrane of Escherichia coli: a B-lymphocyte mitogen. Exp Med 1975;142:473–482.
54. Norgard MV, Arndt LL, Akins DR, Curetty LL, Harrich DA, Radolf JD: Activation of human monocytic cells by *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides proceeds via a pathway distinct from that of lipopolysaccharide but involves the transcriptional activator NF-[kappa]B. Infect Immun 1996;64:3845–3852.
55. Kreutz M, Ackermann U, Hauschildt S, et al: A comparative analysis of cytokine production and tolerance induction by bacterial lipopeptides, lipopolysaccharides and staphyloccocus aureus in human monocytes. Immunology 1997;92:396–401.
56. Wiesmuller KH, Bessler W, Jung G: Synthesis of the mitogenic S-[2,3-bis(palmitoyloxy)propyl]-N-palmitoylpentapeptide from *Escherichia coli* lipoprotein. Hoppe Seyler Physiol Chem 1983;364:593–606.
57. Hoffmann P, Heinle S, Schade UF, et al: Stimulation of human and murine adherent cells by bacterial lipoprotein and synthetic lipopeptide analogues. Immunobiology 1988;177:158–170.
58. Seifert R, Schultz G, Richter-Freund M, et al: Activation of superoxide formation and lysozyme release in human neutrophils by the synthetic lipopeptide Pam3Cys-Ser-(Lys)4. Involvement of guanine-nucleotide-binding proteins and synergism with chemotactic peptides. Biochem J 1990;267:795–802.
59. Berg M, Offermanns S, Seifert R, Schultz G: Synthetic lipopeptide Pam3CysSer(Lys)4 is an effective activator of human platelets. Am J Physiol 1994;266:C1684–C1691.
60. Wiesmuller K-H, Jung G, Hess G: Novel low-molecular-weight synthetic vaccine against foot-and-mouth disease containing a potent B-cell and macrophage activator. Vaccine 1989;7:29–33.
61. Re F, Strominger J-L: Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem 2001;276:37692–37699.
62. Takeda K, Kaisho T, Akira S: Toll-like receptors. Annu Rev Immunol 2003;21:335–376.
63. Jackson DC, Lau YF, Le T, et al: A totally synthetic vaccine of generic structure that targets Toll-like receptor 2 on dendritic cells and promotes antibody or cytotoxic T cell responses. Proc Natl Acad Sci USA 2004; 101:15440–15445.
64. Zeng W, Ghosh S, Lau YF, Brown LE, Jackson DC: Highly immunogenic and totally synthetic lipopeptides as self-adjuvanting immunocontraceptive vaccines. J Immunol 2002;169:4905–4912.
65. Tam JP: Synthetic peptide vaccine design: synthesis and properties of a high–density multiple antigenic peptide system. Proc Natl Acad Sci USA 1988;85:5409–5413.
66. Olive C, Batzloff MR, Toth I: Lipid core peptide technology and group A streptococcal vaccine delivery. Expert Rev Vaccines 2004;3:43–58.
67. McGeary RP, Olive C, Toth I: Lipid and carbohydrate based adjuvant/carriers in immunology. J Pept Sci 2003;9:405–418.
68. Olive C, Toth I, Jackson D: Technological advances in antigen delivery and synthetic peptide vaccine developmental strategies. Mini Rev Med Chem 2001;1:429–438.
69. Wong A, Toth I: Lipid, sugar and liposaccharide based delivery systems. Curr Med Chem 2001;8:1123–1136.
70. Hayman WA, Toth I, Flinn N, Scanlon M, Good MF: Enhancing the immunogenicity and modulating the fine epitope recognition of antisera to a helical group A streptococcal peptide vaccine candidate from the M protein using lipid-core peptide technology. Immunol Cell Biol 2002;80:178–187.
71. Olive C, Batzloff MR, Horvath A, et al: A lipid core peptide construct containing a conserved region determinant of the group A streptococcal M protein elicits heterologous opsonic antibodies. Infect Immun 2002;70: 2734–2738.
72. Olive C, Batzloff M, Horvath A, et al: Potential of lipid core peptide technology as a novel self-adjuvanting vaccine delivery system for multiple different synthetic peptide immunogens. Infect Immun 2003;71:2373–2383.
73. Olive C, Hsien K, Horvath A, et al: Protection against group A streptococcal infection by vaccination with self-adjuvanting lipid core M protein peptides. Vaccine 2005;23:2298–2303.
74. Lowell G, Burt DS, White GL, Fries LF: Proteosome™ technology for vaccines and adjuvants; in: Levine MM, Kaper JB, Rappuoli R, Liu MA, Good MF (eds). New Generation Vaccines, 3rd ed. Marcel Dekker, New York, 2004; pp. 271–282.
75. Massari P, Henneke P, Ho Y, Latz E, Golenbock DT, Wetzler LM: Cutting edge: immune stimulation by neisserial porins is toll-like receptor 2 and MyD88 dependent. J Immunol 2002;168:1533–1537.
76. Mannam P, Jones KF, Geller BL: Mucosal vaccine made from live, recombinant Lactococcus lactis protects mice against pharyngeal infection with *Streptococcus pyogenes.* Infect Immun 2004;72:3444–3450.
77. Ferretti JJ, McShan WM, Ajdic D, et al: Complete genome sequence of an M1 strain of Streptococcus pyogenes. Proc Natl Acad Sci USA 2001;98:4658–4663.