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Relationship between the strength of antigen adsorption to an aluminum-containing adjuvant and the immune response

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Abstract

Adsorption of the antigen to an aluminum-containing adjuvant is considered an important aspect of vaccine formulation. Adsorption is described by two parameters: the maximum amount that can be adsorbed as a monolayer, which is characterized by the adsorptive capacity and the strength of the adsorption force, which is described by the adsorptive coefficient. Research to date has focused on the adsorptive capacity with the goal of complete adsorption of the antigen. In this study, the relationship between the adsorptive coefficient and immunopotentiation was investigated. Four vaccines were prepared in which the adsorptive coefficient was varied by altering the number of phosphate groups on the antigen (alpha casein and dephosphorylated alpha casein) or the number of surface hydroxyls on the adjuvant (aluminum hydroxide adjuvant and phosphate-treated aluminum hydroxide adjuvant). In vitro elution upon exposure to interstitial fluid or normal human plasma was inversely related to the adsorptive coefficient. The geometric mean antibody titer in mice was also inversely related to the adsorptive coefficient. T-cell activation was not observed in mice that received the vaccine with the greatest adsorptive coefficient (alpha casein/aluminum hydroxide adjuvant). This suggests that antigen processing and presentation to T-cells is impaired when the antigen is adsorbed too strongly.

Keywords: Antigen adsorption; Aluminum hydroxide adjuvant; Adsorptive coefficient; Strength of adsorption; Ligand exchange; T-cell activation; Elution

1. Introduction

Aluminum-containing adjuvants are used in vaccine formulations to enhance the antibody response. In 1926, Glenny et al. [1] laid the groundwork for the traditional view that adsorption of antigen to aluminum-containing adjuvants prior to administration is essential for the enhancement of immunogenicity. They observed that injecting alum-precipitated diphtheria toxoid led to a significant increase in the immune response. Furthermore, when the precipitate was filtered, the filtrate was devoid of the toxoid. This observation led to the conclusion that the antigen must be adsorbed to the aluminum-containing adjuvant. Thus, a goal of vaccine formulation is to maximize the adsorption of the antigen. Antigen adsorption to aluminum-containing adjuvants occurs by two principle mechanisms. Electrostatic attraction is the most prevalent and occurs when the adjuvant and antigen have opposite charges [2]. Ligand exchange occurs when an antigen contains a phosphate group (e.g. DNA, phosphorylated antigens, phospholipid-bound antigens, and PRP-containing antigens) that can displace a hydroxyl group on the adjuvant surface to form an inner-sphere surface complex with aluminum that is the inorganic equivalent of a covalent bond [3]. Ligand exchange is the strongest adsorption force and can occur even when an electrostatic repulsive force is present [4].

Two parameters are important when considering adsorption: the maximum amount that can be adsorbed as a monolayer, which is characterized by the adsorptive capacity and the strength of the adsorption force, which is characterized by the adsorptive coefficient [5]. The effect of adsorptive capacity on the immune response has been studied. These studies suggest that the percentage of the antigen dose that is adsorbed in the vaccine is not related to immunogenicity.

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For example, equivalent immunopotentiation was observed for three lysozyme vaccines in which the degree of adsorption was 3, 35 or 85% [6]. The degree of adsorption of each vaccine changed to 40% when the vaccines were mixed with the sheep interstitial fluid in vitro. The study concluded that immunopotentiation was not related to the degree of adsorption in the vaccine formulation but was correlated to the degree of adsorption following administration. Another study [7] showed that aluminum phosphate adjuvant potentiated the immune response to alpha casein, ovalbumin or lysozyme when the antigen was not adsorbed in the vaccine formulation nor when mixed in vitro with interstitial fluid. The authors hypothesized that the antigens, even though not adsorbed, were trapped in void spaces within the adjuvant aggregates, resulting in uptake of antigen by dendritic cells. The adsorption of *Bacillus anthracis* recombinant protective antigen by aluminum phosphate adjuvant was not required for immunopotentiation [8].

