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Parvovirus particles as platforms for protein presentation

(capsid/B19/lysozyme/gene therapy/vaccine)

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ABSTRACT **Empty capsids of the human pathogenic parvovirus B19 can be produced in a baculovirus system. B19 capsids are composed mainly of major capsid protein (VP2) and a small amount of minor capsid protein (VP1); VP1 is identical to VP2 but contains an additional 227-aa N-terminal region ("unique" region). A portion of that region of VP1 is external to the capsid, and VP1 is not required for capsid formation. We substituted the unique region with a sequence encoding the 147 aa of hen egg white lysozyme (HEL) and constructed recombinant baculoviruses with variable amounts of retained VP1 sequence joined to the VP2 backbone. After cotransfection with VP2 baculovirus and expression in insect cells, capsids were purified by density sedimentation. Purified recombinant capsids contained HEL. External presentation of HEL was demonstrated by immunoprecipitation, ELISA, and immune electron microscopy using anti-lysozyme monoclonal antibodies or specific rabbit antisera. Empty particles showed enzymatic activity in a micrococcal cell wall digestion assay. Rabbits inoculated with capsids made antibodies to HEL. Intact heterologous protein can be incorporated in B19 particles and presented on the capsid surface, properties that may be useful in vaccine development, cell targeting, and gene therapy.**

B19 parvovirus is a human pathogen, causing the common childhood rash illness called fifth disease; acute and chronic anemias in susceptible hosts; and, after *in utero* infection, nonimmune hydrops fetalis (1). B19 infects erythroid progenitors of marrow, blood, and fetal liver (2). The narrow tropism of the virus is due to both binding to the receptor erythrocyte P antigen (globoside) (3) and full-length transcription of genome only in permissive cells (4).

B19 shares structural features with other animal parvoviruses (5,6). Their single-stranded DNA genome is contained in nonenveloped icosahedral structures of 18–26 nm diameter and with a buoyant density of 1.36–1.43 g/ml in CsCl. The capsid shell is composed of 60 copies of capsid protein in $T=1$ icosahedral symmetry. The atomic structure of canine parvovirus has been solved to 3.4 Å (7). The central structural core motif of eight anti-parallel β-pleated sheets is common to many viruses. Canine parvovirus surface structure is remarkable for a large spike at the threefold axis of symmetry, absent from B19 empty capsids (8, 9).

Two structural proteins are contained in the capsid, a major capsid protein (VP2) of 58 kDa and a minor capsid protein (VP1) of 83 kDa, the difference due to 227 additional aa at the VP1 N terminus ("unique" region) (10). VP1 constitutes <5% of virion (11). Some VP1 unique region is external, as capsids and virions can be immunoprecipitated by specific antiserum (12). Anti-VP1 antisera also neutralize virus (12). The predominant antigenic specificity in human late convalescent sera is to VP1 not VP2 (13). VP1 unique region and the junction between VP1 and VP2 contain multiple linear neutralizing epitopes (14). The ability of empty capsids to elicit neutralizing antibodies in animals correlates with increasing amounts of VP1 (15).

We had previously determined that empty capsids morphologically and immunologically similar to natural capsids formed from VP2 alone (16). This property, plus the external localization of VP1 on the capsid surface and its immunodominance in infected humans and inoculated animals, suggested the possibility and advantage of substitution of a unique region with a sequence of another protein. We chose hen egg white lysozyme (HEL) because of its convenient length, well-defined antigenic sites, and measurable activity.

MATERIALS AND METHODS

Production of Recombinant Baculoviruses. The HEL gene was inserted into baculovirus transfer vector pVL1393 (Invitrogen) (Fig. 1). HEL coding sequence (17) was amplified from an M13mp18 plasmid containing the gene template (18) (a gift of Jack Kirsch, University of California, Berkeley); primers included *Bam*HI sites, allowing insertion of the amplified product into the multiple cloning site of pVL1393. For capsid sequence, we used pVP1/941 (16). To generate truncated versions of VP1, five different 5′ primers incorporating *Sma* I recognition sequences were used to amplify VP1 fragments. The 2.5-kb fragment of pHEL/1393, the 8.9-kb fragment of pVP1/941, and the short amplified fragment were triple ligated by using T4 DNA ligase (New England Biolabs). Recombinant baculoviruses were termed HEL/CP1–5 (capsid proteins 1–5). Sequences of HEL and truncated VP1 products of PCR were confirmed (Sequenase; United States Biochemical).

