Adsorption of a Novel Recombinant Glycoprotein from HIV (Env gp120dV2 SF162) to Anionic PLG Microparticles Retains the Structural Integrity of the Protein, Whereas Encapsulation in PLG Microparticles Does Not

Manmohan Singh,[1,2] James Chesko,[1] Jina Kazzaz,[1] Mildred Ugozzoli,[1] Elaine Kan,[1] Indresh Srivastava,[1] and Derek T. O'Hagan[1]

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Purpose. To evaluate the delivery of a novel HIV-1 antigen (gp120dV2 SF162) by surface adsorption or encapsulation within polylactide-co-glycolide microparticles and to compare both the formulations for their ability to preserve functional activity as measured by binding to soluble CD4.

Methods. Poly(lactide-*co*-glycolide) microparticles were synthesized by a water-in-oil-in-water (w/o/w) emulsification method in the presence of the anionic surfactant dioctylsulfosuccinate (DSS) or polyvinyl alcohol. The HIV envelope glyocoprotein was adsorbed and encapsulated in the PLG particles. Binding efficiency and burst release measured to determine adsorption characteristics. The ability to bind CD4 was assayed to measure the functional integrity of gp120dV2 following different formulation processes.

Results. Protein (antigen) binding to PLG microparticles was influenced by both electrostatic interaction and other mechanisms such as hydrophobic attraction and structural accommodation of the polymer and biomolecule. The functional activity as measured by the ability of gp120dV2 to bind CD4 was maintained by adsorption onto anionic microparticles but drastically reduced by encapsulation.

Conclusions. The antigen on the adsorbed PLG formulation maintained its binding ability to soluble CD4 in comparison to encapsulation, demonstrating the feasibility of using these novel anionic microparticles as a potential vaccine delivery system.

KEY WORDS: CD4; gp120; HIV; microparticle; vaccine.

INTRODUCTION

The development of vaccines to combat infectious diseases such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) requires effective delivery of antigens to the cells of the immune system (1–3). In the case of HIV, it is postulated that both the humoral and cell-mediated responses will need to be induced to develop an effective defense against the invading virus (4,5). For a vaccine against HIV, the formulation will need to present the antigens in a highly effective manner to the most relevant immune cells.

Several formulations including alum, emulsions, and microparticles have been evaluated to deliver HIV antigens (6–11). Biodegradable polymers such as poly(lactide-*co*-glycolide) present an interesting approach for vaccine delivery (6,7,9,10). Cleland *et al.* had previously reported the delivery of HIV-1 gp120 encapsulated in polylactide-co-glycolide microparticles and showed enhanced *in vivo* response to the antigen (6). They also showed that an additional adjuvant (QS21) can be delivered along with gp120 encapsulated in PLG microparticles with improved immunogenicity (7). However, these studies did not conclusively establish whether the integrity of the antigen was maintained after microencapsulation within PLG microparticles. Protein instability during encapsulation is well-known, and many studies have been undertaken to stabilize proteins during the encapsulation process (12,13).

Earlier preclinical HIV vaccine work with encapsulated antigen had been done using envelope proteins derived from T-cell adapted lab isolates (TCLA) (6,7,11). There are significant biochemical differences between the env proteins derived from TCLA isolates *versus* those derived from primary isolates. The antigens that are derived from primary isolates offer a broader neutralizing potential. Therefore, for the current investigation we choose to use env protein derived from primary isolate (i.e., SF162) for enhanced protection (14). To improve further the ability of env protein to induce broadly cross-reactive and neutralizing antibodies, efforts have been focused on designing and evaluating novel env structure such as env containing deletions of the variable loops (15). This new protein (gp120dV2 SF162) is a more optimized structure for broader protection than previous env proteins.

We have recently reported the use of surface adsorption on anionic PLG microparticles for delivery of antigens and shown that antigens from a novel Meningococcus B vaccine can be effectively delivered using this approach (16).This approach (i.e., surface adsorption on PLG) microparticle provides an alternative to the use of alum as a broadly applicable vehicle for vaccine formulations.

