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Adsorption of Globular Proteins to Vaccine Adjuvants

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Abstract : The maximum adsorption/desorption conditions and the adsorption mechanism of globular proteins to vaccine adjuvants were determined. The maximum adsorption ratio of protein to the Al^{3+} content of aluminum oxyhydroxide and the optimal adsorption pH are 2:1 (μg:μg) for bovine serum albumin (BSA) at pH 6.0 and 2.5:1 (μg:μg) for immunoglobulin G (IgG) at pH 7.0, respectively. The maximum adsorption ratio onto aluminum phosphate gel was 1.5:1 (μg Protein:μg Al^{3+}) at pH 5.0 for both BSA and IgG. Adsorption of the native globular proteins, BSA and IgG, to aluminum oxyhydroxide and aluminum phosphate gel was reversible as a function of pH. Complete desorption of these proteins from aluminum phosphate gel was observed at alkaline pH, whereas only 80~90% removal from aluminum oxyhydroxide was achieved with alkaline pH and 50 mM phosphate buffer. We conclude that electrostatic and hydrogen bonding interactions between the native proteins and adjuvants are important binding mechanisms for adsorption, and that the surface charge of the protein and the colloid components control the maximum adsorption conditions.

Key words : adsorption, aluminum phosphate gel, aluminum oxyhydroxide, bovine serum albumin (BSA), desorption, vaccine adjuvant, immunoglobulin G (IgG)

To stimulate an optimal immune response, a vaccine is composed of both antigenic and adjuvant components; the antigen or epitope elicits a specific immune response while the adjuvant enhances this response (Edelman, 1980). Vaccines composed of killed whole viruses or bacteria can be self-adjuvanting owing to the mixture of components present; however, highly purified subunit antigens alone are inactive and require the presence of an effective immunological adjuvant. A wide variety of compounds, such as liposomes, detoxified lipopolysaccharide, peptides, lymphokines and polymeric species (White *et al.*, 1991; Stieneker *et al.*, 1995) are under investigation but the only approved adjuvants in the United States are aluminum-based suspensions such as precipitated alum ($KAl(SO_4)_2 \cdot 12H_2O$) and preformed aluminum hydroxide and aluminum phosphate gels (Schirodkar *et al.*, 1990).

Effective aluminum-based adjuvants adsorb the proteinaceous antigen completely which prevents rapid enzymatic degradation and results in a delayed release after injection. The presence of the aluminum mineral adjuvants also stimulates the presence of immunocompetent cells to the area of injection. Irritation induced by aluminum suspension is a function of the physicochemical properties of the adjuvant alone, while the depot or delayed release 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, few studies have been carried out in order to elucidate the mechanism of antigen adsorption to aluminum oxyhydroxide and aluminum phosphate gel (Sepelyak *et al.*, 1984; Seeber *et al.*, 1991; Callahan *et al.*, 1991; Al-Shakhshir *et al.*, 1994; Al-Shakhshir *et al.*, 1995; Rinella *et al.*, 1996).

Precipitated aluminum oxyhydroxide has variable physical properties depending on the pH and buffer ions present during precipitation (Schirodkar *et al.*, 1990). For this reason, this work focused only on the preformed aluminum oxyhydroxide and aluminum phosphate gel. Commercially available aluminum hydroxide gel is composed of crystalline material known as boehmite with a small particle size and a large surface area and has the empirical formula, Al(O)(OH), (aluminum oxyhydroxide) (Schirodkar *et al.*, 1990). The point of zero charge (PZC) of aluminum oxyhydroxide gel is in the range of 9 to 11 (Schirodkar *et al.*, 1990; Seeber *et al.*, 1991; Callahan *et al.*, 1991). This results in an increasingly positively charged surface below pH 9. The second

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adjuvant studied, aluminum phosphate gel, is an amorphous material (Schirodkar *et al.*, 1990). The surface charge of the aluminum phosphate gel is negative at neutral pH which is a consequence of its PZC ranges from 4.5 to 6.5 (Schirodkar *et al.*, 1990; Seeber *et al.*, 1991; Callahan *et al.*, 1991). These differences in surface area and surface charge of the aluminum oxyhydroxide and aluminum phosphate gels are expected to greatly impact their antigen adsorption behavior.