Preparation of Recombinant Parvovirus Capsids. *Autographa californica* nuclear polyhedrosis virus (Invitrogen) and recombinant viruses were grown in Sf9 cells (16) and constructs were transfected by cationic liposome mediation (Invitrogen) (19). Capsids were purified from Sf9 cells infected with recombinant viruses at a multiplicity of infection of 10 and were harvested 4 days after inoculation (16).

Detection of HEL Antigen on Capsid Surface. B19 capsid proteins were detected with specific rabbit antisera (16) and mouse monoclonal antibody (mAb) (Chemicon) and HEL was detected with rabbit antiserum (a gift of E. Prager, University of California, Berkeley) and mouse mAbs HyHEL-5, HyHEL-10, and HyHEL-15, which recognize three independent determinants (20, 21). For the antibody capture assay, purified capsids or HEL were added at concentrations of 1 fmol to 4.8 pmol to Immulon-2 96-well microtiter plates (Dynach, Chantilly, VA) and incubated for 1 hr at room temperature. Nonspecific binding was blocked with phosphate-buffered saline (PBS) containing 5% dry milk (Bio-

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Abbreviations: HEL, hen egg white lysozyme; VP1, minor capsid protein; VP2, major capsid protein; mAb, monoclonal antibody.

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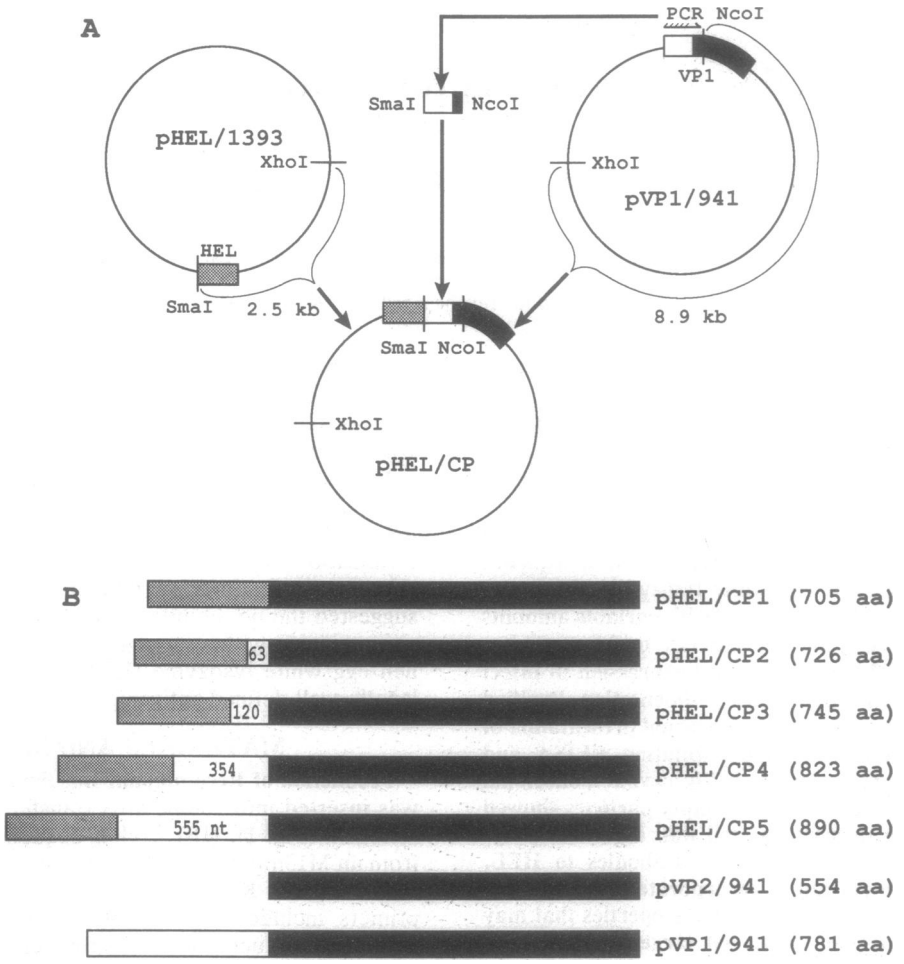

