Characterization of the MN gp120 HIV-1 Vaccine: Antigen Binding to Alum

Robert P. Weissburg, Phillip W. Berman, Jeffrey L. Cleland, Donna Eastman, Francis Farina, Steve Frie, Amy Lim, Joyce Mordenti, Michael R. Peterson, Kalvin Yim, and Michael F. Powell[1]

Received December 13, 1994; accepted June 6, 1995

Purpose. The characterization of recombinant MN gp120/alum vaccine requires the study of the gp120-alum interaction for the successful formulation of an alum-based HIV-1 vaccine.
Methods. Several observations suggest that the gp120-alum interaction is weak, wherein buffer counterions such as phosphate, sulfate, bicarbonate may cause the desorption of gp120 from alum. Comparison of gp120 with other proteins using particle mobility measurements shows that the weak binding of gp120 to alum is not an anomaly. Serum and plasma also cause desorption of gp120 from alum with a half-life of only a few minutes, wherein this half-life may be faster than the in-vivo recruitment of antigen presenting cells to the site of immunization.
Results. Immunization of guinea pigs, rabbits and baboons with gp120 formulated in alum or saline demonstrated that alum provides adjuvant activity for gp120, particularly after early immunizations, but the adjuvant effect is attenuated after several boosts.
Conclusions. These observations indicate that both the antigen *and* the adjuvant require optimization together.

KEY WORDS: gp120; AIDS-HIV-1 vaccine; alum adjuvant; aluminum hydroxide.

INTRODUCTION

The design of an effective HIV-1 vaccine for the prevention of HIV-1 infection or the immunotherapeutic treatment of AIDS requires an appropriate antigen from a relevant serotype (1) presented in a functionally relevant conformation (2). The HIV-1 surface glycoprotein gp120 is an excellent candidate for vaccine design because either a humoral or cell-mediated immune response to this protein may neutralize HIV-1. Many of the HIV-1 subunit vaccines currently in clinical trials use alum as adjuvant because alum is the only adjuvant in vaccines currently approved in the US. Because the adjuvant is crucial in determining the magnitude, breadth and duration of the immune response to the administered antigen, we sought to characterize our alum-containing gp120 formulations in order to ensure their chemical and physical formulation stability. This study is particularly germane because a complex particulate formulation such as an alum-based vaccine needs to consider several factors including: kinetic and equilibrium factors affecting the binding of the protein to the alum, the effect of alum-aging wherein recrystallization of the alum occurs over time, and the in-vivo desorption of the protein from alum. 'Alum' has several different forms, including aluminum hydroxide and oxyhydroxides, aluminum phosphate gels (3,4,5) and precipitated alum [$KAl(SO_4)_2 \cdot 12H_2O$]. We focused our studies on the commercially available aluminum hydroxide products Resorptar, Rehydragel HPA and Alhydrogel with the goal of determining excipient effects on the physical nature of the formulation, as well as determining if different alum types affect the immunogenicity of gp120.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals used were ACS grade or better, and were used without further purification. All solutions were prepared using deionized water that was further purified with a Millipore Milli-Q water purification system. Gp120 was obtained from a CHO cell fermentation process, and was purified until it was endotoxin-free. Several proteins were studied for comparison to gp120 (see Figure 4), and were obtained from Genentech or Sigma. Protein stock solutions were prepared in MES buffer [2–10 mg/mL protein, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) (U.S. Biochemical Corporation) adjusted to pH 6.00 with sodium hydroxide (Sigma)]. Alum was obtained as a 2% suspension (Alhydrogel, E. M. Sergeant Chemical Co., Clifton, New Jersey, Rehsorptar or Rehydragel HPA, Reheis, Berkeley Heights, New York) and a 3 mg/mL working alum stock suspension was prepared with MES buffer for further use. Alhydrogel is crystalline aluminum oxyhydroxide (AlOOH, known mineralogically as boehmite), and consists of corrugated sheets of aluminum-containing octahedra. It is obtained by precipitation of aluminum hydroxide under alkaline conditions. Rehsorptar and Rehydragel HPA are structurally similar, and are synthetic oxyhydroxides of aluminum (aluminum hydroxide) prepared by acid-base precipitation. Rehydragel HPA, because of its crystalline morphology, has a higher surface area/volume ratio than the other forms of 'alum', and so usually demonstrates higher protein loading.

Measurement of Adsorbed and Desorbed Protein from Alum

Adsorption experiments were carried out by adding a known volume of alum suspension to a stock solution of gp120 in tris buffer (20 mM tris, 120 mM NaCl, pH 7.4) with gentle stirring. Aliquots were withdrawn at known times after mixing, and immediately centrifuged at 10,000g for 2 minutes. The supernatants were removed and assayed for total protein content by using either the Bio-Rad protein micro assay (Bio-Rad Laboratories, Richmond, California), or the bicinchoninic (BCA) protein assay (Pierce, Rockford, Illinois). The amount of protein bound to alum was usually obtained by subtraction from the amount found in the supernatant.

$$[\text{Protein}]_{\text{alum-adsorbed}} = [\text{Protein}]_{\text{initial}} - [\text{Protein}]_{\text{supernatant}} \quad (1)$$

[1] To whom correspondence should be addressed: Pharmaceutical R&D, Genentech, Inc., MS #82, 460 Point San Bruno Blvd., South San Francisco, California 94080.