Previous adsorption studies of model proteins (Seeber *et al.*, 1991; Al-Shakhshir *et al.*, 1994; Al-Shakhshir *et al.*, 1995; Rinella *et al.*, 1996) and malaria antigens (Callahan *et al.*, 1991) to aluminum-containing adjuvants have demonstrated that the electrostatic attractive forces are important in maximizing the adsorption of antigens. Furthermore, if there are electrostatic repulsive forces, the attractive forces may not be sufficient to promote adsorption of proteins by aluminum-containing adjuvants (Al-Shakhshir *et al.*, 1995). The observation was also made that the optimum conditions for antigen-adjuvant interactions were a function of surface charge of both the ionization state of protein and for the aluminum adjuvants.

Because of the colloidal nature of the adjuvant gels, 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.

The objectives of this work are: 1) to determine the binding mechanism and the extent of reversibility of interactions; 2) to quantitate maximum adsorption conditions for the next following Raman studies. In this work we have used two model antigens (bovine serum albumin and immunoglobulin G) of a distinctly different secondary structure to probe their adsorption characteristics.

Materials and Methods

Materials

The mineral adjuvants used in this study were obtained commercially: aluminum oxyhydroxide gel (Alhydrogel) and aluminum phosphate gel (Adju-Phos) were obtained from Seargent (Clifton, USA). The buffers and proteins used were obtained from Sigma (St. Louis, USA).

Adsorption isotherm experiment

The concentration dependence of adsorption was monitored in the following manner. After the protein and vaccine adjuvant were mixed and incubated for 15 minutes, the samples were centrifuged for 15 minutes at 12,400 rpm in a Beckman microfuge. The supernatant was removed and assayed in triplicate with the Pierce standard (37°C, 30 minutes) bicinchoninic acid (BCA) dye binding assay using a Hitachi U-3100 spectrophotometer. The amount of adsorbed protein was determined by the difference between initial and final protein concentration. The ionic strength was held approximately constant at 150 mM for all the experiments. Concentrations of the aluminum oxyhydroxide and aluminum phosphate gels are reported as total concentration of the aluminum ion, Al^{3+}.

pH dependent adsorption experiment

The pH dependent adsorption experiments were performed according to the procedure of Callahan *et al.* (1991); the method is summarized here. BSA or IgG in 150 mM NaCl was added to a suspension of adjuvant gel to make an ionic strength of 150 mM at 25°C. The pH was varied by addition of 1 N NaOH or 1 N HCl. The initial, unbuffered pH was around 6 and the sequence of pH values started at approximately pH 4, increased by single pH units to 10, and then was reversed through several different pH values to a final pH of approximately 4. The pH of the antigen-adjuvant solutions was held constant by pH electrode. The solution was maintained at each pH point for 15 minutes with continuous stirring, and aliquots were removed for assay. The samples were centrifuged for 15 minutes at 12,400 rpm in a Beckman microfuge. The supernatant was removed and assayed in triplicate with the Pierce standard BCA assay. The volume of acid or base needed to vary the pH was recorded and typically around 0.1 ml of titrant was added during the course of an experiment. The reported protein concentrations have been corrected for the dilution that occurred. Control experiments to monitor protein solubility and the sensitivity of the dye binding assay as a function of pH were performed: no pH sensitivity of the protein concentration was observed in the absence of the adjuvant.

Measurement of point of zero charge (PZC)

The PZCs of Alhydrogel and aluminum phosphate gel used in this study were determined by using a Doppler electrophoretic light scattering analyzer (DELSA 440, Coulter Electronics, Inc., Hialeah, FL, USA). The PZC measurements of the mineral adjuvants were obtained. The PZC of aluminum oxyhydroxide and aluminum phosphate gel were determined to be 10 and 5.3, respectively.

