

Toward selective elicitation of T_H1-controlled vaccination responses: vaccine applications of bacterial surface layer proteins

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Abstract

Bacterial surface layer proteins have been utilized as combined vaccine carrier/adjuvants and offer a number of advantages in these applications. The crystalline protein arrays contain functional groups in precisely defined orientations for coupling of haptens. Conventional applications of S-layer vaccines do not cause observable trauma or side effects. Depending on the nature of the S-layer preparations, antigenic conjugates will induce immune responses of a predominantly cellular or predominantly humoral nature. Immune responses to S-layer-hapten conjugates are also observed following oral/nasal application. In the present contribution, the status of investigations with S-layer conjugates in three main immunological projects is reviewed. In a project aimed at immunotherapy of cancer, conjugates of S-layer with small, tumor-associated oligosaccharides have been found to elicit hapten-specific DTH responses. An enlarged program of chemical synthesis has now been initiated to prepare a complete set of mucin-derived, tumor-associated oligosaccharides and their chemically modified analogues for elicitation of cell-mediated immune responses to certain tumors in humans. In another application, oligosaccharides derived from capsules of *Streptococcus pneumoniae* type 8 have been linked to S-layer proteins and have been found to elicit protective antibody responses in animals. Most recently, allergen-S-layer conjugates have been prepared with the intention to suppress the T_H2-directed, IgE-mediated allergic responses to Bet v1, the major allergen of birch pollen. In the former two applications, the S-layer vaccine technology appears to offer the versatility needed to direct vaccination responses toward predominant control by T_H1 or T_H2 lymphocytes to meet the different therapeutic or prophylactic requirements in each case. In the third application, work has progressed to a preliminary stage only.

Keywords: S-layer; Vaccine; Immunotherapy; Tumor-associated antigen; *Streptococcus pneumoniae*; Birch pollen allergen; T-Helper subset

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1. Introduction

1.1. Crystalline bacterial cell surface layers (S-layers)

Bacterial S-layers have been successfully applied in a number of technical areas including the production of isoporous ultrafiltration membranes (Sára et al., 1993a), affinity chromatography (Sára et al., 1993b), production of Langmuir-Blodgett films (Pum and Sleytr, 1994) and as carrier/adjuvants for vaccine development (Messner et al., 1992). Together with progress in basic exploration, the status of these applications has been recently reviewed (Messner and Sleytr, 1992; Sleytr et al., 1993). Presently, we wish to present a progress report concerning three projects wherein S-layers have been utilized as carrier/adjuvants for vaccination and immunotherapy. These projects have reached various degrees of maturity; no applications to humans have as yet been performed.

2. Applications of S-layer technology for vaccination and immunotherapy

2.1. Induction of T-cell immunity to tumor-associated oligosaccharide antigens immobilized on S-layers

The identification of certain mucin-associated oligosaccharides as tumor-associated antigens

prompted investigations aimed at utilization of these determinants as target haptens for immunotherapy (MacLean et al., 1992, 1993). While oligosaccharides per se are too small to effectively engage the immune system, their attachment to carrier proteins results in immunization antigens that are both sufficiently large and capable of involving macrophages and lymphocytes (Jennings, 1983; Schneerson et al., 1987). Using S-layer carrier/adjuvants, it was found that the quantities of haptenated S-layer preparations required to prime and challenge mice for a strong, hapten-specific DTH response (assayed by determination of footpad swelling) to small carbohydrate haptens are not larger than those needed to generate similar DTH responses to complex protein antigens such as measles or herpes simplex type 1 virus (Smith et al., 1993; Smith and Ziola, 1986).

Effective immunotherapeutic responses against tumor-associated antigens are generally assumed to be dependent upon cell-mediated immunity, consisting of CD4⁺ T-cells that mediate DTH, CD8⁺ cytotoxic lymphocytes, and macrophages among other types of cells. Following the investigations of Mosmann and his associates, such cellular responses are mediated primarily by a T-helper cell subset referred to as T_H1 (Mosmann and Coffman, 1989). Conversely, such immune responses that depend upon the effector capabilities of antibodies (humoral immune responses) are mediated primarily by the T_H2 subset. When T-disaccharide (βGal1 → 3αGalNAc) and the

Table 1

Specificity of the DTH response to carbohydrate antigens following immunization with carbohydrate haptenated S-layers (after Smith et al., 1993)

