Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4646–4650, June 1991
Microbiology

Self-assembled B19 parvovirus capsids, produced in a baculovirus system, are antigenically and immunogenically similar to native virions

(fifth disease/transient aplastic crisis/vaccine/enzyme immunoassay)

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Communicated by Helen Ranney, February 1, 1991 (received for review November 15, 1990)

ABSTRACT **B19 parvovirus is pathogenic in humans, causing fifth disease, transient aplastic crisis, some cases of hydrops fetalis, and acquired pure red cell aplasia. Efforts to develop serologic assays and vaccine development have been hampered by the virus's extreme tropism for human bone marrow and the absence of a convenient culture system. We constructed recombinants containing either the major (VP2) or minor (VP1) structural proteins of B19 in a baculovirus-based plasmid, from which the polyhedrin gene had been deleted; these recombinant plasmids were used to generate recombinant infectious baculovirus. Subsequent infection of insect cells *in vitro* resulted in high-level expression of either B19 VP1 or VP2. Parvovirus capsids were obtained by self-assembly in cell cultures coinfected with either VP1- and VP2-containing baculoviruses or, surprisingly, VP2-containing baculoviruses alone. Empty B19 capsids composed of VP1 and VP2 could replace serum virus as a source of antigen in a conventional immunoassay for detection of either IgG or IgM antiparvovirus antibodies in human serum. Immunization of rabbits with capsids composed of VP1 and VP2 resulted in production of antisera that recognized serum parvovirus on immunoblot and neutralized parvovirus infectivity for human erythroid progenitor cells. Baculovirus-derived parvovirus antigen can substitute for scarce viral antigen in immunoassays and should be suitable as a human vaccine.**

B19 parvovirus is the only member of the family Parvoviridae pathogenic in humans (1, 2). Acute infection results in fifth disease, a common childhood exanthem that in adults more usually manifests as an arthralgia/arthritis syndrome. In persons with underlying hemolysis, acute infection produces transient aplastic crisis, precipitous anemia due to hypoproliferative erythropoiesis. B19 infection can be persistent (3). In the setting of immunodeficiency, due to congenital, acquired, or iatrogenic immunodeficiency, persistent parvovirus results in chronic pure red cell aplasia. *In utero* infection causes hydrops fetalis, with fetal death due to severe anemia and congestive heart failure (4).

Clinical studies of B19 parvovirus infection have been hampered by limited supplies of viral antigen. The virus has extraordinary tropism for human erythroid progenitor cells and has only been propagated in explanted human bone marrow (5–8), fetal liver (9), and (to a lesser degree) erythroleukemia cells (10). We describe here expression of the virus's structural proteins in a baculovirus system. Large quantities of empty viral capsids were produced, which can substitute for serum virus in clinical assays and stimulate the production of neutralizing antibodies in animals. Only the major protein was required for capsid assembly; the minor capsid protein serves an important role in virus function independent of virion assembly.

MATERIALS AND METHODS

Cell Culture and Virus Stocks. *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and recombinant viruses were grown in monolayers of Sf9 cells. Sf9 cells (American Type Culture Collection), derived from *Spodoptera frugiperda* (fall army worm) ovary, were maintained in Grace's insect culture medium containing 10% heat-inactivated fetal bovine serum, 2.5 μg of fungizone per ml, 50 μg of gentamicin per ml, 3.33 mg of lactalbumin hydrolysate per ml, and 3.33 mg of yeastolate per ml (Gibco BRL Life Technologies, Gaithersburg MD) at 100% room air, 95% humidity, at 27°C.