In this paper, we will add to this previous work by demonstrating how the formulated, anionic microparticles can deliver an important HIV-1 antigen gp120dV2 with retention of conformational epitopes as measured by the protein's ability to bind the soluble CD4 ligand. We will also compare the results obtained from PLG adsorbed formulations with those obtained with a standard encapsulated gp120 formulation prepared using a solvent evaporation technique.

MATERIALS AND METHODS

Materials

RG503, poly(D,L-lactide-*co*-glycolide) 50:50 copolymer composition (intrinsic viscosity 0.4 from manufacturers specifications) was obtained from Boehringer Ingelheim (Petersburg, VA, USA). Dioctylsulfosuccinate (DSS), USP grade mannitol, sucrose, and trehalose came from Sigma-Aldrich Chemical (St. Louis, MO, USA). Chinese hamster ovary cells derived recombinant gp120dV2 was synthesized and purified in house (Chiron Vaccine Research in Emeryville, CA).

Methods

Preparation of Anionic PLG Microparticles for Protein Adsorption

Anionic microparticles were prepared by a solvent evaporation technique (16). Briefly, microparticles were pre-

[1] Chiron Vaccines, Chiron Corporation, 4560 Horton Street, Emeryville, California 94608, USA.

[2] To whom correspondence should be addressed. (e-mail: manmohan_singh@chiron.com)

pared by homogenizing 10 ml of 6% w/v polymer solution in methylene chloride, with 2.5 ml PBS using a 10-mm probe (Ultra-Turrax T25 IKA-Labortechnik, Wilmington, NC, USA), thus forming a water-in-oil emulsion that was then added to 50 ml of distilled water containing 6 μg/ml DSS and homogenized at very high speed using a homogenizer with a 20-mm probe (ES-15 Omni International, Waterbury, CT, USA) for 25 min in an ice bath. This resulted in water-in-oil-in-water emulsion that was stirred at 1000 rpm for 12 h at room temperature, and the methylene chloride was allowed to evaporate. The resulting microparticles had 0.05% DSS w/w.

The size distribution of the resulting microparticles was determined using a particle size analyzer (Master Sizer, Malvern Instruments, UK). The zeta potential was measured using a Malvern Zeta analyzer (Malvern Instruments). PLG content of the suspension was measured by aliquoting a 1 ml suspension into each of 3 pre-weighed vials, which were lyophilized and weighed again, and the average net weight was used as PLG content/1 ml suspension.

Adsorption of Protein to Microparticles

To prepare microparticles with adsorbed protein, a suspension containing 100 mg of PLG was incubated with 1 mg protein in 10 ml total volume of 10 mM of PBS buffer and left on a lab rocker (aliquot mixer, Dubuque, IA, USA) at 4°C overnight. Formulation stabilizers (mannitol and sucrose) were then added to the suspension. The suspension was then lyophilized.

Adsorption Efficiency

To determine the actual amount of adsorbed protein, the microparticles were separated from the incubation medium before the final lyophilization step, by centrifugation, and the pellet washed three times with distilled water, then lyophilized. The loading level of protein adsorbed to the microparticles was determined by dissolving 10 mg of the microparticles in 2 ml of 5% SDS-0.2 M sodium hydroxide solution at room temperature. Protein concentration was measured both by size exclusion chromatography (HPLC-SEC method, Tosoh-BioSep SW3000XL 4.6 mm × 30 cm column, in PBS) and the BCA method (Pierce Chemical Company kit, Rockford, IL).

Adsorption Isotherm for gp120dV2

The adsorption efficiency for gp120dV2 at increasing concentration was evaluated by adsorbing gp120dV2 at 0.5%, 1.25%, or 2.5% w/w target load using the adsorption method described above. The level of adsorption was determined by base hydrolysis, followed by BCA protein assay. The percentage of adsorbed protein and the efficiency of adsorption at each loading level was averaged from three independent determinations, with 95% confidence limits assigned from the variance of the measurements.