Immunization antigen ^a	Challenge antigen	Footpad swelling (mm ⁻¹)
T-3a 10 μg per mouse	PBS	0.8 ± 0.8 ^b
T-3a 10 μg per mouse	EDC, L111 10 μg per mouse ^c	0.4 ± 0.4
T-3a 10 μg per mouse	T-L111 10 μg per mouse	3.8 ± 0.8 ^d
T-3a 10 μg per mouse	Y-L111 10 μg per mouse	1.2 ± 0.8
Y-3a 10 μg per mouse	Y-L111 10 μg per mouse	3.2 ± 0.8 ^d
Y-3a 10 μg per mouse	T-L111 10 μg per mouse	1.1 ± 0.3
PBS and DDA	T-L111 10 μg per mouse	1.9 ± 0.6
NT ^e	T-L111 10 μg per mouse	1.3 ± 0.4

^a CP-pretreated mice were immunized with antigen containing 100 μg DDA (dimethyl dioctadecyl ammonium bromide).

^b Mean footpad swelling ± standard deviation.

^c L111 was sham treated with EDC to control for the possible modification of L111 by the EDC treatment.

^d Significantly different from all other groups (*P* < 0.001).

^e Not treated.

Lewis-Y (Le^Y) tetrasaccharide ($\alpha\text{Fuc1} \rightarrow 2\beta\text{Gal1} \rightarrow 4(\alpha\text{Fuc1} \rightarrow 3)\beta\text{GlcNAc}$) were used as haptens on cross-linked S-layer preparations, the following features of the respective immune response were observed. (i) The response was purely a DTH response with practically no antibody observed. (ii) Responses were hapten-specific; following priming with T-haptenated S-layer, only baseline responses were measured when animals were challenged with Y-haptenated S-layer, and vice versa (Table 1). (iii) Strong DTH responses were obtained only in animals that had been pre-treated with cyclophosphamide. (iv) The results of the following adoptive transfer experiment indicate that T-helper cells are involved in mediating the observed DTH responses. Groups of mice were pretreated with cyclophosphamide and were then immunized with $10\text{ }\mu\text{g}$ of a preparation of T-disaccharide bound to S-layer from *Bacillus stearothermophilus* NRS 2004/3a (T-3a). Seven days later, the mice were killed, draining lymph nodes and spleens removed, lymphocytes purified and cultured with non-haptenated S-layers from *Thermoanaerobacter* (*Clostridium*) *thermohydrosul-*

furicus L111-69 (as controls) or with T-disaccharide-L111 S-layer conjugate (T-L111). Four days later, all cells were washed with PBS, and the cells stimulated with T-L111 were divided into four groups. One group was sham treated with PBS, the others, incubated with monoclonal antibodies L3T4, Ly2.2 or Thy1.2. These antibodies were at the time considered directed against markers specific for helper T-cells, suppressor T-cells, and all T-cells (in that order). Incubation of the cell preparations in the presence of the respective antibody and complement was therefore expected to deplete them of T-helper, T-suppressor, or of all T-cells (Smith et al., 1993). Following the depletion step, the cells were mixed with either PBS (as control) or with the T-disaccharide-S-layer conjugate with the S-layer from *Bacillus alvei* CCM 2051 (T-2051) and injected into naive mice. When T-3a-primed cells stimulated with T-L111 were depleted with the monoclonals L3T4 or Thy1.2 and then mixed with T-2051, these cells did not transfer the potential to generate a DTH response. However, strong DTH responses were observed in mice adoptively transferred with T-3a-