The strength of adsorption (adsorptive coefficient) by ligand exchange is related to both the number of phosphate groups on the antigen [9], and the number of surface hydroxyl groups on the aluminum-containing adjuvant [10]. No reports were found in the literature of the relationship between the adsorptive coefficient of the antigen to an aluminum-containing adjuvant and the immune response. Thus, a study was undertaken using alpha casein (CAS), which contains eight phosphate groups and dephosphorylated alpha casein (DP-CAS) with two phosphate groups [11]. The two adjuvants used in the study were aluminum hydroxide adjuvant (AH) in which all of the surface groups were hydroxyls, and phosphate-treated aluminum hydroxide adjuvant (PT-AH) which contained a mixture of hydroxyl and phosphate groups on the surface. Four vaccines were prepared by combining these two antigens and two adjuvants. The vaccine composed of CAS and AH had the greatest potential for adsorption by ligand exchange and the vaccine composed of DP-CAS and PT-AH had the least potential for adsorption by ligand exchange. The immunogenicity of the four vaccines was tested in mice.

2. Materials and methods

2.1. Materials

Alpha casein, DP-CAS, Incomplete Freund's adjuvant (IFA), MOPS [3-(*N*-morpholino) propanesulfonic acid], MOPS sodium salt, were ACS grade or better, and were used as supplied (Sigma, St. Louis, MO). The bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) was used to measure the concentration of antigen in vitro. The aluminum hydroxide adjuvant (Rehydragel HPA, Reheis, Berkeley Heights, NJ) was an aqueous suspension containing 2.2% (w/w) equivalent Al_2O_3. Endotoxin was removed from CAS and DP-CAS by EndoClean™ Endotoxin Removal Kit for Protein Solution (Biovintage, Inc., San Diego, CA). Limulus Amebocyte Lysate, ENDOSAFE® KTA (Charles River ENDOSAFE, Charleston, SC) was used for quantitative detection of endotoxin. Pooled normal human plasma in sodium citrate (Innovative Research, Southfield, MI) was used for the in vitro elution study.

2.2. Preparation of aluminum hydroxide adjuvants

A stock AH suspension containing 3.06 mg Al/mL in doubly distilled water (dd H_2O) was prepared by diluting the commercial aluminum hydroxide adjuvant. The pH was adjusted to 7.4 by the addition of 0.1N NaOH. A stock PT-AH suspension containing 3.06 mg Al/mL in dd H_2O was prepared by mixing 26 mL of 0.5 M KH_2PO_4 (adjusted to pH 7.4) with 43.4 g of the commercial AH, adjusting the pH to 7.4 with 1N NaOH and diluting with dd H_2O to 150 mL. The suspension was magnetically stirred for 1 week at room temperature to allow for maximum ligand exchange of phosphate for hydroxyl. Each adjuvant was sterilized at 121 °C for 20 min.

2.3. Antigen preparation

Alpha casein and DP-CAS were used as model antigens. The protein stock solutions, 200 μg/mL, were adjusted to pH 7.4 with 0.01N NaOH, and filtered using a sterile 0.22 μm membrane filter. The protein concentration of the sterile stock solution was determined by the BCA assay.

2.4. Preparation of vaccines

Four vaccines were prepared for the immunization studies: CAS and AH, CAS and PT-AH, DP-CAS and AH, and DP-CAS and PT-AH. Protein stock solutions and adjuvant stock suspensions were prepared at a concentration twice that of the desired final concentration. Equal volumes of the protein stock solution and the adjuvant stock suspension were combined such that the final protein concentration was 100 μg/mL and the final aluminum concentration was 1.53 mg Al/mL. The vaccines were shaken at room temperature for 1 h before administration. The degree of antigen adsorption was assessed by centrifuging the vaccine at 14,000 × *g* for 5 min and determining the concentration of antigen in the supernatant by the BCA assay.

2.5. Adsorption isotherms

Langmuir adsorption isotherms [5] of CAS and DP-CAS with each adjuvant were prepared. Ten protein working solutions in the range described in Table 1 were prepared with 10 mM MOPS (pH 7.4) buffer for each system. Suspensions of AH and PT-AH were prepared with 10 mM MOPS (pH 7.4) such that the final calculated aluminum concentration was 1.53 mg Al/mL. Equal volumes of the protein working solutions and AH or PT-AH were combined in 2.0 mL