Preparation of Microparticles with Entrapped Protein

The microparticles were prepared by a solvent evaporation technique as previously described (6,7,10,17). Briefly, microparticles were prepared by homogenizing 10 ml of 6% w/v polymer solution in methylene chloride, with 2.5 ml PBS containing 3.0 mg of gp120dV2 using a 10-mm probe (Ultra-Turrax T25 IKA-Labortechnik), thus forming a water-in-oil emulsion that was then added to 50 ml of 10% w/v PVA solution and homogenized at very high speed using a homogenizer with a 20-mm probe (ES-15 Omni International) for 25 min in an ice bath. This resulted in water-in-oil-in-water emulsion that was stirred at 1000 rpm for 12 h at room temperature, and the methylene chloride was allowed to evaporate. The microparticles were washed by centrifugation and lyophylized.

Scanning Electron Microscopy Analysis of PLG/gp120 Adsorbed Formulation

Scanning electron micrographs were recorded on a Hitachi S-5000 (UC Berkeley Electron Microscope Laboratory, Berkeley, CA, USA) through imaging emission of secondary electrons. A dilute suspension of the particles was dried on an adhesive, conductive surface and coated with a 5-nm layer of platinum followed by a 20-nm protective carbon overcoat. A field-emission source was accelerated to 10 keV, and the electron beam was focused on to the coated particles to permit spatial resolution of a few nanometers. Various fields within the same stub were monitored and recorded. The images were digitally scanned and recorded in graphical electronic file format.

Determination of the Functional Integrity of gp120dV2 Through Binding Affinity to CD4 Domains

To determine the capability of purified gp120 to bind to CD4, we have developed an high-performance liquid chromatography (HPLC)-based assay using fluoresceinated CD4. Purified sCD4 was labeled with amine-reactive succinimidyl esters of carboxyfluorescein following the instructions provided by manufacturer (Molecular Probes, Inc., Eugene, OR, USA). Approximately 1 μg of purified gp120 was mixed with 0.33 μg of fluorescinated-CD4 in a reaction volume of 60 μl using 2X phosphate-buffered saline at pH 7.4. After a 15-min incubation at the RT, 50 μl of this sample was injected onto a Bio Sil SEC-250 (Bio-Rad laboratories, Hercules, CA, USA) gel filtration HPLC column using Alliance 2690 HPLC system (Waters Corporation, Milford, MA, USA). Samples were run in 20 mM NaH_2PO_4, 2 mM Na_2HPO_4, and 400 mM ammonium sulfate buffer, pH 6.0, at the flow rate of 1 ml/min. The fluorescence profile was monitored at 490 nm using a 996 fluorescent detector and Millennium software package (Waters Corporation).

RESULTS

Anionic microparticles were prepared with a mean size of 1 μm (size distribution of 0.5–1.9 μm). The scanning electron micrographs show these particles to be spherical in shape, with a smooth outer surface (Fig. 1A). After protein adsoption one can see distinct patches of protein distributed uniformly on the PLG surface (Fig. 1B). Anionic PLG/DSS microparticles had a zeta potential of –55 mV, whereas PLG/DSS particles with adsorbed gp120dV2 protein had a zeta potential of –25 mV in PBS, indicating that charge neutralization at the protein/polymer interface had occurred due to the protein adsorption process. The mean size of the entrapped microparticles prepared for comparison was 3.0 ±