FIG. 1. Plasmid construction. (*A*) The coding sequence of HEL (shaded bar) was introduced into the multiple cloning site of pVL1393 to create pHEL/1393. pVP1/941 contains the coding sequence of VP1 [consisting of the VP2 sequence (solid bar) and the unique region sequence of VP1 (open bar)]. To create truncated versions of VP1, N-terminal fragments were amplified by PCR. An 8.9-kb fragment of pVP1/941 containing the C-terminal portion of the capsid protein genes, a 2.5-kb fragment of pHEL/1393, and the PCR fragments were generated by appropriate enzymatic cleavage and ligated to create the various pHEL/CP recombinant baculoviruses. (*B*) Coding sequence of each plasmid. Total number of amino acids in each construct is shown in parentheses; number in the open rectangle refers to the nucleic acids of the VP1 unique region only (4 aa were introduced between the VP and HEL coding sequences during construction of the plasmids). Native B19 VP1 and VP2 are shown below for comparison.

Rad) and 0.1% Tween 20. One microgram of mouse anti-HEL mAb in 100 μl of blocking buffer was added to each well for 1 hr at room temperature. Plates were washed with PBS and incubated with peroxidase-labeled goat anti-mouse antibody (Promega) for 1 hr at room temperature. After washing, 100 μl of substrate solution (1-Step Turbo TMB-ELISA; Pierce) was added for 5 min, the reaction was stopped by 100 μl of 2 M H_2SO_4, and light absorbance at 450 nm was measured (model 3550 Microplate Reader; Bio-Rad). Two-antibody assays were performed by binding 1 μg of HyHEL-5 per well followed by addition of capsid protein at 0.01–64 μg per 100 μl of blocking buffer. Plates were incubated with rabbit antiserum to capsid protein and binding was detected with labeled anti-rabbit antiserum.

One microgram of purified capsid protein was precleared by addition of protein A-Sepharose (CL-4B; Pharmacia LKB), and immunoprecipitation was performed by addition of either anti-HEL mouse mAb or rabbit antiserum; rabbit anti-mouse $F(ab')_2$ (Pierce) was added to the sample of mouse mAb. Incubations were for 1 hr at 4°C with constant agitation. Antibody was attached to 10% (vol/vol) protein A-Sepharose in PBS/10% fetal calf serum (FCS) for 30 min; the beads were washed in PBS/10% FCS and boiled for 10 min in loading buffer (22). The supernatant was then subjected to immunoblotting (14). Membranes were incubated with antiserum or mAb at various dilutions (1:1000–1:5000). For slot blot analysis of protein, 1 μg of capsid protein was applied to a nitrocellulose membrane in a Minifold II slot blot (Schleicher & Schuell) followed by immunoblotting with mouse mAbs.

Electron Microscopy. ImmunoGold labeling was performed with rabbit anti-HEL antiserum or preimmune serum diluted 1:50 followed by 5-nm colloidal gold goat anti-rabbit antiserum (Ted Pella, Redding, CA) (16, 23, 24).

Enzyme Assay. HEL activity was assayed by bacteriolysis (21). Ten microliters of *Micrococcus luteus* cell (Calbiochem) at 1.5 mg/ml and 90 μl containing the test capsid preparation or HEL, both in 0.1 M potassium phosphate buffer (pH 6.3), were mixed and added to each well of a 96-well microtiter plate. The reaction was monitored at 450 nm with readings every 1 min.

Inoculation of Animals and Antisera Preparation. Rabbits were injected subcutaneously with 300 μg of capsid in complete Freund's adjuvant followed at 3, 6, and 9 weeks by injection of 200 μg of capsid in incomplete Freund's adjuvant. Sera were collected 3 weeks after each booster injection.

RESULTS

Preparation and Expression of Recombinant HEL/CP Baculoviruses. Five recombinant baculoviruses were generated. All contained the entire coding sequence of HEL located 5′