Table 1
Langmuir adsorption isotherm parameters at pH 7.4, 4 °C

Antigen	Adjuvant	Protein concentration (mg/mL)	Adsorptive capacity (mg/mg Al) (95% confidence interval)	Adsorptive coefficient (mL/mg) (95% confidence interval)
CAS[a]	AH[b]	0.5–2.0	2.4 (2.2–2.6)	2409 (2235–2585)
CAS	PT-AH[c]	0.15–2.0	0.9 (0.7–1.1)	419 (212–626)
DP-CAS[d]	AH	0.15–1.5	0.4 (0.3–0.5)	59 (55–63)
DP-CAS	PT-AH	0.025–1.0	0.03 (0.02–0.04)	0.8 (0.5–1.0)

[a] Alpha casein.
[b] Aluminum hydroxide adjuvant.
[c] Phosphate-treated aluminum hydroxide adjuvant.
[d] Dephosphorylated alpha casein.

microcentrifuge tubes. The suspensions were mixed gently by end-over-end rotation at 4 °C for 1 h as preliminary experiments indicated that adsorption was complete within 30 min. The protein adsorption level was determined by centrifuging the samples at 14,000 × *g* for 5 min and analyzing the supernatant in triplicate by the BCA assay. The amount of protein adsorbed onto AH or PT-AH was determined by subtracting the amount found in the supernatant from the amount present initially.

2.6. In vitro elution

Elution experiments were carried out by diluting the four model vaccines with sheep lymph fluid or pooled normal human plasma in sodium citrate in the ratio of 1:3. Lymph fluid is identical to interstitial fluid, and was harvested using the method described by Chang et al. [6].

The vaccine samples were mixed at 180 rpm with sheep lymph fluid or normal human plasma at 37 °C for 24 h (Environmental Incubator Shaker G24, New Brunswick Scientific, Edison, NJ). The samples were withdrawn at 15 min intervals for the first hour and then every hour. The supernatant was separated by centrifugation and the protein concentration was determined by ELISA. Rabbit anti-casein bovine antibody (Gene Tex, San Antonio, TX) was used to coat ELISA plates (0.1 μg of protein in 0.1 mL of 0.1 M $NaHCO_3$ at pH 9.6) by overnight incubation at 4 °C. Nonadsorbed antibody was removed by washing with washing buffer (PBS/0.05% Tween 20) and the wells were blocked with SuperBlock (Pierce, Rockford, IL). Standard CAS or DP-CAS solutions containing 1.0–100 ng/mL of protein were prepared in the washing buffer. Samples were diluted in the washing buffer to the concentration range of the standard solution. The standards and the samples of CAS were added to the plates in triplicate and incubated at 37 °C for 1 h. The standards and the samples of DP-CAS were added to the plates in triplicate and incubated overnight at 4 °C. Unbound CAS or DP-CAS was removed by washing with a washing buffer five times. HRP-conjugated sheep anti-bovine casein IgG (Immunology Consultants Laboratory, Newberg, OR) (1:25,000 for CAS and 1:45,000 for DP-CAS, diluted in PBS-T, 100 μL/well) was added to the plates. The CAS plates were incubated at 37 °C for 1 h and the DP-CAS plates were incubated overnight at 4 °C and then washed with the washing buffer as before. The liquid substrate (3,3′,5,5′-tetramethylbenzidine (TMB) 100 μL/well) was added to the plates and incubated at room temperature for 20 min. The reaction was stopped with 2N H_2SO_4 (50 μL/well). The absorbance was measured at 450 nm in an automatic microplate reader. The protein concentration was calculated from the standard curve and the results are the average of triplicate samples.

2.7. Evaluation of humoral immune responses

Eight groups of four or 12 BALB/c female mice 6–8 weeks old (Harlan Sprague Dawley, Indianapolis, IN) were immunized subcutaneously with 100 μL of the appropriate treatment (Table 2). The immunized mice were euthanized on day 21, and sera were collected. Serum IgG titers were determined by ELISA as previously described [7]. Titers were expressed as end-point titers, i.e. the reciprocal dilution higher than the mean + 2 S.D. of the optical density of preimmune sera at 1:100 dilution. The data were calculated as geometric mean titers (GMT) ± 95% confidence interval.

