Pharmaceutical Research, Vol. 8, No. 7, 1991

Research Article

The Importance of Surface Charge in the Optimization of Antigen–Adjuvant Interactions

Patricia M. Callahan,[1,2] Andrew L. Shorter,[1,4] and Stanley L. Hem[1,3]

Received July 5, 1990; accepted February 28, 1991

The adsorptive behavior of the recombinant malarial antigens R32tet32, R32NS181 and NS181V20 to aluminum hydroxide and aluminum phosphate gels was studied as a function of pH and buffer ions. The *Plasmodium falciparum* antigen, R32NS181, and the *P. vivax* antigen, NS181V20, with isoelectric points (*p*I) of 5.9 and 5.5, respectively, adsorbed readily to the positively charged boehmite form of aluminum hydroxide gel. These two antigens displayed reversible, linear adsorption behavior in the pH range 5–9, with maximal adsorption observed at the lowest pH studied. The addition of acetate buffer ions had little effect on adsorption, while the presence of phosphate decreased adsorption for R32NS181 and NS181V20 by 25 and 40% respectively. The adsorptive behavior of these two antigens with the negatively charged adjuvant, aluminum phosphate, was markedly decreased. The converse situation was observed with the R32tet32 antigen, whose *p*I is estimated to be 12.8. There was minimal interaction of this antigen with aluminum hydroxide gel except in the presence of phosphate counter ions and significant, nonreversible adsorption with aluminum phosphate gel. Enhanced adsorption of R32tet32 to aluminum hydroxide gel in the presence of phosphate is suggested to be the result of a covalent bond between a surface aluminum and a phosphate anion that modifies the surface charge of the aluminum hydroxide gel. These results indicate that the role of complementary surface charges, both for the ionization state of the protein and for the aluminum adjuvants, is the key in optimizing conditions for significant antigen-adjuvant interactions.

KEY WORDS: antigen; adjuvant; aluminum hydroxide; aluminum phosphate; adsorption.

INTRODUCTION

To stimulate an optimal immune response, a vaccine is usually comprised of both antigen and adjuvant components, the antigen or epitope eliciting a specific immune response, while the adjuvant usually enhances this response (1,2). Vaccines composed of killed whole viruses or bacteria can be self-adjuvanting owing to the mixture of biologically active components present. Highly purified subunit antigens require the presence of an effective immunological adjuvant. A wide variety of adjuvants, such as liposomes, detoxified lipopolysaccharide, peptides, lymphokines, and polymeric species, is under investigation. The only approved adjuvants in the United States, however, are the aluminum-based suspensions such as precipitated alum [$KAl(SO_4)2 \cdot 12H_2O$] and preformed aluminum hydroxide and aluminum phosphate gels (3,4). Recent advances in vaccine development have been made through a more thorough understanding of the immune process. Current research has also been stimulated in part by the difficulty in preparing consistent vaccine formulations with aluminum-based adjuvants (2,5).

Mineral adjuvants are thought to adsorb the antigen completely, resulting in a delayed release of antigen after injection. The presence of the aluminum mineral adjuvants also stimulates the presence of immune-competent cells to the area of injection. Irritation induced by the aluminum suspension is a function of the physicochemical properties of the adjuvant alone, while the depot effect depends on complete antigen adsorption and desorption without chemical modification of the antigenic protein molecule. In spite of the importance of the antigen–adjuvant interactions, very few studies have been carried out in order to elucidate the mechanism of antigen adsorption to aluminum hydroxide and aluminum phosphate gels (6,7). In this work we have used three recombinant malarial antigens to probe their adsorption characteristics to both aluminum hydroxide and aluminum phosphate gels.

Precipitated aluminum hydroxide has variable physical properties depending on the pH and buffer ions present during precipitation (8,9). This work focused only on the preformed aluminum hydroxide and aluminum phosphate gels. The form of aluminum hydroxide gel which is commercially available as an adjuvant is composed of a crystalline material known as boehmite and has the empirical formula Al(O)(OH)

[1] Department of Pharmaceutical Research, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939.

[2] Present address: Department of Chemistry, University of Missouri—Rolla, Rolla, Missouri 65401.

[3] Department of Industrial and Physical Pharmacy, Purdue University, West Lafayette, Indiana 47907.

[4] To whom correspondence should be addressed at SmithKline Beecham Pharmaceuticals, L-921, P.O. Box 1530, King of Prussia, Pennsylvania 19406-0939.

0724-8741/91/0700-0851$06.50/0

[aluminum oxyhydroxide] (9). This gel has a small particle size and a large surface area and displays a scroll-like three-dimensional structure by transmission electron microscopy (TEM) (9). The surface charge of this colloidal gel is described by the point of zero charge (PZC), the pH at which the net charge on the surface is zero. The PZC of the aluminum hydroxide gel has been determined to be 9.4 (10). This results in an increasingly positively charged surface below pH 9.4. The second adjuvant studied, aluminum phosphate gel, is an amorphous material that has a plate-like appearance by TEM and a smaller surface area (9). The surface charge of the aluminum phosphate gel is negative at neutral pH, which is a consequence of its PZC of 4.5 (see below). These differences in surface area and surface charge of aluminum hydroxide and aluminum phosphate gels were expected to impact greatly on their antigen adsorption behavior.