Results and Discussion

pH dependent adsorption

The pH of maximum adsorption of BSA and IgG to Alhydrogel were pH 6.0 and 7.0, respectively (Fig. 1). These pH values are close to the isoelectric point of

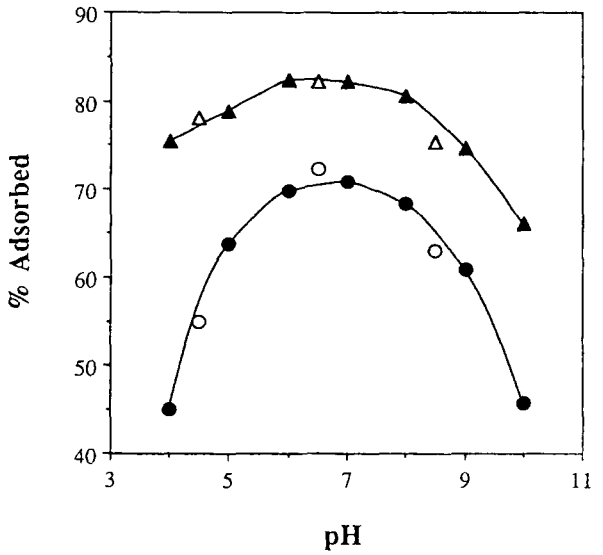

Fig. 1. Effect of pH on the adsorption of BSA and IgG to Alhydrogel: BSA:Al^{3+}=2 μg:1 μg and IgG:Al^{3+}=2.5 μg:1 μg in 150 mM NaCl. Key: (▲) BSA: (△) reversibility of BSA: (●) IgG: (○) reversibility of IgG.

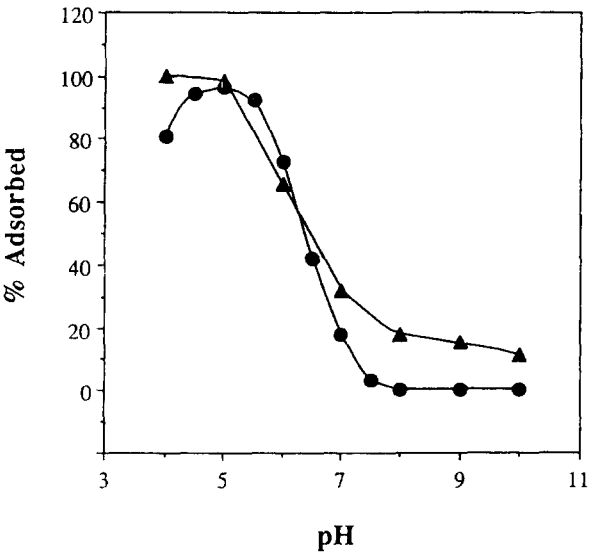

Fig. 2. Effect of pH on the adsorption of BSA and IgG to aluminum phosphate gel: BSA & IgG:Al^{3+}=1 μg:1 μg in 150 mM NaCl. Key: (▲) BSA: (●) IgG.

these two proteins. Under these conditions. BSA adsorption is only weakly sensitive to pH. whereas IgG displays a greater pH sensitivity. as evidenced by ~45% adsorption levels at the pH extremes. At the high pH extreme. the minimum adsorption occurred at pH levels greater than 9.0: which approximates the PZC of the Alhydrogel suspension. At the PZC. the net surface charge is zero. and there is minimal electrostatic attraction between oppositely charged species. In the case of adsorption of IgG to aluminum oxyhydroxide gel. the minimum adsorption was observed at a pH lower than 5. At the low pH extreme. both IgG and Alhydrogel have positively charged surfaces which can cause electrostatic repulsion. The interactions between these moedel antigens and the Alhydrogel vaccine adjuvant were reversible as a function of pH which indicates the presence of titratable charges and possible hydrogen bonding interactions. For adsorption of these two proteins to Alhydrogel. the behavior is governed in large part by the pI of the antigens.