Construction of Recombinant Plasmids and Baculoviruses. Two plasmids were constructed for the full-length minor (VP1) and major (VP2) capsid protein genes of B19 parvovirus. For plasmid pVP1/941, a segment corresponding to the mRNA coding sequence of the VP1 gene was excised from pYT103c, a molecular clone of B19 (11, 12), by digestion with *Hin*dIII (which cuts at map unit 45) and *Eco*RI (which cuts at map unit 95) and treatment with mung bean nuclease to blunt single-stranded ends. The DNA fragment was inserted into the *Bam*HI site (made blunt-ended with the Klenow fragment of DNA polymerase) of the baculovirus transfer vector pVL941, derived by deletion of the polyhedrin gene of AcMNPV and cloning into pUC8 plasmid (13). Construction of pVP2/941 was performed by the insertion of a *Pst* I–*Eco*RI digest fragment of pYT103c (map units 58–95; *Eco*RI site blunt-ended) and a synthetic DNA fragment of 20 nucleotides corresponding to the *Sst* I–*Pst* I region (again with the *Sst* I site blunt-ended) into the *Bam*HI site of pVL941 (Fig. 1).

Recombinant plasmids were used to generate recombinant baculoviruses. Eight micrograms of each plasmid was cotransfected into Sf9 cells with 2 μg of wild-type AcMNPV DNA, using calcium phosphate-mediated precipitation (13). Six days after transfection, progeny virus was harvested and replaqued onto fresh Sf9 cells. Recombinant viruses were recognized by the absence of nuclear occlusion bodies; stocks were prepared after three cycles of plaque purification.

Analysis of Protein Expression and Capsid Structure. Sf9 cells were infected with recombinant viruses at multiplicity of

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Abbreviations: AcMNPV, *Autographa californica* nuclear polyhedrosis virus; EIA, enzyme immunoassay; CFU-E, colony-forming unit, erythroid.

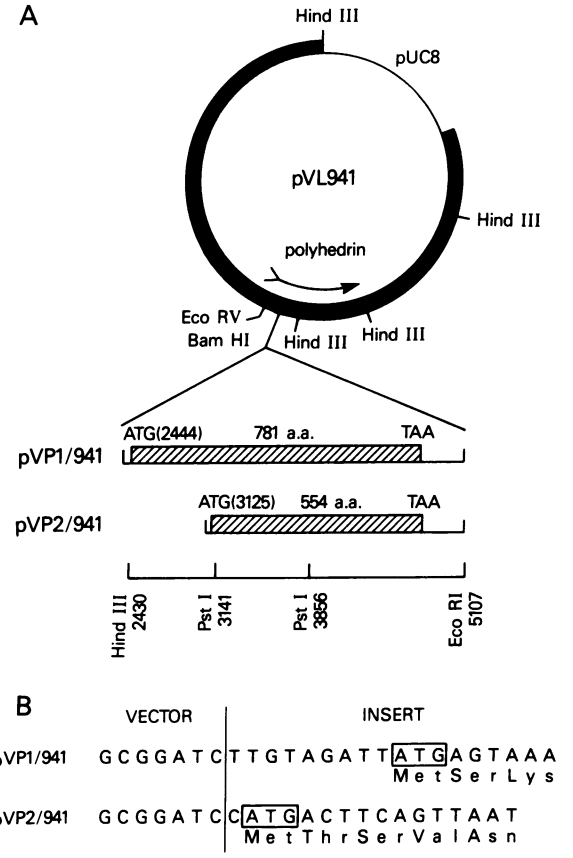

FIG. 1. Plasmid constructions containing the major (VP2) and minor (VP1) capsid genes of B19 parvovirus. (*A*) Diagram outlining relationship of inserts derived from pYT103c, a nearly full-length molecular clone of parvovirus B19, and the baculovirus vector pVL941. (*B*) Synthesized regions of DNA used to complete the gene sequences.

infections ranging from 10 to 50. Cells were harvested and examined for expression of VP1 and VP2 at variable times; 4 days after infection was optimal for recombinant protein expression. Cytocentrifuge preparations of recombinant or wild-type virus-infected cells were fixed in acetone at −20°C for 30 sec, washed twice in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin, and blotted dry. Cells were stained with convalescent phase human anti-B19 parvovirus antiserum (diluted 1:20), followed by application of fluorescein isothiocyanate-conjugated goat anti-human IgG (1:50; Kierkegaard and Perry Laboratories, Gaithersburg, MD).