An additional experiment was conducted to compare the immunogenicity of CAS and DP-CAS in combination with IFA. Three groups of female BALB/c mice were immunized with 100 μL of IFA ($n = 4$), CAS/IFA ($n = 6$), or DP-CAS/IFA ($n = 6$) to investigate the immunogenicity of CAS and DP-CAS. Protein stock solutions were prepared at a concentration of 200 μg/mL. Equal volumes of the protein stock solution and IFA were homogenized such that the final protein concentration was 100 μg/mL. The immunized mice were

Table 2
Experimental design of vaccines for immunization study

Group	Number of mice	Treatment
1	4	10 μg CAS[a]
2	4	10 μg DP-CAS[b]
3	4	AH[c] (153 μg Al)
4	4	PT-AH[d] (153 μg Al)
5	12	10 μg CAS + AH (153 μg Al)
6	12	10 μg CAS + PT-AH (153 μg Al)
7	12	10 μg DP-CAS + AH (153 μg Al)
8	12	10 μg DP-CAS + PT-AH (153 μg Al)

[a] Alpha casein.
[b] Dephosphorylated alpha casein.
[c] Aluminum hydroxide adjuvant.
[d] Phosphate-treated aluminum hydroxide adjuvant.

euthanized on day 21 and sera were collected. Serum IgG titers were determined by ELISA as previously described [7]. Alpha casein was used for CAS immunized mice and DP-CAS for DP-CAS immunized mice. Lower titers were found in heterologous ELISAs. The significance of differences in geometric mean titers among groups was determined by ANOVA followed by the Tukey multiple comparison test. This research was approved by the Purdue Animal Care and Use Committee.

2.8. T-cell proliferation

Activation of T-cells was measured in splenocytes from mice injected with the following vaccines: CAS/AH, CAS/PT-AH and DP-CAS/PT-AH. Spleens from mice immunized with antigen solution, adjuvant suspension and vaccines were removed on day 21 (four mice per group assessed individually). Spleens were homogenized to obtain a single-cell suspension and depleted of erythrocytes by treatment with ACK lysing buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, and 0.1 mM Na_2EDTA). The cells were cultured for 5 days in serum free media (EX-CELL^{TM} Hybridoma Medium, SAFC Biosciences, KS), supplemented with 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 1% mouse serum at 37 °C in 5% CO_2. Proliferation was measured by incubating 2×10^6 cells/well in 0.2 mL serum free medium in a 96-well flat bottom plate (Falcon, Lincoln Park, NJ) in the presence of CAS (for CAS immunized mice) or DP-CAS (for DP-CAS immunized mice) at four concentrations (0, 5, 10, 15 μg/mL). Concanavalin A (Sigma, St. Louis, MO) (1 μg/mL) was used as a positive control. Each of the samples was tested in triplicate wells. Methyl-^{3}H thymidine (Amersham Biosciences, UK) was added to the culture at a final concentration of 0.5 μCi/well 24 h before cell harvesting. Thymidine incorporation was measured by using a microplate scintillation luminescence counter (Topcount, Downers Grove, IL). Proliferative responses were determined from triplicate samples and expressed as a stimulation index, SI (mean cpm of cultures with antigen/mean cpm of cultures with medium only). The significance of differences in SI among groups was determined by ANOVA followed by the Tukey multiple comparison test.

3. Results

Two phosphorylated proteins were selected for this study: CAS (eight phosphates, 26,000 Daltons, iep ~4.6) and DP-CAS (two phosphates, 25,500 Daltons, iep ~4.6) [11]. All the surface sites in AH contain a hydroxyl that is available for ligand exchange. Phosphate treatment of AH reduces the number of surface sites that contain a hydroxyl and thereby reduces the potential for ligand exchange [12]. The four vaccines studied were designed to have different adsorptive coefficients. Adsorption forces that are believed to be operating in each vaccine are presented in Table 3. Alpha casein was completely absorbed in the vaccines containing AH or PT-AH. Dephosphorylated alpha casein was completely adsorbed by AH, while 60% of DP-CAS was adsorbed by PT-AH.