The pH dependent adsorption of BSA and IgG to aluminum phosphate gel are shown in Fig. 2 The pH of maximum adsorption of BSA to aluminum phosphate gel was pH 4.0~5.0. Near pH 5. BSA is slightly positively charged or near zero net charge. As noted above. the maximum adsorption level is observed at the pI of BSA. For IgG. the pH range of maximum adsorption is pH 4.5~5.5. In this pH range. the net surface charge of aluminum phosphate gel is small or slightly positive while IgG is net positively charged. This result does not follow the previously established pattern. that of maximum adsorption near the protein pI. In this case. however. maximum adsorption is observed at approximately the PZC of the Adju-Phos adjuvant. Closer to the pI of IgG. markedly decreased adsorption levels (0~30%) are observed. At pHs greater than 7. both protein and Adju-Phos are negatively charged. Therefore. both the PZC and pI are important for maximum adsorption conditions of BSA and IgG to aluminum phosphate gel. Reversible adsorption/desorption is observed for both proteins as a function of pH. These results indicate that an electrostatic or hydrogen bonding interaction is also important for these antigen-adjuvant systems. This is consistent with the observation of recent studies which have reported the importance of electrostatic attraction in the adsorption of model proteins as antigens by aluminum-containing adjuvants (Seeber *et al.*, 1991: Callahan *et al.*, 1991: Al-Shakhshir *et al.*, 1994: Al-Shakhshir *et al.*, 1995: Rinella *et al.*, 1996). Furthermore. the hydrophobic forces also contribute to adsorption of lysozyme onto aluminum phosphate gel in the presence of ethylene glycol. However. in the adsorption of the pepsin to aluminum hydroxide gel example. a ligand-exchange mechanism was the basis for protein adsorption (Sepelyak *et al.*, 1984).

The nonspecific nature of the pH dependent adsorption observed here is an indication that an electrostatic or hydrogen bonding mechanism is operative for the protein-adjuvant combinations in 150 mM NaCl.

Fig. 3 graphically describes the charge variation with pH of BSA and Alhydrogel and Adju-Phos. The zero crossings are determined by the pI of BSA and the PZC of aluminum adjuvants. The magnitude and slope of the protein line is calculated from the overall amino acid content of BSA. Fig. 3 shows that as the net

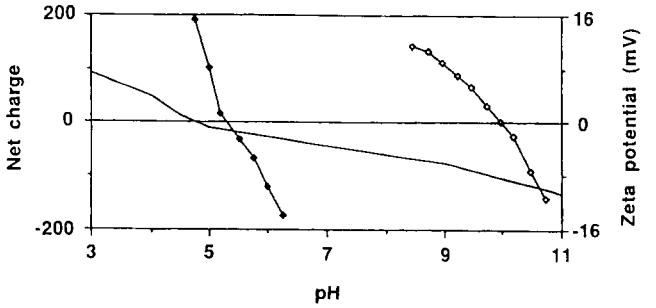

Fig. 3. Effect of pH on the surface charge of BSA and the vaccine adjuvants. Key: (—) BSA: (□) Alhydrogel: (■) aluminum phosphate gel. The Alhydrogel and aluminum phosphate gel data are experimentally determined. The magnitude and slope of BSA line is calculated from the pI of 4.7.

charge on BSA decreases toward its pI. the net surface charge on the Alhydrogel adjuvant remains significantly positive. It is at this pH that the maximum adsorption of BSA onto Alhydrogel is observed. The inverse situation is observed for BSA adsorption to Adju-Phos. In this case. the minimum charge on the adjuvant and maximum surface charge on the protein at pH 10 results in very little (~10%) protein adsorption. This example is distinct from the Alhydrogel results in that the net surface charge on the Adju-Phos adjuvant becomes small upon reaching the pI of BSA. Although the charge on the aluminum phosphate surface approaches a minimum at pH 5 the reduced electrostatic barriers to interaction of BSA at its pI (near pH 5) results in maximum adsorption conditions of BSA to aluminum phosphate gel.

Fig. 4 depicts similar pH-potential plots for IgG interaction with Alhydrogel and Adju-Phos. This figure predicts unfavorable adsorption conditions at pH levels below and above the pI of IgG where there is a similar surface charge of IgG and Alhydrogel adjuvant. Therefore. in the case of IgG adsorbed to Alhydrogel. the adsorption behavior is dependent on the isoelectric point.

