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

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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 countersuch 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 [KA1(SO4)2 · 12H2O]. We focused our studies on the commercially available aluminum hydroxide products Resorpa, Rehydrogel 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, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) (U.S. Biochemical Corporation) adjusted to pH 6.0 with sodium hydroxide (Sigma)]. Alum was obtained as a 2% suspension (Alhydrogel, E. M. Sergeant Chemical Co., Clifton, New Jersey, Rehsorpt or Rehydrogel 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 (A1OH, known mineralogically as boehmite), and consists of corrugated sheets of aluminum-containing octahedra. It is obtained by precipitation of aluminum hydroxide under alkaline conditions. Rehsorpt and Rehydrogel HPA are structurally similar, and are synthetic oxyhydroxides of aluminum (aluminum hydroxide) prepared by acid-base precipitation. Rehydrogel 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.

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

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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 125I-MN gp120 used in these desorption experiments was prepared by the standard Enzymobead (BioRad) method, and purified over a desalting column. The radiolabel 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 vials by combining sufficient protein solution with MES buffer, filtering with 4 mm Milex GV syringe filters (Millipore), and adjusting the final volume with sufficient albumin stock suspension to make the final albumin 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 albumin. 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 (kDesorp), the intramuscular (i.m.) absorption rate constant (kAbs), 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 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 III) showed rapid binding, regardless of the type of alum used (Rehydragel, Rehysorptar 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...