to the B19 parvovirus VP2 gene, with variable amounts of intervening VP1 sequence (Fig. 1). For all baculovirus, chimeric VP1 of appropriate length was expressed in insect cells, and the ratio chimeric VP1/VP2 in cell lysates was similar to the ratio of coinfecting viruses (data not shown). To determine whether chimeric capsids assembled, we coinfected Sf9 cells with recombinant baculoviruses containing VP2 and HEL-VP1 genes. Lysates were subjected to conventional virus purification by sequential sedimentation steps over sucrose and density-gradient sedimentation through CsCl. [When only the VP1 chimeric capsid baculovirus was used for infection of insect cells, most HEL/CP was found above the sucrose cushion, indicating that protein was predominantly not in capsid form (data not shown).] By immunoblotting, capsid protein was detected in specific fractions of the CsCl gradient at densities appropriate for empty capsids (1.30–1.31 g/ml) (Fig. 2*A*); these fractions also reacted with antibody to HEL (Fig. 2*B*). By this criterion, each of the HEL-containing baculoviruses participated in capsid, as chimeric protein was detected in discrete CsCl fractions (Fig. 2 *C* and *D*). Yield of capsid was consistently highest for HEL/CP4; lower for HEL/CP2, -3, and -5; and lowest for HEL/CP1. Optimal capsid formation was obtained with a ratio HEL/CP4 baculovirus /VP2 baculovirus of ≥4. We estimated that each capsid contained one or two HEL molecules, based on comparison of the density of VP2 and chimeric VP1 bands after SDS/PAGE of serially diluted purified capsids (data not shown). Immunoblotting of chimeric capsids under nondenaturing conditions showed HEL and VP2 also restricted to this high molecular weight region (data not shown). Recombinant capsids were stable to warming (42°C × 2 hr or 37°C × 5 hr) or vigorous Vortex mixing (data not shown).

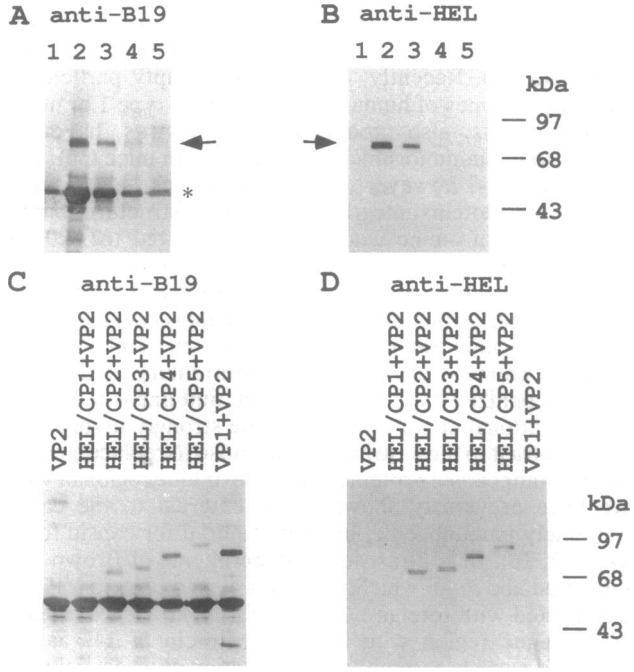

FIG. 2. Detection of lysozyme in chimeric capsids by immunoblotting. (*A* and *B*) Empty capsids from cells coinfected with recombinant VP2 baculovirus (major capsid protein) and HEL/CP1–5 baculovirus (chimeric minor capsid protein) at a ratio of 1:4 were purified. (The protein products are referred to as VP2 and HEL/CP1–5, respectively.) After CsCl density sedimentation a band was visible at a refractive index of 1.363 (density, 1.309 g/ml); 22 500-μl fractions were analyzed by immunoblotting with appropriate antibodies to capsid or HEL; results from five consecutive fractions about the band for HEL/CP4 and VP2 are displayed. Numbers refer to the CsCl density, ranging from 1.324 (lane 1) to 1.281 (lane 5). Capsids peaked in fraction 2 (1.309 g/ml). Asterisk, location of VP2; arrows, VP1 chimera. (*C* and *D*) Immunoblot of capsids containing different HEL–CP chimeric proteins after two CsCl density-gradient purification steps. HEL was detected only in chimeric capsids.

External Localization of HEL in Recombinant Capsids. To localize HEL in chimeric capsids, we used several assays. Binding of anti-HEL antibodies HyHEL-5, -10, and -15 is highly dependent on the conformation of antigen and was used to detect native protein. First, capsids twice purified by CsCl sedimentation were reacted with antibody to HEL, and the antibody-bound precipitate was subjected to SDS/PAGE; protein of appropriate size was detected only when specific antibody reacted with HEL-containing capsids (Fig. 3*A*). Second, antibody-reactive HEL was also present in capsids subjected to slot blot analysis, a method that should minimally disrupt capsids; under these circumstances, binding was found only for antibody to HEL in combination with HEL capsids (Fig. 3*B*). Third, HEL on capsids was visualized by electron microscopy using ImmunoGold-labeled goat anti-rabbit IgG (Fig. 3*C*).