The antigens used in this study were three recombinantly derived proteins that have as their antigenic sites the repeat regions of the sporozoite coat protein of the malarial parasites *Plasmodium falciparum* and *Plasmodium vivax*. This repeat region was selected as a possible vaccine candidate based partly on protection against malarial challenge exhibited by healthy volunteers vaccinated with irradiated sporozoites. The repeat region for the *P. falciparum* antigens, R32tet32 and R32NS181, consists of multiple repeats of the sequence (Asn–Ala–Asn–Pro) plus different nonsporozoite-derived tail regions. The longer multiple-repeat sequence of *P. vivax* plus a nonsporozoite tail sequence was cloned and expressed as NS181V20. The high content of proline in these repeat regions confers a large amount of extended-chain, random-coil structure to these protein conformations (11). The stretched-out nature of the polypeptide structure has been previously noted in the chromatographic and membrane filtration behavior of R32tet32 (11). Although the repeat region sequence is fairly similar for these three antigens, their tail regions result in distinctive isoelectric points (*p*I). The R32NS181 and NS181V20 polypeptides are acidic in nature, with isoelectric points of 5.9 and 5.5, respectively, while the R32tet32 antigen is basic, with a *p*I of 12.8.

The types of adsorption mechanisms previously established for protein–surface interactions are electrostatic, hydrogen bonding, hydrophobic, and covalent or ligand exchange interactions (12–15). Previous protein adsorption studies to finely divided solid surfaces such as silica and alumina reported maximal adsorption at pH levels near the polypeptide isoelectric point, with much less adsorption observed away from the *p*I (16). These results indicate that the driving force for protein adsorption was due predominantly to the minimization of repulsive protein–protein interactions observed at the minimum pH solubility point or isoelectric point. Similarly, in the case of polypeptide adsorption to polymer surfaces, maximal adsorption is typically observed near the protein isoelectric point and less surface denaturation detected for adsorption to hydrophilic than hydrophobic polymer surfaces (12,13).

The study of protein adsorption to mineral adjuvant surfaces requires that the charge of both the polypeptide chain and the aluminum surface be taken into account due to the colloidal nature of the adjuvant gels. Sepelyak *et al.* (6,7) investigated a very acidic protein, pepsin (*p*I = 1) and its interaction with aluminum hydroxide gel [PZC = 9.4 (10)]. Over the pH range 1–10, maximal adsorption was observed at approximately pH 4.0. Infrared spectra taken of pepsin adsorbed to aluminum hydroxide suggested that a carboxylate ligand exchange reaction with the positively charged aluminum surface sites was the dominant adsorption mechanism in this case.

We have extended this previous study by investigating the adsorption of three antigens with varied isoelectric points to the commercially available adjuvants known as aluminum hydroxide and aluminum phosphate gels. In addition to the surface charge of the aluminum hydroxide and aluminum phosphate adjuvants, the effect of competing buffer ions for sites on their reactive surfaces was evaluated. Anions such as carbonate, sulfate, phosphate, and fluoride are known to interact strongly with aluminum hydroxide gel surfaces (17), thereby limiting the available buffers to be used for baseline measurement. In this work, the pH of the antigen and adsorbent was maintained constant with the use of a pH stat titrator in an unbuffered isotonic medium, 150 m*M* NaCl. Therefore, we were able to distinguish between adsorption mechanisms caused by electrostatic effects and those caused by specific ligand effects.

MATERIALS AND METHODS

The mineral adjuvants were obtained commercially: aluminum hydroxide gel, Rehsorptar, was obtained from Armour Pharmaceuticals Co. and aluminum phosphate as Adju-Phos, from Superfos. The buffers were from the following vendors: sodium chloride (Mallinckrodt 7532Y AB), sodium acetate trihydrate (Sigma 94F-0313), and sodium phosphate dibasic 12-hydrate (J. T. Baker 349091). MilliQ purified water was used. The malarial antigens were prepared and isolated as previously described (11). The purity was checked by SDS-PAGE and estimated to be >90%. Buffer exchange of the malarial antigens into 150 m*M* NaCl was accomplished with a 2.5 × 15.0-cm Sephadex G-25 column. The ionic strength at pH 7 was constant at 0.15 for all the experiments, although the ionic strength varied slightly at the extremes of the pH measured for samples with acetate and phosphate buffer. Concentrations of the aluminum hydroxide and aluminum phosphate gels are reported as the total concentration of aluminum as Al^{3+}. All experiments, unless otherwise noted, were carried out at room temperature at a constant protein-to-Al^{3+} ratio of 1.2:1.0 (mg:mg). This ratio was chosen because preliminary adsorption isotherm results performed in the presence of buffer ions determined the adsorption capacity of aluminum hydroxide gel with these antigens to be approximately 1.0 mg antigen/mg Al^{3+}.

The pH of the antigen–adjuvant solutions was held constant by a Radiometer pH stat titrator (PMH 84 pH meter, TT80 titrator, ABU80 autoburette, and TTA80 titration assembly). Protein concentration was assayed with the Pierce bicinchoninic acid (BCA) dye binding assay. A standard curve was generated for R32tet32 and this curve was used for all subsequent assays.