0724-8741/95/1000-1439$07.50/0

Control experiments were often carried out to ensure protein mass balance; that is, 100% recovery of the protein in the supernatant and bound to alum. Desorption experiments were carried out in a similar manner and the time course was initiated with the addition of the desorbing counterion stock solution to human serum or plasma. In the serum desorption experiments, the serum was diluted 1:1 making a 50% final serum concentration. Radiolabeled ^{125}I-MN gp120 used in these desorption experiments was prepared by the standard Enzymobead (BioRad) method, and purified over a desalting column. The radiogel identification of the product was done using Tris-Glycine Novex gels (Novex, San Diego, California) under denaturing conditions, with radio gel analysis.

Electrophoretic Mobility Determination

Mobility measurements were made by using Doppler electrophoretic laser light scattering as measured by a Coulter DELSA 440 analyzer (Coulter Scientific Instruments, Hialeah, Florida). The protein solutions were prepared in 5 mL volumes by combining sufficient protein solution with MES buffer, filtering with 4 mm Milex GV syringe filters (Millipore), and adjusting the final volume with sufficient alum stock suspension to make the final alum concentration 50 μg/mL. The suspensions were capped, vortexed 5 sec, and allowed to stand at room temperature until measured (0–2 h). Immediately before measurement each sample was sonicated in a Branson Bransonic 220 sonicator bath for 15 sec to disperse the alum. Measurements using the DELSA were normally made in triplicate in rapid succession. The measurement conditions were as follows: 25 °C sample cell temperature, 0.7–1.3 mA current, 36 sec total run time, 2 sec current on and 0.5 sec current off intervals with the maximum frequency set to 500 Hz. All measurements were made at the upper stationary layer of the cell. The light scattering at 17° was used for all quantitative determinations.

Adjuvant Preparation

Recombinant MN gp120 was mixed with alum in tris buffer to make a suspension of known concentration in both alum and MN rgp120. Control experiments were carried out to ensure that the MN rgp120 was bound to the alum before injection. The alum formulation was resuspended by swirling immediately before injection. The gp120 formulations containing alum were tested for stability of gp120 by reversed phase-HPLC, size exclusion HPLC, CD4 binding and MN gp120 ELISA before use (6). For these stability experiments, gp120 was desorbed from alum with 0.1 M phosphate at 45 °C for 1 hour. Control experiments, including circular dichroism, CD4 binding, V3 ELISA and HPLC, demonstrated that this process did not affect the conformational integrity of gp120.

Desorption of gp120 from Alum in vivo

A study was performed in eleven male New Zealand white rabbits (3.0–3.5 kg) (Grimaud Farms, Linden, California) to determine the desorption rate of gp120 from alum (k_{Desorp}), the intramuscular (i.m.) absorption rate constant (k_{Abs}), the elimination rate constants (k_E), and the systemic availability (F) of MN rgp120 following a single i.m. or intravenous (i.v.) injection. Two rabbits received 300 μg MN rgp120 as an i.v. bolus into an indwelling lateral ear vein catheter; the remaining six rabbits (n = 3/group) received 300 μg MN rgp120 in saline or 300 μg MN rgp120 with 600 μg Rehydragel® alum as an i.m. dose into the dorsal lumbar region (volume = 1 mL). Blood samples were collected for 48 h (i.v.) or 192 h (i.m.). Plasma (3.8% sodium citrate) was recovered by centrifugation and assayed for immunoreactive MN rgp120 by ELISA (assay range 0.39 to 40 ng/mL). Data from all studies were analyzed simultaneously with the expert model discrimination system GENES which incorporates ADAPT II for pharmacokinetic modeling (7).

Humoral Immune Response

The humoral response to gp120 was evaluated by immunization of guinea pigs (5/group, s.c., 200 μL), rabbits (2/group, i.m., 1 mL) or baboons (5/group, i.m., 1 mL) with MN gp120 in saline or alum. The MN rgp120 and alum doses, and the schedules, were varied as shown in the figures. Analysis of either individual or pooled sera was done at several time points in order to determine the maximum titers found. Sera were analyzed using an anti-gp120 antibody ELISA. The ELISA data were calculated as endpoint titers, where a cutoff of two-fold over background of preimmune sera at a 1:50 dilution was used. The data were calculated as geometric mean titers (GMT) and GMT standard deviations.

RESULTS AND DISCUSSION

The adsorption characteristics of antigen to alum are integral to designing an optimal alum-based adjuvant formulation. In this paper we describe the binding kinetics of gp120 to alum, as well as the desorption of gp120 from alum by a number of different catalysts, some of which are commonly used formulation buffers. We have further characterized the binding of gp120 to alum by comparing the binding potential using particle surface charge determination with different proteins with varying pI values. We find that the adsorption of gp120 to alum behaves as it should based on its pI. The binding capacity is the amount of protein bound to a given amount of alum, and this was determined for several alum types. The desorption of gp120 from alum was also catalyzed by serum and plasma, both *in-vitro* and *in-vivo*, with unknown immunological consequences.

Adsorption of gp120 to Alum

The adsorption of gp120 to alum in tris buffer at neutral pH is quite rapid. For example, more than 70% of the gp120 adsorbed to alum after 1 min, and 95% by 20 min at room temperature (Figure 1). Both types of gp120 (MN and IIIB) showed rapid binding, regardless of the type of alum used (Rehydragel, Rehsorptar and Alhydrogel). Rapid binding of gp120 to alum was also verified by alum particle mobility experiments. When gp120 was mixed with alum and the particle mobility measured as rapidly as possible (~90 seconds after mixing), the mobilities were different from the initial mobility observed without added protein, and then were static over the next 30 minutes. This rapid change in particle

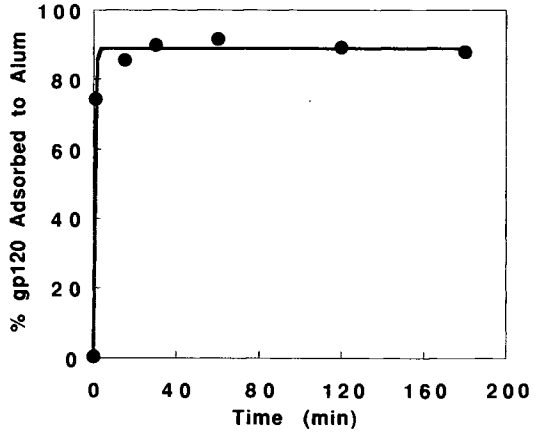


Fig. 1. Adsorption of IIIb gp120 (initial concentration = 300 μg/mL) to 600 μg/mL Resorptar alum in 20 mM Tris buffer (pH 7.4) and 120 mM NaCl at room temperature. The half-life was 3.4 ± 0.4 minutes based on a pseudo first-order fit.