We developed an ELISA for the measurement of HEL in capsids. In the antibody capture assay, anti-HEL mAb reacted specifically with chimeric capsids, and, by compar-

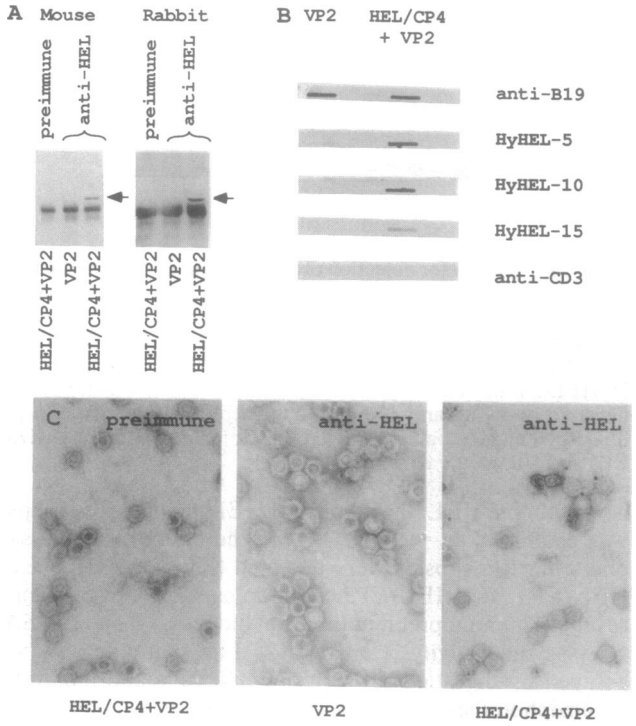

FIG. 3. Detection of lysozyme in HEL/CP4 and VP2 capsids by immunoprecipitation (*A*), slot blot (*B*), and immunoelectron microscopy (*C*). (*A*) Purified capsids (see Fig. 2) were precipitated with murine mAb HyHEL-5 (*Left*) or rabbit anti-HEL antiserum (*Right*). Precipitated proteins were electrophoresed in SDS 8% polyacrylamide gel, transferred to nitrocellulose, and analyzed by immunoblotting with a rabbit anti-B19 capsid antiserum. Arrows, location of VP2. (The low levels of chimeric HEL/CP in these capsids were not detectable.) The broad nonspecific band represents rabbit immunoglobulin bound to anti-rabbit alkaline phosphatase AP-conjugated goat antibody. Anti-HEL antibodies precipitated only chimeric capsids. (*B*) In protein slot blot for HEL and VP2, proteins were detected by application of mouse mAbs directed against B19 capsid protein, conformationally determined epitopes of HEL (HyHEL-5, -10, -15) and, as a control, anti-CD3 antibody. Anti-HEL antibodies bound only to chimeric capsids. (*C*) On immunoelectron microscopy, ≈30% of empty capsids were labeled by gold-conjugated anti-HEL antibodies (*Right*). Chimeric capsids were not labeled in the presence of either preimmune serum (*Left*) or irrelevant antiserum (data not shown), nor were VP2 capsids labeled with anti-HEL antiserum (*Center*). (×97,200.)