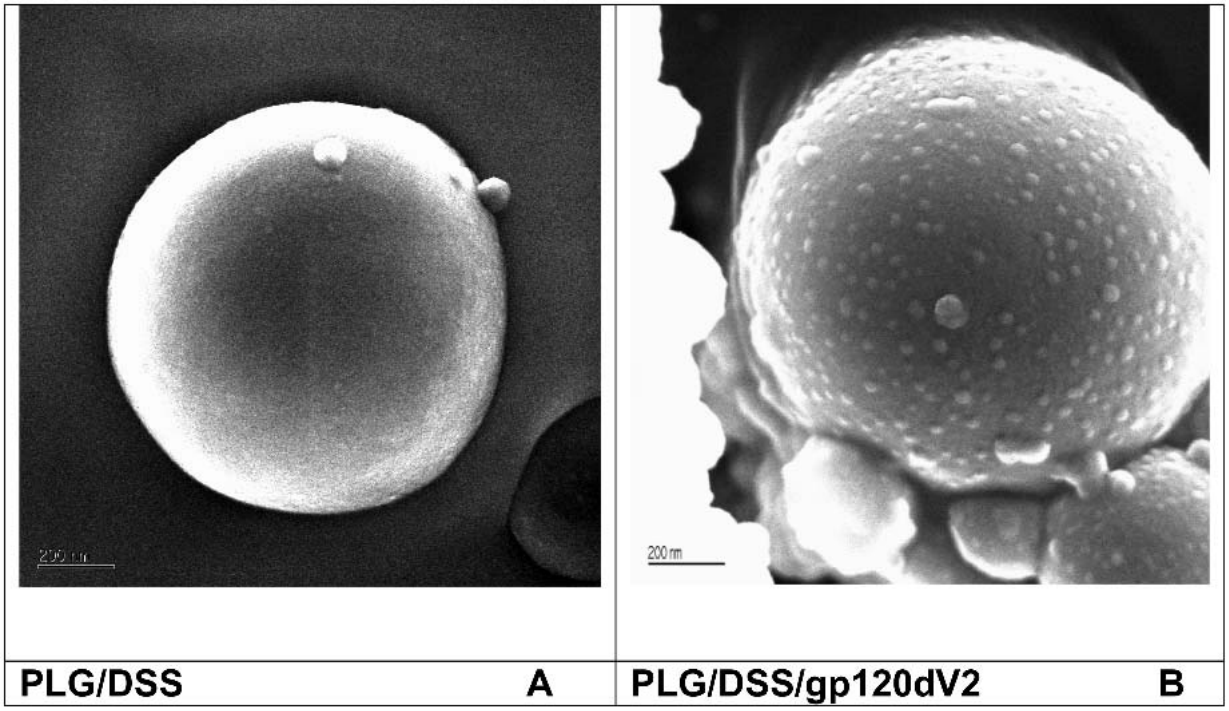

Fig. 1. SEM images of PLG/DSS microparticle formulation (A) before and (B) after gp120dV2 SF162 protein adsorption.

0.72 μm. The zeta potential of the entrapped microparticles was estimated to be between –10 to –12 mV.

Figure 2 shows the adsorption isotherm for gp120dV2 on PLG/DSS microparticles in phosphate buffered saline solution. As expected for a surface adsorption process mathematically described by the Langmuir equation (18), binding efficiency decreased with increasing protein input. This curve is consistently reproduced for numerous proteins that we have studied, including lysozyme, ovalbumin, and carbonic anhydase (data not shown) and typically finds an asymptote between 1% and 2% w/w protein to polymer.

The data in Table I suggest that the amount of protein released rapidly following lyophilization is complementary to the adsorption efficiency. In case of gp120dV2, an isoelectric point around 8.0 is suggested by the data, close to the experimentally determined value of 8.5. Highest efficiency of gp120 adsorption though is seen between pH 5 to 7.