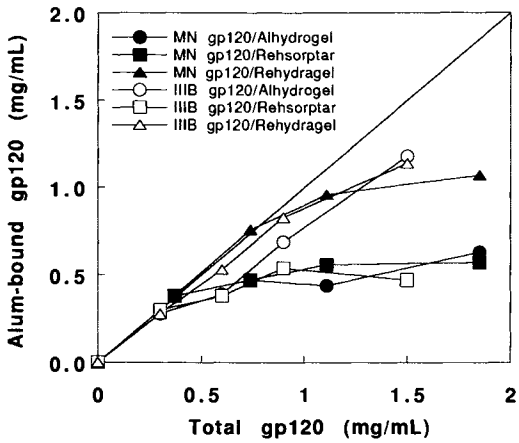


Fig. 3. Comparison of the gp120-binding capacities of different alum types. The alum concentration used throughout was 0.6 mg/mL. The reaction was carried out to equilibrium (several hours) in 20 mM tris buffer (pH 7.4) and 120 mM NaCl at room temperature. The 100% binding curve is shown as a line of unit slope, and represents the maximum binding possible.

mobility (in the oscillating electric field of the DELSA) was due to a change in the overall particle charge after protein binding and is indicative of rapid protein binding to alum. This result indicated that gp120 reached equilibrium binding with the alum in less than the mixing and measurement time (<90 sec, data not shown). In general, as more protein was added the charge on the alum particle was more effectively shielded until the relative mobility (the mobility with added gp120 relative to the mobility of alum alone) decreased to zero. (Figure 2). In this experiment, MN gp120 was slightly less effective in reducing the alum charge (and thus its mobility) than was IIIB gp120. The maximum amount of gp120 bound to alum was dependent on the type of alum used, where Rehydragel showed the highest gp120 loading capacity, and Rehsorptar showed the lowest (Figure 3). In general, the loading capacity of alum for gp120 was approximately 0.5–1 mg protein/mg alum; at higher loading levels only a fraction of the added gp120 was bound to the alum. The

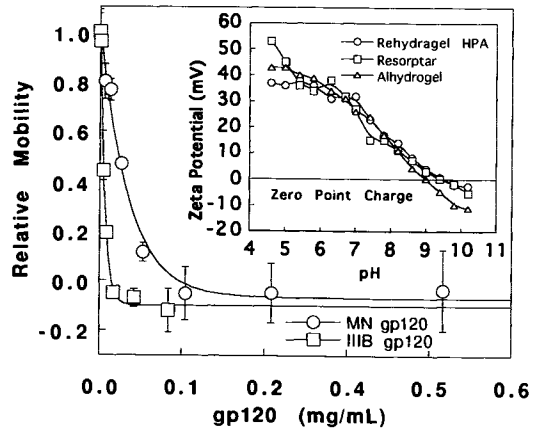


Fig. 2. Effect of gp120 concentration on the relative mobility of alum particles (alum 50 μg/mL) in pH 6 buffer. These measurements are referenced to alum alone. A decrease in the relative mobility below zero occurs when the particle charge changes sign (from net positive to net negative). The inset shows the effect of pH on the zeta potential of three alum types: Rehydragel, Rehsorptar and Alhydrogel. The estimated ZPC of these alum formulations is 9–10.

different alum types showed only slightly different gp120 binding ability, presumably due to their similarity in surface charge. The surface charge potential (zeta potential) obtained from the particle mobility measurements of the three alum types is the same, regardless of the buffer pH (inset, Figure 2). These results indicated that gp120 interacts with alum in tris buffer at neutral pH—a proposed requirement of alum as an adjuvant for gp120.

Comparison of MN and IIIB gp120 with Other Proteins

In order to put into perspective the alum adsorption and desorption of gp120 with other proteins, a study of several other proteins was made using protein loading and particle mobility measurements. Proteins with a wide range in pI were selected. Unfortunately, few proteins with pIs greater than that of alum (pI or ZPC ~9–10) interacted with the alum sufficiently well to provide either particle charge neutralization (zero point particle charge) or observable protein binding to the alum, even at high protein concentrations. There is a reasonable correlation between the pI of a protein and the amount of protein required to effect complete charge neutralization of the alum particle upon binding (Figure 4). Proteins with a low pI (such as pepsin, alpha-1 acid glycoproteinand gp120) have a high negative charge density at pH 6 and so only a small amount of protein (<100 μg/50 μg of alum) is required to effect neutralization of the positively charged alum particle. In contrast, proteins with higher pI (such as lysosyme, trypsinogen and IGF-1) are positively charged at pH 6 and interact weakly with the positively charged alum particles. An elegant set of experiments showing the importance of electrostatic interactions on the binding of proteins to alum has been reported previously, and these results are in general agreement with the binding of other proteins to alum (8). The results herein show that gp120 behaves similarly (at least in terms of particle mobility) to other proteins of comparable pI. Slightly more MN gp120 is required to effect charge neutralization than IIIB gp120, but this difference is probably not of mechanistic sig-