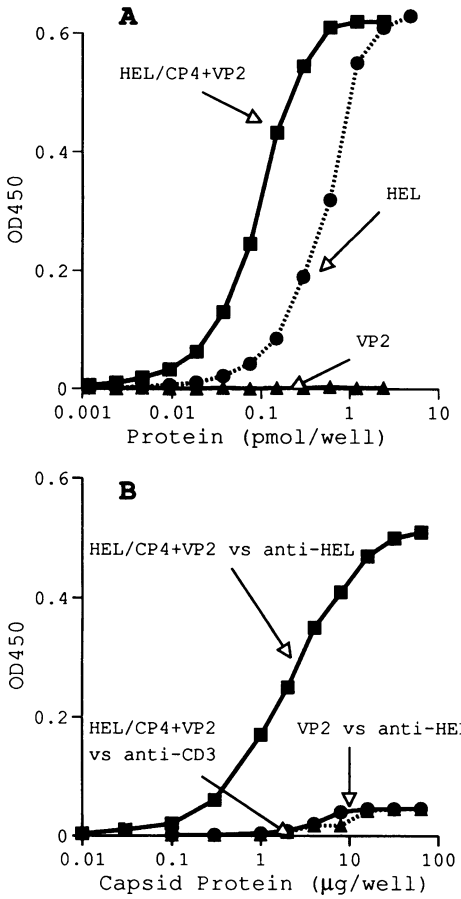

FIG. 4. ELISA for detection of HEL in chimeric capsids. (*A*) For antibody capture, plates were coated with capsid preparations and treated with anti-HEL antibody followed by horseradish peroxidase-conjugated goat anti-mouse IgG. HEL was detected in chimeric capsids. (*B*) In the two-antibody assay, plates were coated with HyHEL-5 followed by addition of capsid protein, which was then detected by rabbit anti-B19 capsid antiserum and horseradish peroxidase-conjugated anti-rabbit IgG. Only chimeric capsids (HEL/CP4 and VP2) were detected.

ison with free HEL, the number of HEL molecules per capsid was estimated at ≈5 (Fig. 4*A*). In the two-antibody assay, only chimeric capsids were detected (Fig. 4*B*). (HEL/CP2, HEL/CP3, and HEL/CP5 chimeric capsids showed similar results in immunoprecipitation, slot blot assays, and ELISA.)

Lysozyme Activity of HEL/CP4. Enzymatic activity was assayed by digestion of bacterial cell walls and monitored spectrophotometrically (Fig. 5). Empty capsids composed of only VP2 were inactive. Addition of HEL/CP4 and VP2 capsids led to digestion of micrococcal cell walls; activity was approximately proportional to the quantity of chimeric capsids. By comparison with HEL, we estimated by enzymatic activity of this preparation of HEL-containing capsids the presence of 0.5 HEL molecule per capsid.

Immunogenicity of HEL Recombinant Capsids. Two rabbits were immunized with chimeric capsids; we estimated that the booster dose of 200 μg of capsids contained <1 μg of HEL protein. After three injections, anti-HEL activity was detected by ELISA in the sera of both animals with specimens diluted 1:500–1:1000 (data not shown).

DISCUSSION

We have produced recombinant B19 parvovirus capsids in which a portion of the minor capsid protein was replaced with lysozyme that was externally expressed, correctly folded, enzymatically active, and immunogenic. Our results can be

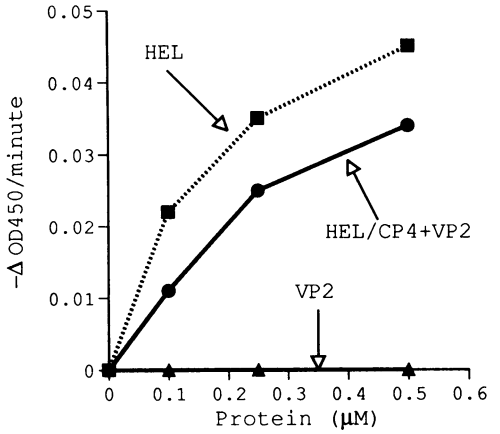

FIG. 5. Lysozyme enzymatic activity of chimeric capsid. Micrococcal cell wall digestion was measured spectrophotometrically; decline in optical density was linear during the first 2 min of the reaction. One of three similar experiments is shown.

compared with other methods of producing chimeric viruses or capsids. Poliovirus was first used to produce composite particles by site-directed mutagenesis of epitopes within antigenic site 1 of the virus VP1 gene. Chimeric live polioviruses (25) and poliovirus incorporating epitopes of other viruses (26, 27) elicit antibody responses in animals. Viral capsids have also been used to present foreign proteins. Peptides fused to the N terminus of the hepatitis B core antigen and expressed in *Escherichia coli* appear on the surface of self-assembled particles (28). Chimeric rotavirus proteins, expressed in a baculovirus system, also self-assemble (29). Recently, chimeric B19 empty particles expressing epitopes of human herpes simplex type 1 or murine hepatitis virus, also produced in baculovirus, have been shown to be immunogenic and protective in mice (30). In all these systems, however, foreign sequences have been inserted into proteins integral to the capsid structure, limiting the number of amino acids to be substituted to <20, and usually the equivalent of a single antigenic epitope; position constraints, as well as the nature of the inserted sequences, affect not only efficiency of capsid assembly but also expression of antigenic fragments. The same constraints affect the ability of chimeric capsids to elicit neutralizing antibodies or to be recognized by specific antisera, and linear rather than conformational epitopes appear to be strongly favored (28).