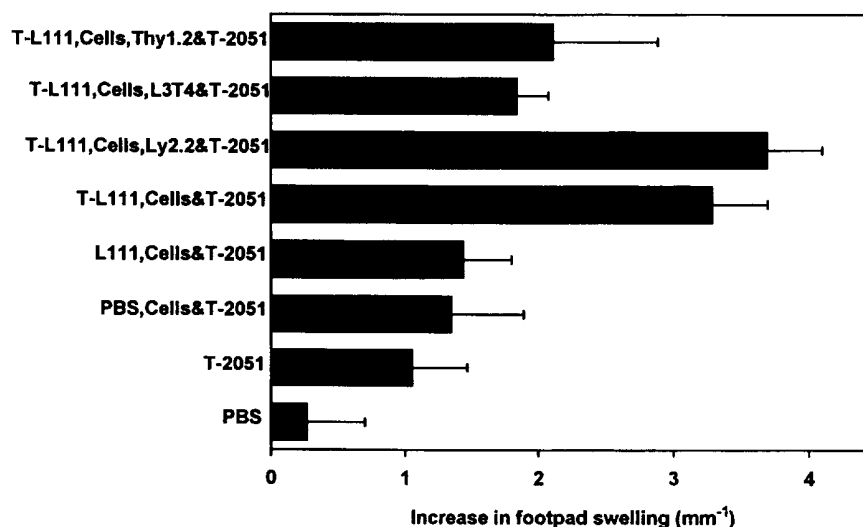


Fig. 1. Transfer of T-disaccharide DTH response. Groups of mice were pretreated with cyclophosphamide, then immunized with $10\text{ }\mu\text{g}$ T-disaccharide-3a-S-layer conjugate (T-3a). Seven days later, the mice were killed, draining lymph nodes and spleens were collected, lymphocytes purified and cultured with S-layers from *T. thermohydrosulfuricus* (L111), or T-disaccharide-L111-S-layer conjugate (T-L111). Four days later, the stimulated cells were washed with PBS. The cells which had been stimulated with T-L111 were divided into four groups and sham treated (PBS) or treated with monoclonal antibodies, L3T4, Ly2.2 or Thy1.2. These cells were then incubated with complement, washed and counted. The primed cells (5×10^5) were then mixed with either PBS or T-disaccharide-2051 conjugate (T-2051), and injected into naive mice. Footpad swelling (mm^{-1}) was measured 24 h later (after Smith et al., 1993).

primed cells stimulated in vitro with T-L111 and mixed with T-2051. Primed lymphocytes which had been bulk-stimulated with the non-haptenated S-layer L111 alone, then transferred with T-2051, did not enable a DTH response beyond the level of control cells. Moreover, cells treated with antibody Ly2.2 (for depletion of T suppressor cells) also enabled a full DTH response (Fig. 1).

Strong DTH responses were also observed in mice primed by oral/nasal application of T-L111 conjugates. As seen in Fig. 2, such responses are comparable to those achieved following intramuscular immunization.

Presently, a program of chemical synthesis has been initiated to prepare a complete set of mucin-derived, tumor-associated oligosaccharides. These and their chemically modified analogs will be attached to S-layers and examined for their ability to induce a predominantly T_H1 -controlled immune response against tumor-associated mucins in humans.

2.2. Elicitation of immunoprotective cellular and antibody responses against *Streptococcus pneumoniae* type 8 using conjugates of type 8-oligosaccharide and the S-layer from *Bacillus alvei* CCM 2051

Successful vaccination against *S. pneumoniae* requires elicitation of protective antibody titers specific for the capsular polysaccharides of these pathogenic organisms. To date, 83 serotypes have been reported

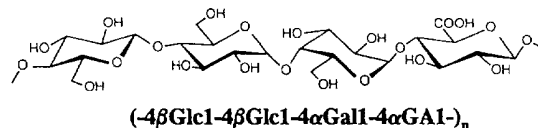


Fig. 3. Structure of *Streptococcus pneumoniae* serotype 8 capsular polysaccharide.

(Eskola and Käyhty, 1995). Thus, a typical anti-pneumococcal vaccine contains capsular polysaccharide material from 23 serotypes of *S. pneumoniae* (for a review see Shapiro, 1991). While many of the capsular polysaccharides elicit protective immunity in healthy adult vaccinees, several of them elicit poor responses in infants or immune-compromised patients. To overcome these difficulties, *S. pneumoniae* type 8 was chosen as a model target (Malcolm et al., 1993). Type 8 polysaccharide material, which is used in the commercially available pneumonia vaccine, did not elicit bactericidal antibody responses in mice in our hands (for the structure of the capsular polysaccharide, see Fig. 3). Therefore, oligosaccharides (1–8 repeat units) derived from type 8 capsular polysaccharide by controlled, acid-catalyzed hydrolysis were coupled to non-crosslinked S-layer from *B. alvei* CCM 2051 and injected into mice. Conjugates comprising relatively small oligosaccharide haptens elicited good antibody responses as determined by EIA (Fig. 4). Serum from the mice thus immunized had immunoprotective properties as re-