The continuous titration method to determine the point of zero charge (18) of aluminum phosphate gel was not pos-

sible because the aluminum phosphate began to dissolve at low pH. However, the addition of KCl caused the pH of the suspension to decrease. This effect was used to determine the PZC because the pH of the gel medium shifts toward the PZC as its surface charge is masked by the addition of excess salt. Therefore, the pH of the suspension was monitored as a function of added KCl and the pH asymptotically approached 4.5. This is the experimentally determined PZC of the aluminum phosphate gel used in this analysis. The PZC of Rehsorptar (lot A) used in this study was determined to be 9.8 by using a Doppler electrophoretic light-scattering analyzer (DELSA 440, Coulter Electronics, Inc. Hialeah, FL.). The PZC of Rehsorptar (lot B) was not measured due to insufficient supplies.

Samples for X-ray diffraction were prepared as random powder mounts after gently grinding freeze-dried adjuvants in an agate mortar and pestle. The diffraction patterns were recorded from 4 to 40° 2θ using CuK_α radiation.

The pH-dependent adsorption experiments were performed in the following manner. Malaria antigen in 150 m*M* NaCl was added to a suspension of adjuvant gel, NaCl, and sodium acetate or sodium phosphate (dibasic) to make an ionic strength of 0.15 at pH 7.0 and 25°C. The pH was varied by the addition of 0.01 *N* NaOH and 0.01 *N* HCl with the use of the pH stat titrator. The initial, unbuffered pH was typically 6 and the sequence of pH values started at approximately pH 5, increased by single pH units to 9, and then reversed through several different pH values to a final pH of approximately 5.5. The solution was maintained at each pH point for 15 min with continuous stirring. An aliquot was then removed for assay (preliminary experiments indicated that adsorption occurred in less than 15 min). Samples were centrifuged for 30 min at 10,000 rpm (Beckman Microfuge) and the supernatant removed and assayed in triplicate with the Pierce enhanced (60°C, 30 min) BCA assay. The volume of acid or base needed to vary the pH was recorded. Typically 0.2–0.5 ml of titrant was added during the course of an experiment. The reported protein concentrations have been corrected for the dilution that occurred. A control experiment was run on R32NS181 to monitor protein solubility and the sensitivity of the dye binding assay as a function of pH. No pH sensitivity of the protein concentration was observed in the absence of the adjuvant.

RESULTS

Reversibility of Antigen Binding

A separation of the effects of electrostatic and ligand-mediated binding of the malarial antigens to aluminum hydroxide is possible with the use of the pH stat titrator technique. Electrostatic effects can be monitored by varying the pH of the suspension in an unbuffered medium which modifies the adjuvant surface charge. In this manner, adsorption can be studied in the absence of added buffer ions. The pH dependence of the extent of adsorption of the malarial antigens R32NS181, NS181V20, and R32tet32 is presented in Fig. 1. These samples were adjusted to constant ionic strength in 150 m*M* NaCl. The data include experimental points obtained for both forward and reverse pH steps as indicated by the numerical sequence. A wide range of adsorption levels was observed for these antigens. The acidic proteins, R32NS181 and NS181V20, adsorbed to a considerable extent at this ratio of protein to aluminum hydroxide. A linear and reversible dependence on adsorption with pH was observed for both antigens. Maximal adsorption levels of 70 and 90% were obtained for R32NS181 and NS181V20, respectively, at the lowest pH studies, pH 5.0. These antigens showed the same general trend, i.e., maximum adsorption at the lowest pH studied, a gradual decrease in adsorption as the pH is raised, followed by a reversible rebinding as the pH is reduced. The magnitude of adsorption for NS181V20 was 20% higher, however. This difference may be due to other adsorption mechanisms such as hydrophobic bonding and hydrogen bonding.

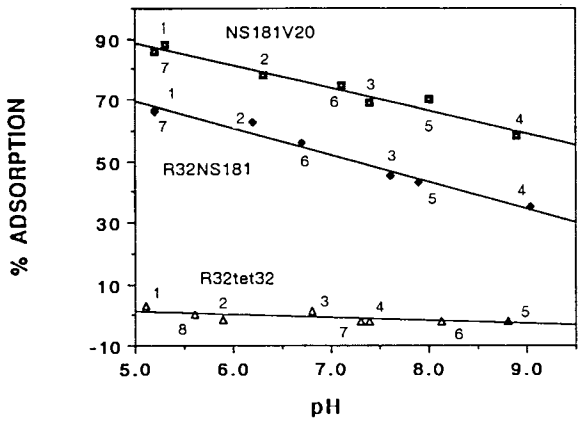

Fig. 1. Effect of pH on the adsorption of NS181V20, R32NS181, and R32tet32 malaria antigens to aluminum hydroxide gel; protein/Al^{3+} ratio, 1.2/1.0, in 150 m*M* NaCl. The numbers indicate the sequence in which the experiments were performed.

The basic antigen, R32tet32, was markedly different in its adsorption properties to the aluminum hydroxide gel. Poor adsorption was observed over the entire pH range studied. The time for adsorption of R32tet32 to aluminum hydroxide gel was extended from 15 min to 7.5 hr to determine if adsorption was time dependent (data not shown). None of the adsorption values in either the forward or the reverse pH direction were significantly different from zero.