3.1. Adsorption isotherms

The adsorption behavior of each system was studied by constructing adsorption isotherms that were found to follow the Langmuir equation [5]. The adsorption data were analyzed by linearizing the Langmuir equation to calculate the adsorptive capacity and the adsorptive coefficient (Table 1). The adsorptive capacity and adsorptive coefficient of CAS/AH were the largest at 2.4 mg CAS/mg Al and 2409 mL/mg, respectively. At the other extreme, DP-CAS/PT-AH had a very small adsorptive capacity and adsorptive coefficient. The adsorptive capacities and adsorptive coefficients of CAS/PT-AH and DP-CAS/AH were intermediate. The adsorptive coefficients followed the order predicted by the analysis of the adsorption forces in Table 3. Both the adsorptive capacity and adsorptive coefficient were directly related to the degree of antigen phosphorylation, whereas the degree of phosphate substitution for hydroxyl on the adjuvant was inversely related. The results from the Langmuir adsorption isotherms provide evidence that the antigens in the four vaccines have different adsorptive coefficients reflecting adsorption forces that range from strong to very weak in the following order: CAS/AH > CAS/PT-AH > DP-CAS/AH > DP-CAS/PT-AH.

3.2. In vitro elution

The in vitro elution of CAS or DP-CAS from each vaccine was determined by exposure to sheep lymph fluid and normal human plasma to simulate the changes in adsorption that may occur when a vaccine is administered intramuscularly or subcutaneously [4,9,13]. Normal human plasma was selected as a commercially available alternative to sheep lymph fluid. The two vaccines with the greatest adsorptive coefficients, CAS/AH and CAS/PT-AH, exhibited no measurable elution of CAS during the 24 h exposure period to either sheep lymph fluid or normal human plasma. DP-CAS exhibited approx-

Table 3
Adsorption forces in vaccines

		Adsorption force	
Antigen	Adjuvant	Electrostatic	Ligand exchange
CAS[a]	AH[b]	Attractive	Strong
DP-CAS[c]	AH	Attractive	Weak
CAS	PT-AH[d]	Repulsive	Weak
DP-CAS	PT-AH	Repulsive	Very weak

[a] Alpha casein.
[b] Aluminum hydroxide adjuvant.
[c] Dephosphorylated alpha casein.
[d] Phosphate-treated aluminum hydroxide adjuvant.

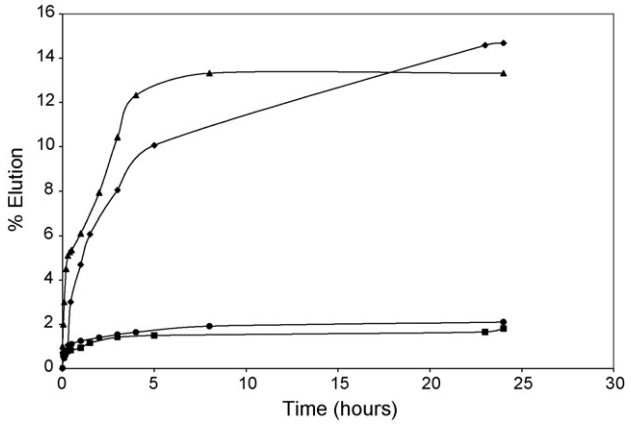

Fig. 1. In vitro elution of dephosphorylated alpha casein from aluminum hydroxide adjuvant and phosphate-treated aluminum hydroxide upon exposure to sheep lymph fluid or normal human plasma at pH 7.4, 37 °C. Alpha casein adsorbed to either aluminum hydroxide adjuvant or phosphate-treated aluminum hydroxide adjuvant exhibited no detectable elution. *Key*: (▲) DP-CAS/PT-AH in sheep lymph fluid; (♦) DP-CAS/PT-AH in normal human plasma; (●) DP-CAS/AH in sheep lymph fluid; (■) DP-CAS/AH in normal human plasma.

imately 1–2% and 13–15% elution from AH and PT-AH, respectively, during a 24 h exposure period (Fig. 1). Thus, the in vitro elution profiles of the four vaccines were inversely related to the adsorptive coefficients presented in Table 1. It is important to note that the elution profiles were virtually the same for sheep lymph fluid and normal human plasma.