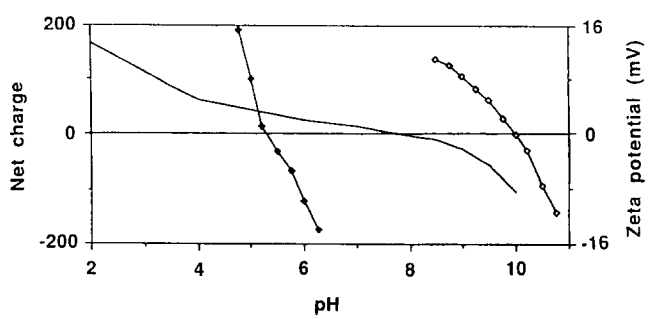

Fig. 4. Effect of pH on the surface charge of IgG and the vaccine adjuvants. Key: (—) IgG: (□) Alhydrogel: (■) aluminum phosphate gel. The Alhydrogel and aluminum phosphate gel data are experimentally determined. The magnitude and slope of IgG line is calculated from the pI of about 7.5.

For the second adjuvant. the charge on the aluminum phosphate surface approaches a minimum at pH 5 which is several pH units away from the pI of the antigen and is the pH of maximum adsorption of IgG. When the net surface charge (above pH 7) on both aluminum phosphate gel and IgG both become negative. the minimum interaction is observed. Therefore. for this combination. the minimum net charge on the Adju-Phos surface governs the maximum adsorption conditions. Similarly. repulsive electrostatic interactions above pH 7 govern the minimum adsorption conditions.

Adsorption isotherms

By working at the pH of maximum adsorption. the concentration dependence of BSA and IgG adsorption to aluminum oxyhydroxide gel was determined (Fig. 5). After a linear region of adsorption. both proteins adsorb to a level of approximately 2 μg protein/μg Al^{3+}. although IgG displays higher adsorption at higher bulk solution concentrations. The maximum adsorption ratio of BSA and IgG to the Al^{3+} content of Alhydrogel was 2.0 and 2.5 μg protein/μg Al^{3+}. respectively (Fig. 5). At high solution concentrations. the adsorption of BSA decreases. This may be a consequence of a change in the surface orientation of BSA on the Alhydrogel surface. The slight dip in the adsorbed amount of BSA at 600 μg/ml is reproducible.

Conditions (pH 10 and 50 mM phosphate buffer) were established to desorb 80~90% of the adsorbed polypeptides from Alhydrogel. Circular dichroism spectra of the soluble proteins were obtained both prior to adsorption and after desorption (date not shown).

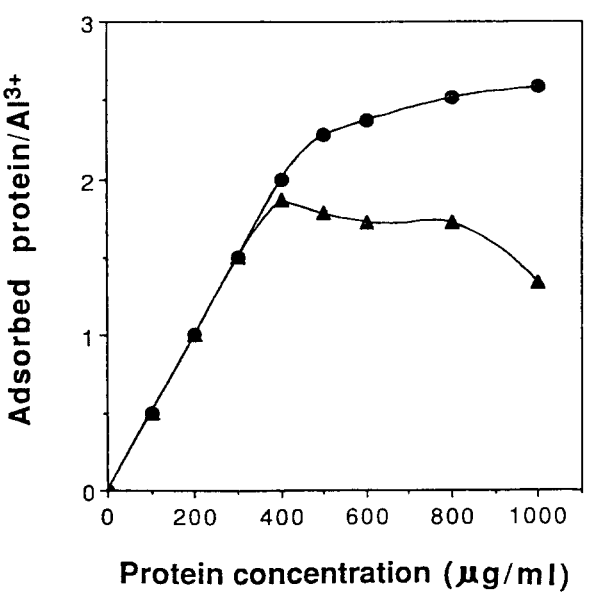

Fig. 5. Adsorption isotherm of BSA and IgG to Alhydrogel: Al^{3+} (200 μg/ml) Key: (▲) BSA: (●) IgG.