The adsorptive characteristics of a different lot of commercial aluminum hydroxide adjuvant (labeled lot B) was also studied by adsorption of R32NS181. Figure 2 displays a

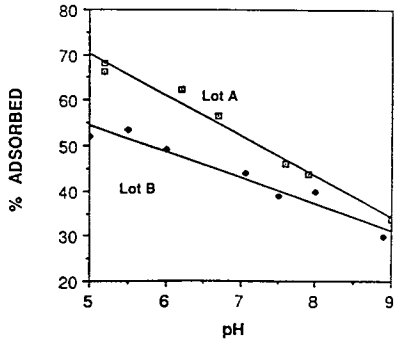

Fig. 2. Effect of pH on the adsorption of R32NS181 to two different lots of aluminum hydroxide gel; protein/Al^{3+} ratio, 1.2/1.0, in 150 m*M* NaCl.

linear, reversible relationship between adsorption to lot B and pH. Less antigen was adsorbed to lot B than to lot A, however. This difference in adsorption is statistically significant between pH 5 and pH 6. Above this pH value, the adsorption curves lie within the experimental error. The lower adsorption exhibited by lot B may be due to a lower surface area.

The surface area of these colloidal gels is related to the primary crystallite size of the boehmite particles. This parameter can be quantified by monitoring the powder X-ray diffraction bandwidth at half-height of the 020 reflection (6.46 Å). A sharp X-ray diffraction band (i.e., small width at half-height) characterizes highly ordered material with a large crystallite size, while poorly ordered material has a small crystallite size and a broad X-ray diffraction band. The commercial aluminum hydroxide used in the adsorption study presented in Fig. 2 (lot A) has a diffraction bandwidth at half-height of the 020 reflection (6.46 Å) of 2.99° 2θ and that measured for lot B is 2.59° 2θ. The crystallite size for these two adjuvants can be compared to the crystallite size observed for 32 synthetic boehmites (19). The line broadening seen in the samples studied by Tettenhorst and Hofmann ranged from 5.45 to 0.10° 2θ. Thus, the two lots of commercial aluminum hydroxide adjuvants are in the middle of the possible range of boehmite crystallite size. The smaller X-ray diffraction bandwidth indicates that lot B has a larger crystallite size than lot A and therefore a smaller surface area for antigen adsorption. The cause of this difference in crystallite size is not clear. All further adsorption studies were conducted with the higher surface area lot A commercial aluminum hydroxide.

Effect of Buffer Ions on Antigen Binding

Having established the binding of the proteins in the absence of buffer ions, it was possible to study the effect of acetate and phosphate buffer ions on adsorption of the malarial antigens as shown in Figs. 3 and 4 for the R32NS181 and NS181V20 antigens. Figure 3 exhibits the results for the control experiment (150 m*M* NaCl) and shows that in the presence of 20 m*M* acetate, the results are within experimental error. The addition of 20 m*M* phosphate lowers the amount of adsorbed protein by 20% over the pH range 5–7.5. Above pH 7.5, the binding of protein to aluminum hydroxide is essentially independent of pH.

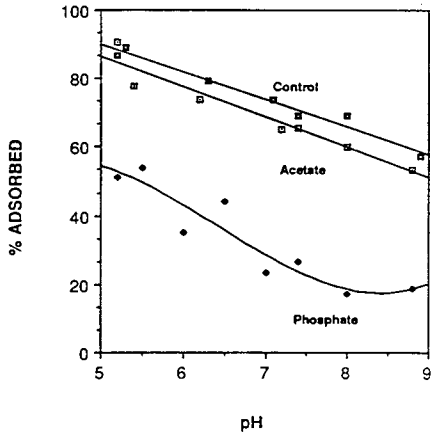

Fig. 4. Effect of pH on the adsorption of NS181V20 to aluminum hydroxide gel in the presence of buffer ions; protein/Al^{3+} ratio, 1.2/1.0. Control, 150 m*M* NaCl. Acetate, 20 m*M* sodium acetate and 130 m*M* NaCl. Phosphate, 20 m*M* sodium phosphate and 110 m*M* NaCl.

Figure 4 shows a similar set of experiments for the NS181V20 malarial antigen and aluminum hydroxide. Again, there was little difference between the extent of protein binding in the presence and that in the absence of 20 m*M* acetate ion. The addition of 20 m*M* phosphate diminished the amount of adsorbed protein by more than 20%. The pH independence of protein binding at pH's greater than 7 suggests that electrostatic effects do not play a major role in the presence of phosphate and that a specific ligand-mediated binding may be operative in this case.

The pH dependence of adsorption of R32tet32 to aluminum hydroxide gel in the presence of 20 m*M* acetate and several phosphate ion concentrations is shown in Figure 5. Adsorption of this antigen in 20 m*M* sodium acetate and 130 m*M* NaCl was similar to the results observed in 150 m*M* NaCl and does not significantly differ from zero. However, the presence of 20 m*M* phosphate greatly enhanced the adsorption level, up to 50% antigen adsorption being observed.

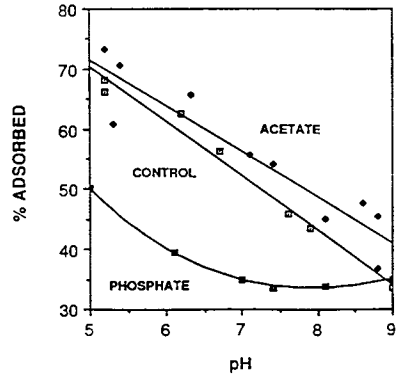

Fig. 3. Effect of pH on the adsorption of R32NS181 to aluminum hydroxide gel in the presence of buffer ions; protein/Al^{3+} ratio, 1.2/1.0. Control, 150 m*M* NaCl. Acetate, 20 m*M* sodium acetate and 130 m*M* NaCl. Phosphate, 20 m*M* sodium phosphate and 110 m*M* NaCl.