3.3. Humoral immune response

The antibody titer was determined in mice injected with the four vaccines, CAS and DP-CAS (Fig. 2). Data showed that the mice immunized with DP-CAS/PT-AH elicited the highest antibody response of 50,817, followed by the DP-CAS/AH at 23,622 and CAS/PT-AH at 1869. The titer obtained following immunization with CAS/AH was low (124) and similar to that of CAS (123). The antibody titers for DP-CAS/PT-AH, DP-CAS/AH and DP-CAS were significantly different ($p < 0.0001$). The antibody titer for CAS/PT-AH is significantly different from CAS/AH and CAS ($p < 0.0001$). Comparison of the antibody titers produced by the four vaccines and the adsorptive coefficients revealed an inverse relationship.

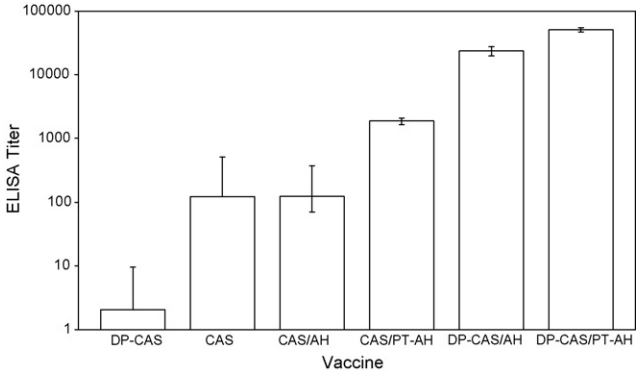

Fig. 2. Immune responses of BALB/c female mice injected with various vaccine preparations. Each bar represents the geometric mean IgG titer and 95% confidence intervals from 4 (CAS, DP-CAS) or 12 (CAS/AH, CAS/PT-AH, DP-CAS/AH, DP-CAS/PT-AH) mice. *Treatments*: DP-CAS, 10 μg; CAS, 10 μg; CAS/AH, 10 μg CAS and 153 μg AH; CAS/PT-AH, 10 μg CAS and 153 μg PT-AH; DP-CAS/AH, 10 μg DP-CAS and 153 μg AH; DP-CAS/PT-AH, 10 μg DP-CAS and 153 μg PT-AH.

A study was undertaken to determine if CAS and DP-CAS were equivalent immunologically. It has been reported that antigen dephosphorylation reduces immunogenicity [14]. Three groups of mice where immunized with IFA, CAS/IFA, or DP-CAS/IFA. The antibody titer of CAS/IFA was significantly higher (9005) than DP-CAS/IFA (2647) ($p < 0.001$). These results indicate that CAS is more immunogenic than DP-CAS when administered with IFA. Antigen dephosphorylation may cause a loss of phosphorylated epitopes that are immunogenic.

Examination of the results from the geometric mean antibody titers for the four vaccines presented in Fig. 2 revealed that DP-CAS adsorbed to AH or PT-AH induced higher antibody titers than the two CAS vaccines even though CAS was more immunogenic than DP-CAS. This apparent contradiction emphasized the importance of the adsorptive coefficient. It is likely that the two DP-CAS vaccines produced higher antibody titers because they were not as strongly adsorbed by the aluminum-containing adjuvant. Regardless of whether the two CAS or the two DP-CAS vaccines are compared to each other or whether the four vaccines are considered as a group, the strength of antigen adsorption had a greater impact on antibody titer than the immunogenicity of the antigen.

3.4. Proliferative response of spleen cells from immunized mice

An experiment was designed to investigate whether a high adsorptive coefficient adversely affected antigen processing and presentation to T-cells leading to a low antibody response. The ability of the CAS/AH, CAS/PT-AH and DP-CAS/PT-AH vaccines to induce activation of antigen-specific T-cells was evaluated. Fig. 3 shows that CAS and CAS/AH produced SI values less than three at three different antigen doses. Thus, neither CAS nor CAS/AH induced significant T-cell activation. In contrast, CAS/PT-AH produced SI values of 7.2, 7.5, and 7.6, which were significantly higher than CAS and CAS/AH ($p < 0.001$), indicating T-cell activation. The vaccine, DP-CAS/PT-AH, produced the highest antibody titer and the highest SI values of 10.8, 16.3, and 14.0, which were significantly higher than CAS/PT-AH, CAS/AH and CAS ($p < 0.001$). Tukey pairwise comparisons showed that the means are significantly different for DP-CAS/PT-AH, CAS/PT-AH, CAS/AH and CAS. However, the means for CAS/AH and CAS are not significantly different.