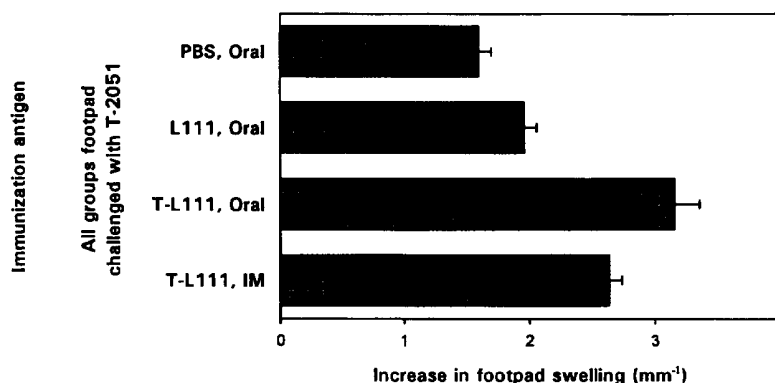


Fig. 2. Comparison of oral and intramuscular immunization with T-S-layer conjugates. Groups of mice were sham immunized with 10 μ g of a preparation of T-L111 either orally or intramuscularly. Seven days after immunization each group of mice were footpad challenged with 10 μ g of a preparation of T-2051. Footpad swelling (mm⁻¹) was measured 24 h later (after Smith et al., 1993).

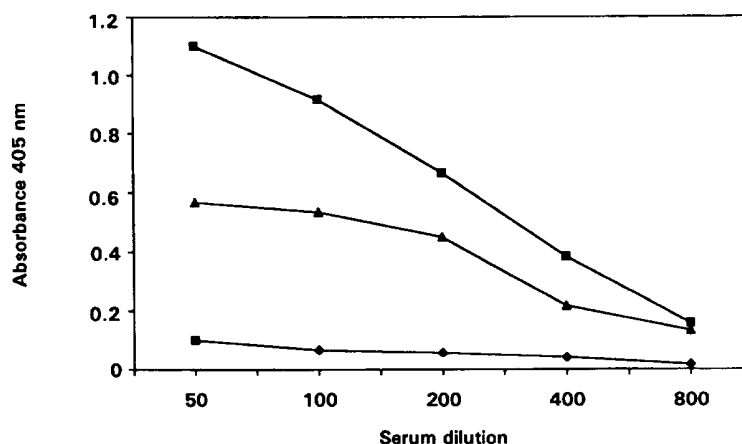


Fig. 4. Tertiary antibody response of pneumococcal S-layer conjugates. IgA, IgG and IgM response of Balb/c mice injected with either *S. pneumoniae* serotype 8 oligosaccharide-S-layer 2051 conjugate (●), serotype 8 polysaccharide (■) or with two injections of polysaccharide followed by a single injection of the type 8-2051 conjugate (▲) (after Malcolm et al., 1993).

vealed in a serum killing assay, as follows (Table 2). On blood agar plates, the mouse sera reduced *S. pneumoniae* serotype 8 colony forming units by 99%. No reduction was observed with sera from mice that had been immunized with type 8 capsular polysaccharide. However, injection of conjugates into mice that had previously been immunized with capsular polysaccharide produced sera capable of reduc-

ing colony forming units by 65%. In rabbits, results were similar, except for the finding that unconjugated type 8-oligosaccharide alone elicits a level of IgG response somewhat below that of the oligosaccharide-2051 conjugates (Table 2).

The type 8-oligosaccharide-2051-conjugates also elicited DTH in mice, as shown by the following experiment. Mice were primed intramuscularly with