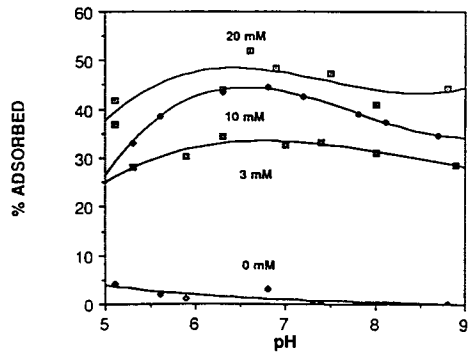

Fig. 5. Effect of pH on the adsorption of R32tet32 to aluminum hydroxide gel in the presence of buffer ions at a protein/Al^{3+} ratio of 1.2/1.0. Zero phosphate concentration corresponds to a solvent composition of both 150 m*M* NaCl and 130 m*M* NaCl plus 20 m*M* sodium acetate. The increasing phosphate concentration curves were obtained on samples with constant ionic strength at pH 7.0.

There was an increase in adsorption levels from pH 5 to pH 7. Above pH 7, the level of adsorption was approximately constant. The increasing phosphate concentration curves appear to be converging to the 20 m*M* phosphate curve. This is more clearly displayed in Fig. 6, where the adsorption level at pH 7.0 at each concentration of buffer is plotted against the buffer concentration. This curve shows a maximum level of adsorption for 20 m*M* phosphate.

Antigen Binding to Aluminum Phosphate

Aluminum phosphate gel has an oppositely charged surface relative to aluminum hydroxide gel at physiological pH as determined by its PZC of 4.5. The pH-dependent adsorption behavior of the two acidic antigens, R32NS181 and NS181V20, with aluminum phosphate gel in 150 m*M* NaCl is presented in Fig. 7. Antigen R32NS181, with an isoelectric point of 5.9, did not adsorb to any measurable extent over the pH range studied. In comparison, this protein displayed an adsorption level of 70% at pH 5.0 to the aluminum hydroxide gel (Fig. 1). The adjuvant surface charge seems to be the controlling factor in this case.

Antigen NS181V20, with an isoelectric point of 5.5, displayed a higher degree of adsorption to aluminum phosphate gel with respect to R32NS181, but its adsorption was decreased by approximately 50% relative to its interaction with the aluminum hydroxide gel (Fig. 1). Both acidic antigens displayed markedly diminished binding to the aluminum phosphate adjuvant but the NS181V20 polypeptide still exhibited some reversible adsorption.

The basic antigen, R32tet32, displayed very different adsorption characteristics to aluminum phosphate gel (Fig. 8) as follows: (i) there was significant binding in isotonic saline in the absence of added buffer ions, (ii) the extent of adsorption increased as the solution became more alkaline, and (iii) nonreversible binding was observed, as indicated by data points 6 and 7. This nonreversible binding was similar to that observed for pepsin adsorption to aluminum hydroxide gel by Sepelyak *et al.* (6,7). No other desorption studies were conducted on the R32tet32/aluminum phosphate system other than the pH variation.

DISCUSSION

Reversible Adsorption

Data in Fig. 1 demonstrate that adsorption of the acidic malarial antigens to aluminum hydroxide is reversible. Additionally, the acidic antigens are maximally adsorbed at the lowest pH studied, pH 5.0. The surface charge of aluminum hydroxide gel is large and positive at pH 5.0 due to the PZC of 9.8. Conversely, pH 5 approximates the isoelectric points of the two acidic antigens, R32NS181 and NS181V20. Therefore these polypeptides display minimal net surface charge at this pH. It is known that protein adsorption tends to be maximal at the isoelectric point, as protein–protein electrostatic interactions are at a minimum (12–15). However, this reasoning alone does not account for the difference between the adsorption of the acidic antigens to aluminum hydroxide and that to aluminum phosphate gels (compare Figs. 1 and 7). Under optimal conditions, i.e., near the antigen *p*I, the driving force for adsorption appears to be the surface charge (including both sign and magnitude) of the aluminum-based adjuvant gel. The reversibility of this effect with changes in pH is indicative of an adsorption mechanism that is noncovalent and electrostatic in origin.

Reversibility of protein adsorption to solid surfaces has also been suggested to be dependent on the nature of the polypeptide. Time-dependent conformational changes can lead to multiple attachment sites and result in irreversible adsorption (14,20). The reversible adsorption studied here

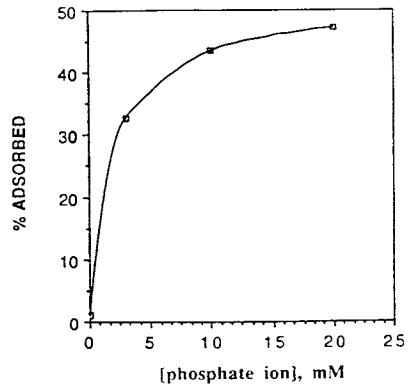

Fig. 6. Effect of phosphate concentration on the adsorption of R32tet32 to aluminum hydroxide gel at pH 7.0 and constant ionic strength.