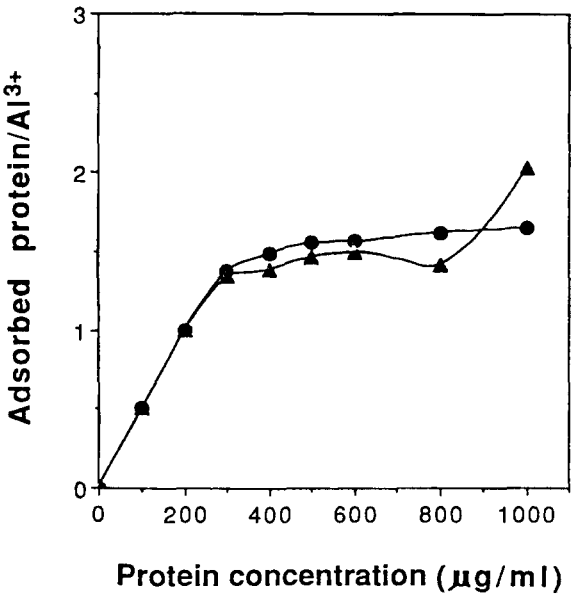

Fig. 6. Adsorption isotherm of BSA and IgG to aluminum phosphate gel at pH 5.0: Al^{3+} (200 μg/ml). Key: (▲) BSA; (●) IgG.

There was no observable difference in ellipticity for the two sample conditions, which indicate that there are no irreversible structural modifications imposed by the adjuvant system.

The adsorption behaviors of BSA and IgG to aluminum phosphate gel are presented in Fig. 6. The maximum adsorption levels are observed to be at 1.5 μg protein/μg Al^{3+} content of Adju-Phos for both BSA and IgG. Similar to the Alhydrogel results, the adsorption behavior of IgG follows an approximate Langmuir isotherm. The adsorption of BSA onto aluminum phosphate gel levels off with increasing protein concentration and a slight dip is observed at 800 μg/ml. Above this concentration, the adsorption level of BSA shows a sudden increase. The greater adsorption of these two proteins to Alhydrogel rather than to Adju-Phos may be a result of the higher surface area of Alhydrogel.

The adsorbed forms of BSA and IgG do not display even the slightly modified conformations of the solid forms of these proteins. This indicates that negligible protein-protein interactions are induced by adsorption to Alhydrogel and Adju-Phos. Similarly, the completely reversible nature of protein binding to Adju-Phos surface upon pH variation suggests that few, if any, of the protein molecules undergo multi-site attachment to the hydrophilic adjuvant surface. In the case of Alhydrogel, pH modification alone could remove the majority of the adsorbed protein, but 50 mM phosphate buffer was needed to remove up to 80~90% of the remaining polypeptides. Phosphate anion is known to specifically adsorb to aluminum hydroxide by anionic ligand exchange (Liu *et al.*, 1984; Sepelyak *et al.*, 1984); therefore it is able to displace the protein molecules by competitive binding at the adjuvant surface. It has also been shown that phosphate anions desorb protein from the surface of aluminum oxyhydroxide (Nishida *et al.*, 1992; Rinella *et al.*, 1995). These data describe the adsorbed environment of BSA and IgG to the aluminum vaccine adjuvants as one in which single-site attachment or a small number of attachment sites is present. The hydrophilic surface environment prevents protein aggregation behavior as seen for the dried, solid state forms of the proteins. The combined electrostatic and possible hydrogen bonding and ligand exchange binding mechanism present in this system result in mild, solution-like conditions for the adsorbed proteins with the end result of essentially constant secondary structures for solution and adsorbed states of BSA and IgG.

A summary of this study is that the adsorption behavior between both BSA and IgG, and Alhydrogel is governed in large part by the isoelectric point of the antigens. In the case of the interaction between these proteins and aluminum phosphate gel, both the point of zero charge and the isoelectric point are important for maximum adsorption conditions. However, low net charges on either the protein or the surface result in maximum adsorption for either combination. Both the pH range and the nature of the molecule govern the adsorption behavior of proteins onto the Alhydrogel surface.

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