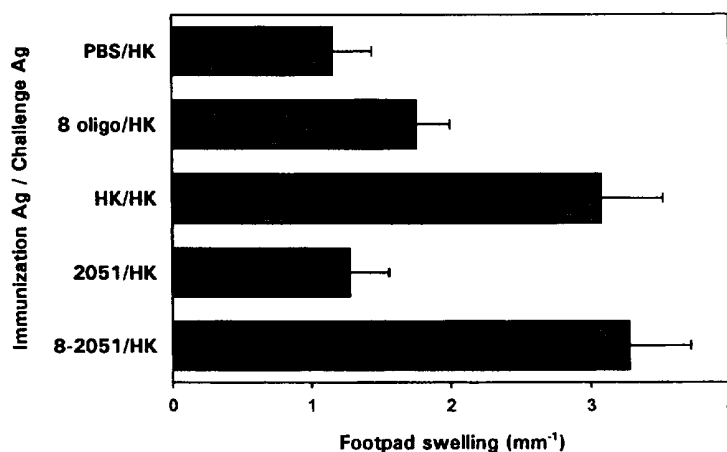


Fig. 5. Cellular immune response of *S. pneumoniae* serotype 8 oligosaccharide-S-layer 2051 conjugate as measured by DTH reaction in Balb/c mice. One week after intramuscular immunization of *S. pneumoniae* type 8 oligosaccharide alone or coupled to 2051 S-layer, or with heat-killed (HK) *S. pneumoniae*, 2051 alone or PBS as controls, all mice were footpad challenged with heat-killed *S. pneumoniae* type 8. The delayed type inflammatory reaction at the challenge site was measured 24 h later (after Malcolm et al., 1993).

Table 2
Bactericidal assay ^a

Immunogen	% Reduction
1. Oligosaccharide conjugate/polysaccharide immunizations	
a. 8-2051/8-2051/8-2051	99
b. 8 poly/8 poly/8 poly	0
c. 8 poly/8 poly/8-2051	65
d. PBS	0
2. Rabbit immunizations	
a. 8-2051	88
b. 8 oligo	49
c. 8 poly	55
d. NRS	0

^a The ability of mouse and rabbit antisera to inhibit growth of *S. pneumoniae* serotype 8 as measured by the blood bactericidal assay. Three sets of antisera of different immunization experiments were mixed with heparinized naive mouse blood containing live *S. pneumoniae*. After an incubation of 1 h at 37°C, samples were plated onto blood agar. Results were expressed as percent reduction of colony forming units when compared with PBS control serum or normal rabbit serum (NRS; after Malcolm et al., 1993).

type 8-oligosaccharide-2051 conjugate, the oligosaccharide alone, or with heat-killed, *S. pneumoniae* serotype 8. Unhaptentated S-layer or phosphate-buffered saline were used as vaccination controls. One week later, all mice were footpad-challenged with heat-killed *S. pneumoniae* type 8. The results (Fig. 5) indicate that the conjugates are as effective in priming for a DTH response as the heat-killed bacteria, whereas the oligosaccharide alone is a considerably less effective primer.

Presently, specialized composite vaccines are being designed for high-risk target groups such as infants or immune-compromised patients. Such vaccines will contain S-layer-oligosaccharide conjugates admixed with purified capsular polysaccharides. S-layer oligosaccharide conjugates will be developed for those target organisms whose capsular polysaccharides afford poor vaccination responses in the respective target population.

2.3. Attempts to use S-layer conjugates for suppression of an IgE response to Bet v 1, the major allergen of birch pollen

Results of immunization experiments with S-layer conjugates have indicated that immune responses in

animals can be modulated toward a T_H1- or a T_H2-directed response mode through the choice and construction of the respective S-layer conjugates. Thus, in the case of small tumor-associated oligosaccharides, cross-linked S-layers effectively induced DTH (presumably a T_H1-directed response) whereas with the *S. pneumoniae* type 8 polysaccharide, non-cross-linked S-layers were more effective in eliciting an immunoprotective antibody response. Presently, different types of Bet v 1-S-layer conjugates are being studied to determine whether they are capable of converting a (T_H2-directed) IgE antibody response into a T_H1-mediated response against Bet v 1. In allergic individuals, the activation of allergen-specific T-cell clones leads to a higher ratio of produced IL-4 vs. interferon- γ (Ebner et al., 1995). This results in a T_H2-directed immune response which is responsible for enhanced IgE production. If Bet v 1-S-layer conjugates were capable of effecting the switch from a T_H2-directed into a T_H1-directed immune response, this would indicate the potential of such conjugates to suppress the manifestations of allergy in patients susceptible to pollen allergies.

These studies have only recently been initiated and have so far resulted in the reproducible preparation of Bet v 1-S-layer conjugates. The allergen-S-layer conjugates are capable of eliciting antibodies in mice and of stimulating human T-cell clones specific for certain Bet v 1 epitopes. Results of experiments directed at IgE suppression by this route will be reported in due course.

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