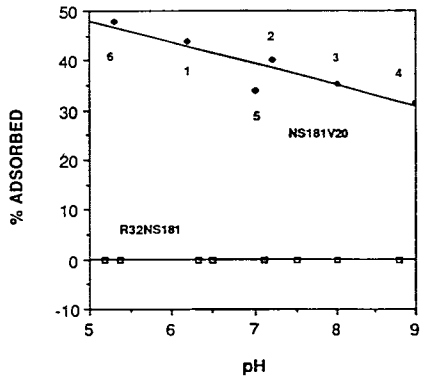

Fig. 7. Effect of pH on the adsorption of NS181V20 and R32NS181 to aluminum phosphate gel; protein/Al^{3+} ratio, 1.2/1.0, in 150 m*M* NaCl. The numbers indicate the sequence in which the experiments were performed.

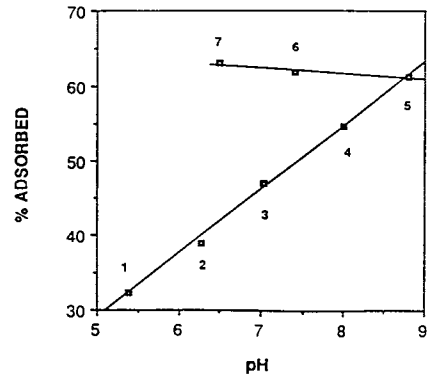

Fig. 8. Effect of pH on the adsorption of the R32tet32 to aluminum phosphate gel; protein/Al^{3+} ratio, 1.2/1.0, in 150 m*M* NaCl. The numbers indicate the sequence in which the experiments were performed.

was determined to occur readily even after three cycles of acid and alkaline pH levels but was not evaluated over extended periods of time (days or weeks). Based on their amino acid sequence, the random coil regions of the malarial antigens contribute very little to their charges but may contribute to the highly reversible adsorption seen for the two acidic polypeptides.

pH Dependence on Binding

The malarial antigens discussed here display a linear pH dependence of adsorption over the pH range 5–9 (see Figs. 1 and 8). This is not a typical titration curve for a single ionic species in solution, either for the aluminum-based adjuvant or for a specific amino acid. Sepelyak *et al.* (6) observed a pH-dependent adsorption of pepsin onto aluminum hydroxide gel that behaved as if a single amino acid or group of amino acids with similar p*K*a's was responsible for the adsorptive behavior. In the pepsin example it was concluded that a ligand-exchange mechanism was the basis for protein adsorption. The nonspecific nature of the pH-dependent adsorption observed here is an indication that an electrostatic mechanism is operative for the antigen–adjuvant combinations in 150 m*M* NaCl.

Figures 9a and b graphically describe the charge variation with pH of the antigens and aluminum hydroxide gel. Figure 9a describes the charge behavior of the acidic antigens, R32NS181 and NS181V20. Figure 9b describes that of the basic antigen, R32tet32. The zero crossings are determined by the isoelectric points of the proteins and the PZC of the aluminum adjuvant with the absolute magnitude and slopes of the lines being approximate. Figure 9a shows that as the net charge on the proteins decreases toward their *p*I's, the net surface charge on the aluminum hydroxide adjuvant remains significantly positive. It is at this pH that the maximum adsorption is observed. The converse situation of minimum charge on the adjuvant and maximum surface charge on the proteins at pH 9.8 results in very little protein adsorption. Figure 9b describes the experimental combination of antigen and adjuvant where no measurable adsorption was observed throughout the pH range 5–9. The basic nature of both the R32tet32 antigen and the aluminum hydroxide gel results in similar surface charges over the pH range studied and electrostatic repulsion prevents intermolecular interactions. This experiment and the experimental results exhibited in Fig. 1 indicate that electrostatic binding is the major mechanism of antigen adsorption under these conditions.

Figures 10a and b depict similar pH–potential plots for the malarial antigens and aluminum phosphate gel. Figure 10a describes an example of unfavorable adsorption conditions for the acidic antigens: similar surface charge of the two components throughout the pH range studied and minimum adjuvant surface charge near the antigen *p*I. The dependence of adsorption on the isoelectric point is observed, however, by reference to Fig. 7, where maximal adsorption is observed at the lowest pH studied. Although the charge on the aluminum phosphate surface is approaching a minimum at pH 5 (near the *p*I of the antigen), this is the pH of maximum, albeit reduced, adsorption of NS181V20.

NS181V20 displays a greater adsorption to both the aluminum-based adjuvants than R32NS181. This difference is greater than would be expected based on its electrostatic properties alone. The chemical differences between these two antigens lie in the different repeat sequences of the *vivax* and *falciparum* parasites and in the N-terminal versus C-terminal placement of the NS181 region. Based on these chemical differences alone, the reason for the greater adsorption levels is not clear. However, structural differences, domain effects, or different adsorption mechanisms such as hydrogen bonding or hydrophobic interactions cannot be ruled out and may account for the observed results.

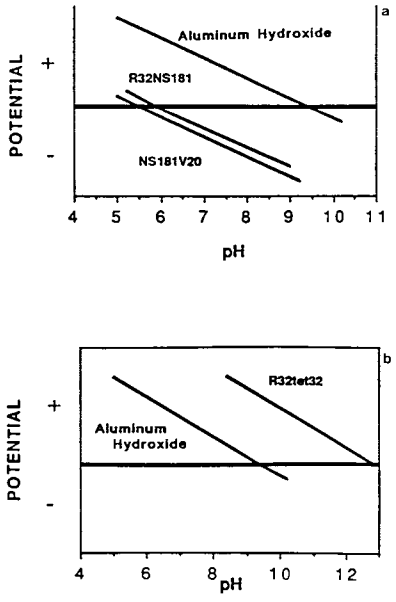

Fig. 9. Schematic of charge potential versus pH for malaria antigen adsorption to aluminum hydroxide gel: (a) R32NS181 and NS181V20 and (b) R32tet32.