In contrast to these strategies we chose to replace a portion of the minor capsid protein of B19, the unique region of which we have previously shown to be external to the capsid, extremely immunogenic, but not required for capsid formation (12, 16). In contrast to the experiments of Brown *et al.* (30) cited above, in which the major capsid protein VP2 was substituted with foreign peptides of 8 or 13 aa, we replaced VP1 unique region with an intact protein of 147 aa. We achieved particle assembly by coinfection of insect cells with recombinant baculoviruses encoding chimeric VP1 and normal VP2 genes. As anticipated, HEL was present external to the capsid surface. Recognition of chimeric particles by antibodies to conformational determinants of HEL was preserved; from the binding of well-characterized mAbs HyHEL-5, -10, and -15, we can infer that >40% of the protein surface, including the active site, is accessible (20). The particles showed enzymatic activity and elicited antibody responses in rabbits. Our method appears to be generalizable to other foreign proteins, as we have achieved capsid assembly with VP1 substituted with >40 aa of human immunodeficiency virus 1 envelope glycoprotein and 219 aa of coding sequence of influenza hemagglutinin (S.K., M. Kawase, G. Franchini, P. Rota, and N.S.Y., unpublished data). Compa-

rable production of virus particles displaying extensive foreign protein has been accomplished only by using retroviral vectors expressing fusion genes in packaging cell lines (31) and for combinatorial antibody libraries on the surface of λ phage (32).

While our data indicate external localization of HEL in B19 capsids, the exact configuration of the HEL substituted region of VP1 is unknown. Not visualized by x-ray crystallography of canine parvovirus were the 40 most N-terminal residues of the major capsid protein or the unique region of VP1 (33). The N terminus of VP1 may reach the capsid surface through a cylindrical tunnel of ≥8 Å diameter at each of the fivefold axes of the icosahedron; in canine parvovirus, aa 38 was sited near the interior of the capsid at the fivefold axis (7). However, only 12 cylinders exist in a capsid; yet the proportion of VP1 in empty capsids, produced by coinfection of insect cells with VP1- and VP2-containing recombinant baculoviruses, can be as high 40–50% (12, 15), exceeding the expected limit of 12 VP1 molecules per 60 capsomeres. Perhaps some VP1 molecules participate in capsid formation but do not appear on the capsid surface. In the current work, we derived various estimates of the number of HEL molecules per capsid: 0.5 by enzymatic activity, 1 or 2 by density-gradient analysis, and 5 by ELISA. The difference might reflect steric hindrance of HEL active sites.

Because the signals required for external localization of VP1 are not known, we analyzed a variety of constructs with variable quantities of VP1 unique region. Capsid formation and encapsidation of chimeric protein were least efficient for the construct that lacked all VP1 unique region sequence, and both were optimal for HEL/CP4, in which most but not all of the unique region was preserved. We have observed increased empty capsid formation by recombinant baculoviruses containing progressively truncated VP1 alone without VP2 (19). Use of modified or substituted constructs should allow definition of the length constraints and sequence determinants necessary for optimal presentation of VP1 native or substituted unique region to the surface. The quantity of immunogenic residual VP1 sequence may also influence the quality of the immune response to HEL or other foreign antigen.

Substituted B19 parvovirus particles should have potential value as platforms for multivalent antigen presentation, although the strength and quality of the immune response in animals requires study. Preliminary results, using capsids containing very small quantities of HEL, suggest that foreign protein in the B19 capsid can be recognized by the host immune system. If substituted unique region is similar to native VP1, epitopes in this region should be highly immunogenic (14). In addition, parvoviruses are extraordinarily heat resistant, a useful quality for vaccination programs in the developing world. B19 capsids may be useful for delivery of intact and active proteins, as, for example, of enzymes into deficient macrophages or of toxins or drugs to erythroid cells. This system might be applied to protein purification. B19 particles might be used in receptor-mediated transfer of genes, as has been described for adenovirus (34). Indeed, parvoviruses are of current interest as vehicles for gene therapy (35–37), and substituted capsids may permit targeting of their recombinant DNA to specific cells or tissues.

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