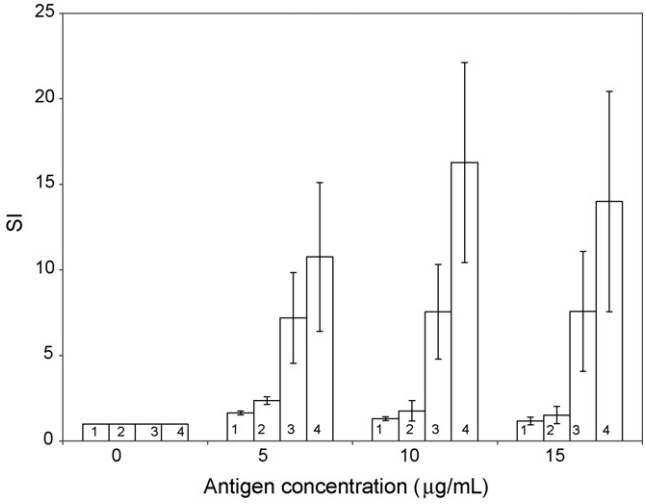

Fig. 3. Antigen-specific T-cell proliferative response (SI) of mouse splenocytes harvested 21 days after immunization. Bars represent the mean SI ± S.D. of four mice. *Treatments*: 1, CAS; 2, CAS/AH; 3, CAS/PT-AH; 4, DP-CAS/PT-AH.

4. Discussion

The activation of naïve vaccine-specific B and T-cells occurs primarily in lymph nodes draining the site of vaccination. Antigens reach the lymph node via the afferent lymphatics either as free antigens or intracellularly in dendritic cells [15]. Protein antigens taken up by dendritic cells are partially degraded by proteolytic enzymes into peptides in acidic endosomal compartments. The peptides bind to MHC II molecules and the MHC II/peptide complexes are presented to antigen-specific T-cells [16]. On the other hand, B-cells bind intact antigens in their native conformation.

Phosphorylated antigens are adsorbed to aluminum-containing adjuvants primarily by ligand exchange [3,9]. This process is nearly irreversible for proteins with multiple phosphate groups, such as casein. As demonstrated here, high adsorptive coefficients interfere with the production of antibodies and with activation of antigen-specific T-cells. When the protein was dephosphorylated, T-cell activation was observed. The binding of antigens to aluminum particles enhances the uptake by dendritic cells [17], but tight binding may interfere with the proteolytic processing and generation of peptides for binding to MHC II molecules. The absence of activation of antigen-specific T-helper cells impairs the B-cell response and results in low antibody production. In addition, tight adsorption of antigen may prevent elution of any antigen from the adjuvant following administration, as was observed for CAS/AH and CAS/PT-AH, and thereby reduce the amount of antigen that reaches the lymph node for recognition by B-cells. After injection of vaccines with aluminum-containing adjuvants, the bulk of the adjuvant and adsorbed antigen are retained at the injection site, but some aluminum-containing particles can be found in the draining lymph nodes [18].

Aluminum-containing adjuvants are believed to potentiate the immune response by retaining antigen at the injection site and by inducing a local inflammatory response with recruitment of dendritic cells [19,20]. The retention of antigen results in a high concentration of antigen and enhances the uptake of antigen by dendritic cells. However, very strong adsorption of the antigen may interfere with the immune response at least in part by interfering with antigen processing in antigen-presenting cells. This indicates that it is important to determine the adsorptive coefficient when a vaccine formulation is being developed. It is also important to know if antigens are phosphorylated and have the potential to bind to aluminum-containing adjuvants by ligand exchange because ligand exchange is a stronger adsorption force than electrostatic attraction. Antigens that are associated with a lipid bilayer that is composed of phospholipids, such as hepatitis B surface antigen [3] or contain polyribosylribitolphosphate (PRP), such as *Haemophilus influenza* [21] may adsorb by ligand exchange as phosphate groups are generated by hydrolysis of phospholipids or PRP. This study demonstrated that modification of the degree of phosphorylation of antigens and pretreatment of adjuvants with phosphate to reduce the number of hydroxyl groups are methods to change the strength of adsorption by ligand exchange and to optimize the immune response.

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