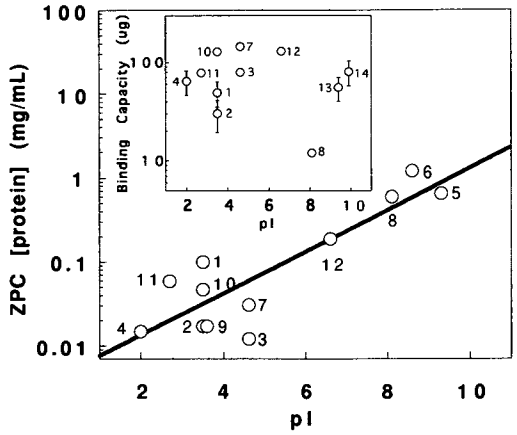


Fig. 4. Correlation of protein pI and the amount of protein required to effect alum particle charge neutralization (zero point charge) as measured by particle mobility measurements. Recombinant MN gp120 (*1*), recombinant IIIb gp120 (*2*), recombinant human deoxyribonuclease I (rDNase I) (*3*), recombinant insulin-like growth factor-1 (IGF-1) (*8*), recombinant CD4-IgG (*13*) and recombinant human gamma interferon-γ (*14*) were produced by Genentech, Inc. Porcine stomach pepsin (*4*), bovine pancreatic trypsinogen (*5*), chicken egg lysozyme (*6*), soybean trypsin inhibitor (*7*), A. niger amyloglucosidase (*9*), fetal calf fetuin (*10*), bovine serum alpha-1 acid glycoprotein (*11*), bovine serum albumin (*12*) and bovine cytochrome C were obtained from Sigma. In this experiment, 50 μg/mL Rehydragel alum in pH 6 imidazole buffer was used. It is interesting to note that several proteins of high pI (such as cytochrome c, IGF-1, CD4-IgG and IFN) were unable to effect charge neutralization (and so are not shown on the plot). The binding capacity (μg protein/50 μg alum) shown in the inset was independent of protein pI. In this plot, error bars are shown for each determination.

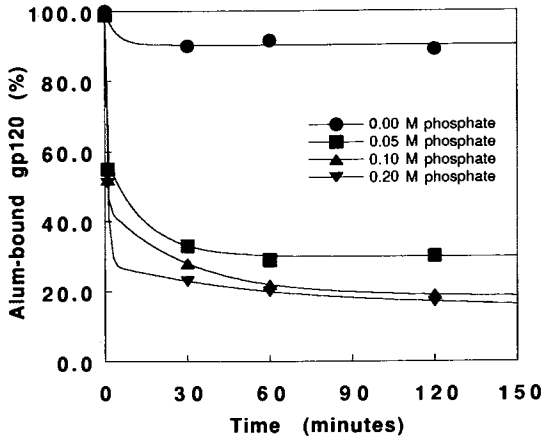


Fig. 5. Effect of phosphate buffer on the desorption of 300 μg/mL IIIB gp120 from Rehsorptar alum (600 μg/mL) at room temperature. Even small amounts of phosphate cause rapid desorption of gp120 from alum. No mechanistic information should be drawn from the curves through the data.

nificance. These data suggest that MN and IIIB gp120 behave as predicted (based on their pIs) to interact with alum particles.

The binding capacity of alum for several different proteins was also tested as a probe for protein-alum interaction (inset, Figure 4). In this case, there was little correlation between the binding capacity of alum and the pI of the protein, except that several proteins of high pI did not show detectable alum binding (and so these points are not shown in the semi-log plot). The binding of gp120 to alum was not unusually low (or high) compared with other proteins of similar pI and, therefore, these results may be directly applicable to other antigens with similar charge properties.

Desorption of gp120 from Alum by Phosphate and Other Ions

It has been reported in the literature (8) that phosphate and other polyvalent anions bind alum, with concomitant displacement of bound proteins. We have also observed this effect for gp120 bound to alum using sodium phosphate (Figure 5). The addition of phosphate to the gp120/alum formulation resulted in a rapid desorption of gp120 from alum. By using low phosphate concentrations (<0.2 M) at room temperature, approximately 80% of the gp120 was displaced from the alum particles. Desorption was also caused by other anions including sulfate and bicarbonate (Figure 6). The extent of desorption was also increased by added sodium chloride (see inset, Figure 6). We also investigated the role of pH on the desorption of gp120 from alum in the presence of 20 mM phosphate or imidazole (Figure 7). When phosphate was used as the buffer, there was less adsorption of gp120 at a higher pH when compared with imidazole as the buffer. These results are consistent with the observations of Callahan et al, where they showed the importance of surface charge in adjuvant-antigen interactions (8). At pH 6 and in the absence of phosphate, negatively charged gp120 (pI ~3.5) binds to the positively charged alum (pI ~9.6). Addition of phosphate causes a masking of the positive charges on alum, with a concomitant decrease in the amount of gp120 bound to alum. As the pH is increased, monobasic phosphate ion, HPO_4^-, is the major phosphate ion, and this ion interacts with the positively charged aluminum sites on the alum surface.