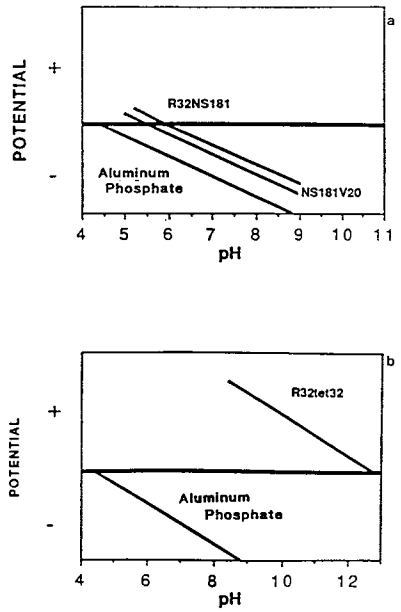

Fig. 10. Schematic of charge potential versus pH for malaria antigen adsorption to aluminum phosphate gel: (a) R32NS181 and NS181V20 and (b) R32tet32.

The irreversible adsorption observed between R32tet32 and the aluminum phosphate gel (Fig. 8) implies that a different adsorption mechanism may be operative. The initial pH-dependent adsorption increases toward alkaline pH as predicted by the pH–potential plot (Fig. 10b). The irreversible adsorption may be the result of a specific protein domain effect. The tail of the R32tet32 antigen contains a highly charged region that contains eight arginine residues. The neutral form of the guanidino groups of these residues may interact strongly with the negatively charged surface phosphate groups of the adjuvant thereby leading to an irreversibly adsorbed system. Phosphate buffer increases the adsorption of this antigen to the aluminum hydroxide gel surface possibly through a similar mechanism (see below).

Effect of Buffer Ions on Adsorption to Aluminum Hydroxide Gel

In Figs. 3–5 the addition of 20 m*M* sodium acetate to the protein–adjuvant suspension does not affect the adsorption level of the antigens. Acetate is predominantly ionized throughout the pH range studied. Although more strongly basic compounds do interact with the positively charged aluminum hydroxide gel (17), the constancy of the antigen adsorption results indicate that the acetate anion does not interact with the aluminum gel surface.

Also in Figs. 3–5, the presence of 20 m*M* sodium phosphate significantly alters the protein adsorptive behavior. The reversible pH dependence of the phosphate adsorption curves corresponds to a titration of $H_2PO_4^-$ (pK_a = 7.2). Therefore the adsorption of the acidic malarial antigens is dependent on the ionization state of the phosphate buffer. The monobasic phosphate ion, HPO_4^{2-}, which is present above pH 7.2, is known to interact covalently with the positively charged aluminum sites on the gel surface (6,17,21). This results in an altered adjuvant surface charge and a lowered PZC. The decreased antigen adsorption is caused by an ionic attachment of monobasic phosphate to the aluminum surface sites, which then results in a more negative surface charge as described by a lower PZC. Although the charge complementarity between antigen and adjuvant is not optimal, as evidenced by the decreased adsorption at pH 5.0, the isoelectric pH is again the pH of maximum adsorption for the acidic antigens.

Conversely, the effect of 20 m*M* phosphate on the adsorption of the basic protein R32tet32 to aluminum hydroxide gel dramatically increases the level of adsorption (Fig. 5). These adsorption curves are indicative of the titration of a species with a pK_a of approximately 7 and the effect of phosphate concentration seems to be saturated at approximately 20 m*M*. Again, monobasic phosphate alters the antigen adsorption level by binding to the positively charged aluminum surface, thereby lowering the PZC of the adjuvant. The resultant more negative surface charge creates a favorable adsorption environment for the basic R32tet32 antigen. A schematic of such a ligand-mediated adsorption process is given in Fig. 11.

This work indicates that there are two possible methods of improving the adsorption levels of a poorly adsorbed antigen. Given a protein with either an acidic or a basic *p*I, the complementary charged surface adjuvant, aluminum hydroxide or aluminum phosphate gel, can be chosen for maximum adsorption. Alternatively, if aluminum hydroxide is the adjuvant of choice because of its higher surface area and because it is a more commonly used vaccine component, the surface charge can be modified *in situ* by the presence of phosphate ions or other divalent anions, thereby producing a fully adsorbed vaccine antigen.

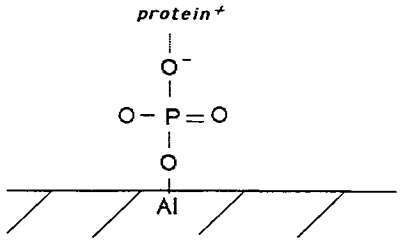

Fig. 11. Schematic model of a basic protein binding to an aluminum hydroxide surface via a covalently adsorbed phosphate anion.