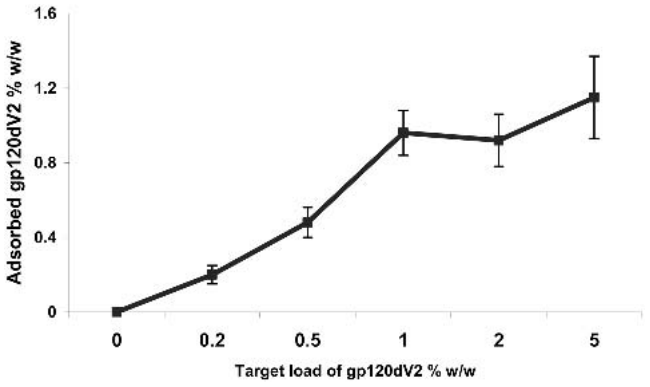

Fig. 2. Adsorption isotherm for gp120dV2 on PLG\/DSS microparticle formulation. Error bars represent the standard deviation from n = 3 independent adsorption experiments.

The ability of gp120dV2 to bind effectively to a CD4 domain is a key measurement of retention of functional activity. A range of chemical and physical formulation steps were taken to the same lot of gp120dV2, followed by a subsequent measurement of CD4 binding. Figures 3 and 4 summarize the inhibition of CD4 binding as measured by the ratio of free to bound CD4 for gp120dV2 proteins subjected to a range of conditions and formulations. By subjecting gp120dV2 to strongly denaturing conditions (pH 2.0 for 45 min), we note that approximately 80% of the binding affinity is lost (negative control). The protein released from encapsulated PLG formulation shows diminished activity in the early release component (4 h), but the extended release (2 days) has lost its ability to bind CD4, suggesting that the protein has drastically changed from its native conformation (i.e., denatured.) Adsorbing gp120dV2 on to PLG and then lyophilizing dramatically improves CD4 binding to about 70%. The best performing groups were those with added excipients (mannitol/sucrose). These stabilizers and cryoprotectants stabilize the protein during the freeze-drying process. These lyophilized

Table I. The Effect of pH on Adsorption of gp120dV2 on PLG/DSS Microparticles

Protein	Buffer	pH	% adsorption efficiency	% (neutral & positively charged protein) to total	% 2-h release
gp120dV2	Citrate	5.0	95	100	8
gp120dV2	PBS	7.0	80	100	21
gp120dV2	Phos	7.0	60	100	25
gp120dV2	Phos	8.0	29	100	59
gp120dV2	Borate	9.0	5	68	96

DSS, dioctylsulfosuccinate; PBS, phosphate buffered saline; Phos, phosphate; PLG, polylactide-co-glycolide.

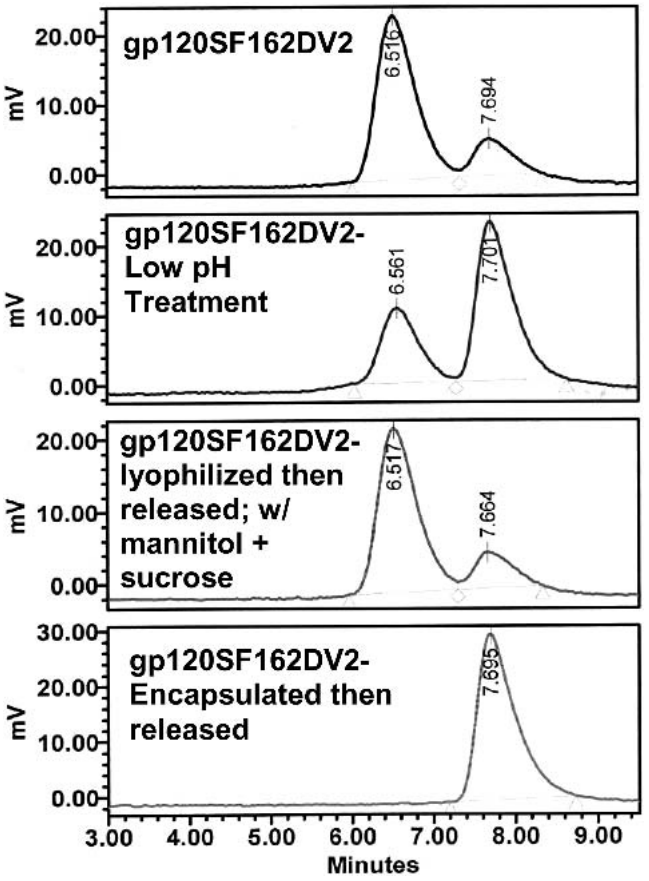

Fig. 3. Chromatograph of bound and free CD4 for various gp120dV2 samples, namely gp120 starting material, pH treated, PLG adsorbed, and PLG encapsulated.

groups showed greater than 80% CD4 binding, comparable to the original purified gp120dV2 protein.