Desorption of gp120 from Alum and 'Aging'

We found that the phosphate-catalyzed desorption of gp120 from alum decreased with the age of the alum prepa-

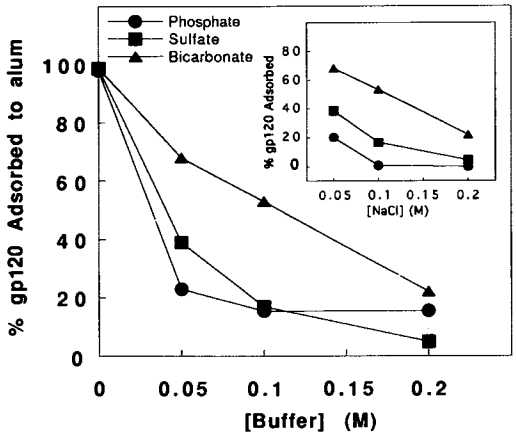


Fig. 6. Effect of phosphate, sulfate and carbonate on the desorption of IIIb rgp120 (300 μg/mL) from Resorptar alum (600 μg/mL). The inset shows that the desorption of gp120 from alum is further exacerbated by added sodium chloride.

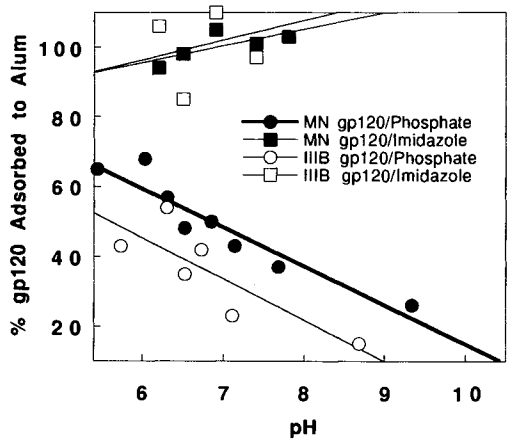


Fig. 7. Effect of pH on the release of MN and IIIB rgp120 (300 μg/mL) from Resorptar alum (600 μg/mL) in 20 mM sodium phosphate or imidazole, 140 mM NaCl.

ration. For example, ~80% of the alum-adsorbed gp120 was desorbed by 20 mM phosphate when the sample was prepared the same day, but only 40% was desorbed when the sample was a year old (Figure 8). This difference was less apparent at higher phosphate concentrations, indicative that the aging effect is a subtle one, and depends on the conditions used to measure 'aging'. The aging of alum is not a new phenomenon and has been well studied by Hem and colleagues (9,10,11). After freshly preparing aluminum hydroxide from aluminum chloride and ammonium hydroxide, Hem found that the pH and acid-consuming capacity of the gels changed markedly over time, and in some instances changes were observed more than 150 days later. These changes were attributed to alum particle growth by a deprotonation-dehydration mechanism, leading eventually to the formation of gibbsite. The aging of alum-protein mixtures is less well described, but the basis for this may be similar to alum aging. Alum particle growth may result in the slower phosphate-catalyzed desorption of gp120 from alum. First, macroscopic particle growth may physically entrap gp120 within the

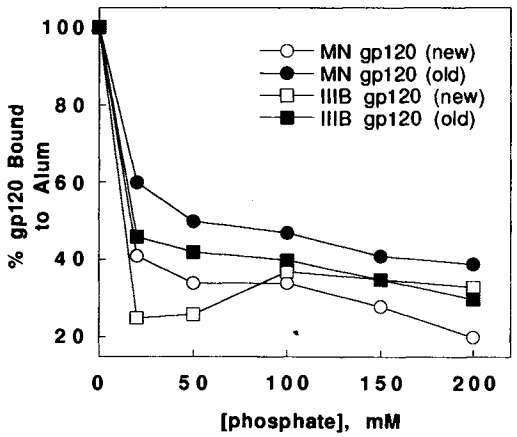


Fig. 8. Effect of formulation age on the phosphate-catalyzed desorption of MN and IIIB rgp120 (300 μg/mL) from Resorptar alum (600 μg/mL). In this experiment, the 'new' formulations were prepared the same day the desorption experiments were done; the aged formulations were approximately one year old.

growing particle matrix, thus giving slower gp120 desorption. Second, the altered crystal morphology of the 'aged' alum may adsorb gp120 more tightly. (This is probably not likely because the alum samples were aged for at least a year before adding gp120). Third, conformational changes of gp120 may occur over time, where alum-denatured conformers of gp120 may bind alum particles more tightly. To date, we have not found evidence for denaturation of gp120 by alum after desorption by phosphate, but we have not studied the conformation of gp120 while bound to alum.

Plasma-Catalyzed Desorption of gp120 from Alum: in vitro *and* in vivo

Blood and interstitial fluid contain numerous anions and proteins that may desorb gp120 from alum (12). Interstitial fluid contains organic acids (acetoacetic, citric, α-ketoglutaric, lactic, maleic, pyruvic and succinic acid) in sufficiently high concentrations (~6.3 mEq/L) to effect alum solubilization with a concomitant loss of alum-bound antigen. Further, other ions including phosphate (2–5 mEq/L) and bicarbonate (24–28 mEq/L), and serum proteins (15–20 mEq/L) also cause desorption of gp120 from alum. The in vitro rate of gp120 desorption from alum by serum or heparinized plasma was measured to determine if desorption is sufficiently fast to compete with the recruitment of antigen presenting cells to the site of immunization. If plasma-catalyzed desorption is very rapid (within a few hours), then there would be little depot effect of alum. Adding 300 μg/mL radiolabeled gp120 in pH 7.4 tris buffer to 600 μg/mL alum resulted in 100% protein binding to alum, as observed for unlabeled gp120. Addition of this formulation to human plasma (1:1 dilution) resulted in rapid desorption of gp120 from the alum, with an initial half-life of approximately 5 minutes at 37 °C (Figure 9). The desorption kinetics were similar for serum (data not shown). The desorption kinetics were also independent of the type of alum used, indicating that the different alum types may not offer different adjuvant action, at least as it pertains to alum acting by a depot effect.