The above discussion has established the importance of the physical properties of the adjuvant on adsorption by several malarial antigen proteins. Any perturbation that can alter the surface charge, such as covalent interactions with buffer ions or changes in the degree of crystallinity, should be monitored for reproducible vaccine preparations. Both of these parameters have been shown to have an influence on the levels of antigen adsorption as can be observed by variation in protein binding to aluminum hydroxide containing different degrees of crystallinity and on the phosphate dependence of the aluminum hydroxide PZC. The choice of buffer can also alter the adjuvant adsorption characteristics.

Different types of protein binding can be observed such as electrostatic or ligand-mediated, reversible or irreversible, depending on the adsorption conditions. Although several different adsorption conditions can lead to a fully adsorbed antigen, the mechanism of binding and the adsorbed antigen structure may be important in controlling the immune process. Therefore, animal studies are needed in conjunction with additional physical characterization studies to understand further adjuvant action and to optimize immune protection.

ACKNOWLEDGMENTS

We would like to thank Donna Dunleavy, John Baldoni, Robin Roman, Bill Vincek, and Don McCarthy for their advice regarding the study and review of the manuscript.

REFERENCES

1. M. A. Aprile and A. C. Wardlaw. Aluminum compounds as adjuvants for vaccines and toxoids in man: A review. *Can. J. Public Health* 57:343–354 (1966).
2. R. Edelman. Vaccine adjuvants. *Rev. Infect. Dis.* 2:370–383 (1980).
3. H. S. Warren, F. R. Vogel, and L. A. Chedid. Current status of immunological adjuvants. *Annu. Rev. Immunol.* 4:369–388 (1986).
4. H. S. Warren and L. A. Chedid. Future prospects for vaccine adjuvants. *CRC Crit. Rev. Immunol.* 8:83–101 (1988).
5. C. Hanndun. Vaccines against influenza. In A. Voller and H. Friedman (eds.), *New Trends and Developments in Vaccines*, MTP Press, Lancaster, England, 1978, p. 65.

6. R. J. Sepelyak, J. R. Feldkamp, T. E. Moody, J. L. White, and S. L. Hem. Adsorption of pepsin by aluminum hydroxide I: Adsorption mechanism. *J. Pharm. Sci.* 73:1514–1517 (1984).
7. R. J. Sepelyak, J. R. Feldkamp, F. E. Regnier, J. L. White, and S. L. Hem. Adsorption of pepsin by aluminum hydroxide II: Pepsin inactivation. *J. Pharm. Sci.* 73:1517–1522 (1984).
8. S. L. Hem and J. L. White. Characterization of aluminum hydroxide for use as an adjuvant in parenteral vaccines. *J. Parent. Sci. Technol.* 38:2–10 (1984).
9. S. Shirodkar, R. L. Hutchinson, D. L. Perry, J. L. White, and S. L. Hem. Aluminum compounds used as adjuvants in vaccines. *J. Pharm. Res.* 7:1282–1288 (1990).
10. G. A. Parks. The isoelectric points of solid oxides, solid hydroxides, and aqueous hydroxo complex systems. *Chem. Rev.* 65:177–198 (1965).
11. G. Folena-Wasserman, R. Inacker, and J. Rosenbloom. Assay, purification and characterization of a recombinant malaria circumsporozoite fusion protein by high performance liquid chromatography. *J. Chromatogr.* 411:345–354 (1987).
12. F. MacRitchie. Proteins at interfaces. *Adv. Protein Chem.* 32:283–326 (1978).
13. W. Norde. Adsorption of proteins at solid surfaces. In L.-H. Y. Lee (ed.), *Adhesion and Adsorption of Polymers, Vol. 2,* Plenum Press, New York, 1980, pp. 801–826.
14. J. D. Andrade. Principles of protein adsorption. In J. D. Andrade (ed.), *Surface and Interfacial Aspects of Biomedical Polymers, Vol. 2,* Plenum Press, New York, 1985, pp. 1–80.
15. T. A. Horbett and J. L. Brash. Proteins at interfaces: Current issues and future prospects. In J. L. Brash and T. A. Horbett (eds.), *Proteins at Interfaces: Physicochemical and Biochemical Studies,* ACS Symposium Series, Vol. 343, ACS, Washington, D.C., 1987, pp. 1–33.
16. W. Norde, F. MacRitchie, G. Nowicka, and J. Lyklema. Protein adsorption at solid-liquid interfaces: Reversibility and conformational aspects. *J. Colloid Interface Sci.* 112:447–456 (1986).
17. R. G. Gast. Aluminum hydroxides and oxyhydroxides. In J. B. Dixon and S. B. Weed (eds)., *Minerals in Soil Environments,* Soil Society of America, Madison, WI, 1977, pp. 99–143.
18. J. R. Feldkamp, D. N. Shah, S. L. Meyer, J. L. White, and S. L. Hem. Effect of adsorbed carbonate on surface charge characteristics and physical properties of aluminum hydroxide gel. *J. Pharm. Sci.* 70:638–640 (1981).
19. R. Tettenhorst and D. A. Hofmann. Crystal chemistry of boehmite. *Clays Clay Miner.* 28:373–380 (1980).
20. B. W. Morrissey and R. R. Stromberg. The conformation of adsorbed blood proteins by infrared bound fraction measurements. *J. Colloid Interface Sci.* 46:152–164 (1974).
21. J. Katz. Desorption of porcine parvovirus from aluminum hydroxide adjuvant with subsequent viral immunoassay or hemagglutination assay. *Vet. Res. Commun.* 11:83–92 (1987).