DISCUSSION

The binding characteristics of proteins to adjuvants such as alum are governed by electrostatic interactions between the ionic salt (aluminum phosphate and hydroxyls) and the protein (19–21). In contrast to alum, PLG microparticles have surface functional groups such as aliphatic chains and ester linkages in addition to ionizable groups such as carboxyls and hydroxyls that will carry a net negative charge at physiologic pH.

Table I shows that gp120 has higher efficiency of adsorption at pH 7 and 5, suggesting that electrostatic attraction is not the driving force in the adsorption process. Hence, the data suggests that it is the additional forces such as hydrophobic (van der Waals) interactions thath are strong enough to drive the adsorption process. The important conclusions to draw from Table I are that the magnitude of the adsorption does follow the trend of pI *versus* pH.

Another important observation is that encapsulation of the model gp120 protein in PLG microparticles did not maintain its CD4 binding ability. This may be due to denaturation of the protein either during processing (high shear and organic solvents) or in the micro-environment within the microparticle (12,13). Clearly, encapsulation of labile and conformationally sensitive antigens within PLG microparticles may not be a process of choice. Surface adsorption of the same antigen on PLG/DSS microparticles on the other hand is a more suitable environment by which it maintains its CD4 binding ability. This is further enhanced by addition of excipients during freeze drying and storage. This demonstrates that surface adsorption of antigens on PLG microparticles (like on alum) is a more preferred approach to retain antigen integrity.

In summary, anionic PLG microparticles can be used as an efficacious delivery system for protein antigens used in HIV vaccine formulations. The microparticles adsorb molecular proteins on their surface through a combination of electrostatic and hydrophobic attraction and can stimulate strong immune responses following vaccination (16). The ability to control the amount and disposition of protein in the vaccine formulation through use of buffers, excipients, adjuvants, and an optimized process can lead to the development of novel vaccine formulation in the future. The propensity of gp120dV2 to bind CD4 is a measure of the protein's retention of functional activity, and the formulations we tested show a dramatic improvement in the binding affinity of excipient stabilized gp120dV2 adsorbed to PLG as compared to encapsulated protein. Generation of potent neutralizing antibodies to the native structure *in vivo* is the real test of such a formulation, and we have initiated rabbit studies to evaluate the of bredth of the immune response with this microparticle formulation.

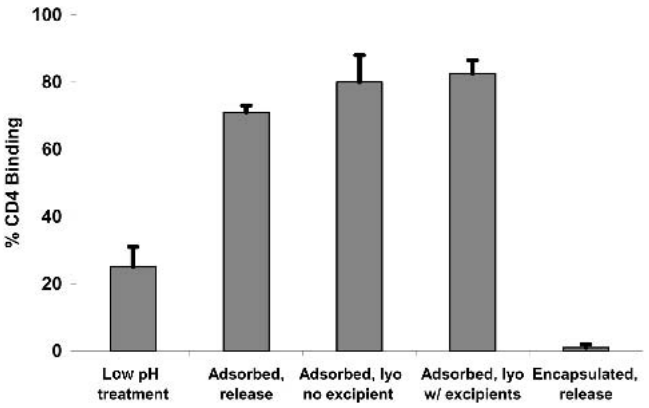

Fig. 4. Percent bound CD4 ± SE for gp120dV2 formulations, namely pH treated, PLG adsorbed (released), PLG adsorbed (extracted, no excipients), PLG adsorbed (extracted, excipients), and PLG encapsulated released.

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