It was also demonstrated that the observed desorption kinetics using radiolabeled gp120 were not due to the loss of the ^{125}I label from the protein, as verified by the radiogel identification of labeled gp120 (inset, Figure 9). In all experiments, the ^{125}I label remained bound to the protein, and the radioactivity desorbed from the alum over time was found in the plasma as radiolabeled protein of ~120 kD. These in vitro experiments strongly suggest that desorption of gp120 from alum may be sufficiently fast such that alum does not provide a significant depot effect for gp120. Further, it is difficult to estimate the fluid volume (over time) surrounding the site of injection, and this dilution may affect the desorption rate. To overcome these shortcomings, the *in vivo* release of gp120 from alum was determined after intramuscular (i.m.) administration of gp120/alum in rabbits.

Rabbits received 300 μg gp120 in saline (i.v. or i.m.) or with 600 μg alum (i.m.). The gp120 plasma concentrations were determined to assess the absorption of free gp120 from the site of injection (Figure 10). The pharmacokinetic model is shown in Scheme 1. This model describes a standard three compartment model (defined by K1 and K2) with additional compartments to describe the amount of gp120 at the injec-

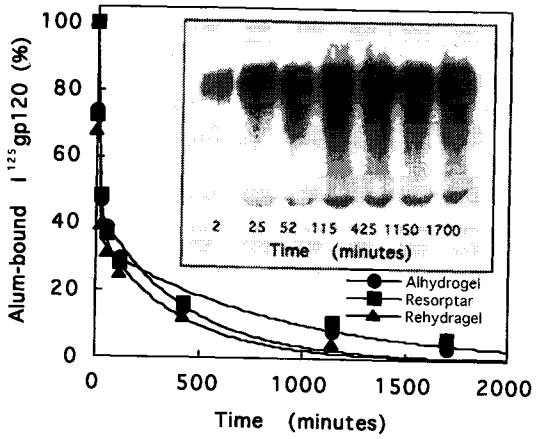


Fig. 9. In vitro desorption of I^{125}-gp120 from alum in human serum at 37°C. The rate of serum-catalyzed desorption is independent of the alum type used. The half-life (calculated for the first half-life during the rapid initial phase) for desorption was found to be approximately 5 minutes. The radiolabel was stable in serum at 37°C over the duration of the experiment as shown by the radiogel of the I^{125}-gp120 in the serum after desorption.

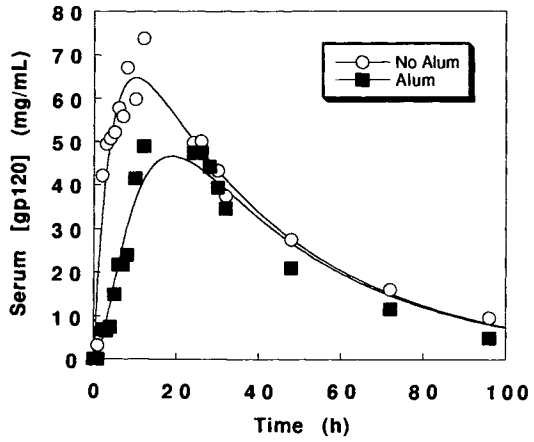
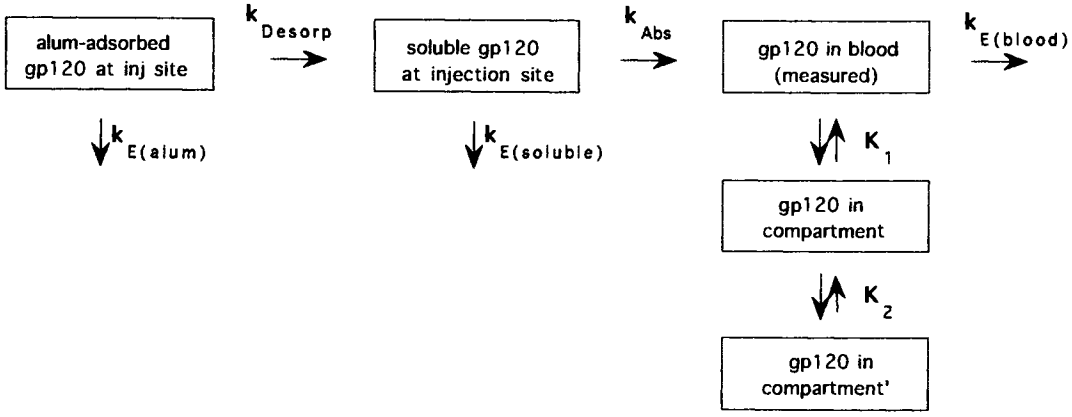


Fig. 10. Effect of alum adjuvant on blood concentration of MN rgp120 after i.v. (n = 2) and i.m. (n = 3) administration to rabbits, the curves shown are the means for all animals per group. The plasma levels of gp120 were determined by MN rpg120 ELISA. Doses were 300 μg MN rgp120 with or without 600 μg/mL alum.

tion site, either bound to alum or as soluble gp120. The rate constants are defined as: k_{Desorp} (the rate of desorption of gp120 from alum at the injection site, $k_{E(alum)}$ (the elimination rate of alum-bound gp120 from the injection site), k_{Abs} (the rate of absorption of gp120 from the injection site), $k_{E(soluble)}$ (the elimination rate of soluble gp120 from the injection site), $k_{E(blood)}$ (the systemic elimination of gp120 from the blood). The simultaneous fitting of the i.v. and the i.m. data was carried out using the GENES program (7,13). The following pharmacokinetic parameter estimates were obtained: $k_{Desorp} = 0.131 \pm 0.001\ h^{-1}$, $k_{Abs} = 0.0187 \pm 0.0004\ h^{-1}$, and $E(k_{E(alum,\ soluble,\ blood)} = 0.189 \pm 0.012\ h^{-1}$ for this rabbit population (13). The k_{Desorp} rate constant corresponds to half-life of ~5 hours. This is significantly longer than observed in vitro, and may be due to the slow rate of gp120 diffusion away from the alum depot, allowing for possible readsorption of gp120 to the alum. This is in agreement with the slow rate of gp120 absorption (k_{Abs}).

These data and the time profiles of Figure 10 show that there is a slight depot effect using alum, but that this depot effect does not last much more than a few hours, in agreement with the *in vitro* data. Systemic availability of gp120 was also determined from the individual pharmacokinetic parameters. Systemic availability of gp120 in saline (defined as $k_{Abs}/[k_{Abs} + k_{E(soluble)}]$) was ~35%; from the alum formulation it was ~29%. This model oversimplifies the 'true' pharmacokinetics from the injection site, in that it does not compensate for readsorption of gp120 to alum, nor does it account for possible elimination from the other systemic compartments, but serves to show that gp120 desorbs from alum *in vivo* somewhat slower than *in vitro*. The depot effect may also depend on the site of injection. Because subcutaneous tissue would be expected to have a lower interstitial fluid volume than muscle tissue, it is predicted that gp120 would reside longer at the site of injection if given s.c. rather than i.m. This longer residence time may result in an increased immune response for a s.c. administered gp120 alum-based vaccine. These results indicate that the immune system is presented with soluble (desorbed) gp120 shortly after immunization, rather than alum-adsorbed antigen. Thus, it is predicted that alum-based gp120 formulations cannot be optimized by using different alum types if the binding of gp120 to alum is important for adjuvant action.



Scheme 1. Pharmacokinetic model for the in vivo desorption of gp120 from alum.

Adjuvant Action of Alum with gp120

It is generally believed that the adjuvant action of alum is due to several factors. Mild irritation by alum at the site of injection causes recruitment of antigen presenting cells and concomitant cytokine up-regulation. Irritation at the site of injection, however, is not likely the major adjuvant mechanism of alum since it is one of the least irritating adjuvants known. Alum potentiates the immune response even though there is negligible local irritation by microscopic histopathological examination. It is thought that the predominant mechanism of adjuvant action by alum may be due to a depot effect caused by antigen adsorption to alum (14). It is possible that this depot effect allows sufficient time for phagocytosis of the alum particles by antigen presenting cells. Unfortunately, depoting may not operate for all proteins, as certain proteins are desorbed from alum in the presence of anionic counterions (15). We found that human serum rapidly displaced gp120 from alum *in vitro*. This also occurred *in vivo*, where the presence of alum provided only a slight delay in the peak blood levels of rgp120, compared with gp120 given in its soluble form without alum. In order to determine if these physical effects alter the immunogenicity of gp120, the adjuvant effect of alum with gp120 was tested by immunizing several species with gp120, either with or without alum.

In general, the titers observed for animals immunized with alum were slightly higher than for soluble antigen alone, especially after early immunizations. After repeated boosting (usually three immunizations), the anti-gp120 antibody titers were very similar for gp120 given either with or without alum. Figure 11 shows a comparison of antibody responses in guinea pigs, rabbits and baboons after immunization with gp120, with and without alum. These data show that alum provides an adjuvant effect for gp120 during the boosting

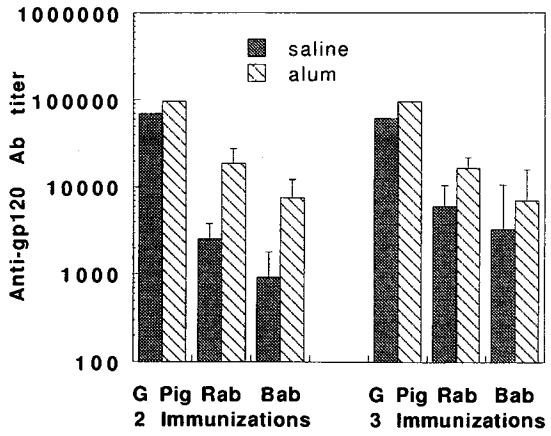


Fig. 11. Effect of alum as adjuvant for MN rgp120 in different species. Guinea pigs (G Pig) were immunized with 120 μg MN rgp120 (with or without 600 μg alum) at t = 0, 4 and 8 weeks (bled at t = 6 and 10 weeks). Rabbits (Rab) were immunized with 300 μg MN rgp120 (with or without 600 μg alum) at t = 0, 3 and 6 weeks (bled at t = 4 and 8 weeks). Baboons (Bab) were immunized with 100 μg MN rgp120 (with or without 200 μg alum) at t = 0, 4 and 24 weeks (bled at t = 6 and 26 weeks). After two immunizations, rabbits and baboons showed a modest adjuvant effect by alum; after three immunizations, there was little difference between saline and alum formulations.

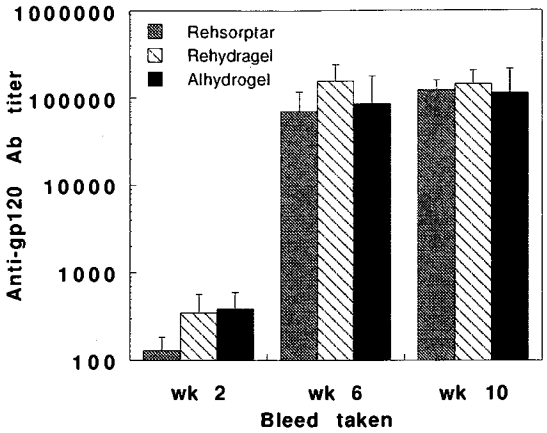


Fig. 12. Effect of different alum types (60 μg/dose) on the immunogenicity of MN gp120 (30 μg/dose) in guinea pigs using a 0, 4, 8 week immunization schedule. Bleeds were taken 2 weeks after each immunization.

phase, but eventually (after three immunizations), the immune response of gp120 alone is similar to that with alum and gp120. The antigen doses used in these studies were fairly large (120 μg/shot in guinea pigs; 300 μg/shot in rabbits; 100 μg/shot in baboons), and likely contribute to the leveling off of the immune response after three immunizations; it is well known that larger doses of antigen often 'push' the immune response to the top of the antigen dose response curve, even without adjuvant. Finally, we determined that the anti-gp120 immune response could not be optimized by using different types of alum, even when these different type exhibited differing propensities to bind gp120. As shown in Figure 12, the anti-gp120 antibody titers elicited in guinea pigs by the three different alum types (that vary in their protein binding capacity, but not in their plasma-catalyzed desorption rates) were the same. Based on these data, it is likely the different alum types gave a similar immune response because gp120 was desorbed from alum shortly after injection.

These immunogenicity results provide compelling evidence that optimization of alum as adjuvant is not straightforward, possibly because of the desorption of gp120 from alum shortly after injection, and must take into account several factors including protein loading, the kinetics of adsorption and desorption, and site of delivery. Modification of the alum crystal state to physically incorporate, rather than adsorb, gp120 may result in a slower release of antigen *in vivo*, with a concomitant greater antibody response. Experiments to test this hypothesis are underway. Alternatively, another strategy may be to combine gp120 and/or alum with other adjuvants that operate by an alternate mechanism than the depot effect.

ACKNOWLEDGMENTS

The superb technical editing by Jessica Burdman is gratefully acknowledged. The authors wish to thank Tim Gregory and Lavon Riddle for a pipeline supply of gp120 and for many helpful comments. Phyllis Mallett, Gary Osaka, Phil Abramowitz and Joe DiStefano provided expert assistance with the in-vivo desorption study and data analysis.

Finally, many thanks to Jack Obijeski, Don Francis, Rodney Pearlman, Dave Lowe, Catherine Lucas, Tom Patapoff, David Giltinan, Linda De Young and Stanley Hem for helpful comments and suggestions.

REFERENCES

1. G. J. LaRosa, J. P. David, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreedsman, R. N. Boswell, P. Sheeduck, L. H. Holley, M. Kamplus, D. P. Bolognesi, T. J. Mathews, E. A. Emini and S. D. Putney. Conserved sequences and structural elements in the HIV-1 principal neutralizing determinant. *Science,* **249**:932–935 (1990).
2. P. W. Berman, T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershberg, E. K. Cobba nd J. W. Eichberg. Protection of chimpanzees from infection by HIV-1 after vaccination with gp120 but not gp160. *Nature,* **345**:622–625 (1990).
3. H. S. Warren and L. A. Chedid. Future prospects for vaccine adjuvants. *CRC Crit. Rev. Immunol.* 8:83–101 (1988).
4. H. S. Warren, F. R. Vogel, and L. A. Chedid. Current status of immunological adjuvants. *Annu. Rev. Immunol.* 4:369–388 (1986).
5. S. Shirodkar, R. L. Hutchinson, D. L. Perry, J. L. White and S. L. Hem. Aluminum compounds used as adjuvants in vaccines. *Pharm. Res.,* 7:1282–1288 (1990).
6. J. L. Cleland, C. R. Kensil, A. Lim, N. E. Jacobsen, L. Basa, M. Spellman, D. Bedore and M. F. Powell. Isomerization and Formulation Stability of the Vaccine Adjuvant QS21. (*J. Pharm. Sci.,* in press, 1995).
7. GENES: J. DiStefano, III, Biocybernetics Laboratory, UCLA, Los Angeles, CA, 1994; ADAPT: D. D'Argenio and A. Schumitzky, Biomedical Simulations Resource, USC, Los Angeles, CA 1992.
8. P. M. Callahan, A. L. Shorter, S. L. Hem. The importance of surface charge in the optimization of antigen-adjuvant interactions. *Pharm. Res.* 8:851–858 (1991).
9. E. M. Morefield, G. E. Peck, J. R. Feldkamp, J. L. White and S. L. Hem. Role of water in the aging of aluminum hydroxide suspensions. *J. Pharm. Sci.,* 75:403–406 (1986).
10. S. L. Nail, J. L. White and S. L. Hem. Structure of aluminum hydroxide Gel II. Aging mechanism. *J. Pharm. Sci.,* **65**:1192–1195 (1976).
11. S. L. Nail, J. W. White and S. L. Hem. IR Studies of development of order in aluminum hydroxide gels. *J. Pharm. Sci.,* **65**:231 (1976).
12. S. J. Seeber, J. L. White and S. L. Hem. Solubilization of aluminum-containing adjuvants by constituents of interstitial fluid. *J. Parenter. Sci. Technol.,* **45**:156–159 (1991).
13. J. Mordenti, T. Nguyen, D. Eastman, G. Osaka, S. Frie, R. P. Weissburg, P. Berman, P. Abramowitz, J. DiStefano, III, and M. F. Powell. unpublished data, 1994.
14. M. A. Aprile and A. C. Wardlaw. Aluminum compounds as adjuvants for vaccines and toxoids in man: A review. *Can. J. Public Health,* 57:343–360 (1966).
15. S. L. Hem and J. L. White. Characterization of aluminum hydroxide for use as an adjuvant in parenteral vaccines. *J. Parenteral Sci. Tech.,* **38**:2–10 (1984).