For analysis of proteins by gel electrophoresis, lysates were prepared by heat disruption at 100°C for 3 min in 100 μl of Laemmli sample buffer (14). Aliquots were applied to 8% polyacrylamide gels (10 μl per lane) in the presence of sodium dodecyl sulfate. Proteins were directly visualized by staining with 0.25% Coomassie brilliant blue dye. For immunoblotting, proteins were transferred by electroblotting (Hoefer) onto nitrocellulose membranes. Specific proteins were detected by sequential application of convalescent phase human antiserum (1:300) and ^{125}I-labeled protein A (Amersham; ref. 15).

Capsids were examined by electron microscopy after equilibrium density gradient sedimentation. Sf9 cells were harvested 4 days after inoculation with recombinant baculoviruses. Cells were lysed by freezing and thawing followed by Dounce homogenization in a solution containing 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein inhibitor units of aprotinin per ml, 5 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 5 mM 2-mercaptoethanol, and 0.2% Triton X-100. Lysates were centrifuged at 100,000 × *g* for 16 hr over 40% (wt/vol) sucrose in Hanks' balanced salt solution. Precipitates were mixed with CsCl in 50 mM Tris·HCl, pH 8.7/5 mM EDTA/0.1% sarcosyl at an initial density of 1.31 g/ml and centrifuged at 100,000 × *g* in a SW41 rotor for 35 hr at 18°C. Transmission electron microscopy was performed after three banding procedures. For direct electron microscopy, pellets were prepared by ultracentrifugation of 50 μl of sample in 3.5 ml of PBS. Immune electron microscopy was performed by incubating 50 μl of sample with 50 μl of human serum containing IgG antibody to B19 parvovirus for 45 min at 20°C prior to dilution in PBS and ultracentrifugation. Pellets were resuspended in 50 μl of distilled water and negatively stained using 3% phosphotungstic acid (pH 6.5). Grids were examined at 60,000 × magnification in a JEOL 1200EX electron microscope.

Antibody Enzyme Immunoassay (EIA). The capture IgG and IgM antibody EIAs were adapted from a previously published B19 EIA (16). Capture antibody was either goat anti-human IgG or IgM antibody (Tago), applied to solid-phase Imunolon II 96 microtiter plates (Dynatech); the biotinylated detector was monoclonal antibody 521-5d, raised against partially purified serum parvovirus and broadly reactive with B19 capsid antigen; peroxidase-conjugated streptavidin (Amersham) was allowed to react with substrate 3,3′,5,5′-tetramethylbenzidine and H_2O_2. Each specimen was tested in duplicate against baculovirus B19 positive (P) and negative (N) control antigens and against human serum-derived B19 positive and negative antigens. A specimen was considered positive if the values for P-N and P/N were ≥ the mean plus three standard deviations for negative specimens. These values for baculovirus antigen IgG EIA were 0.035 for P-N and 2.0 for P/N and in the IgM EIA values were 0.030 and 2.0, respectively; for serum antigen IgG and IgM EIAs, values were 0.30 and 2.5, respectively.

Production of Antisera and Neutralization Assays. Rabbits were immunized with CsCl gradient-purified empty capsids. Animals were inoculated with either 20 or 200 μg of capsid protein by subcutaneous injection, initially in complete Freund's adjuvant, and with booster injections in incomplete Freund's adjuvant at 2- to 4-week intervals. Rabbit sera were analyzed by immunoblot and in neutralization assays. To determine neutralizing activity, sera were heated to 56°C for 30 min to inactivate complement and then incubated with quantities of serum containing B19 parvovirus (583A), at a concentration that completely inhibits erythropoiesis *in vitro*, for 1–2 hr at 4°C. Virus or virus plus serum was then incubated with normal human marrow cells at a concentration of 3 × 10^6 per ml for a further 1 hr at the same temperature; this was followed by dilution in standard tissue culture medium for late erythroid progenitors [colony-forming unit, erythroid (CFU-E)], consisting of 0.8% methylcellulose, 30% fetal calf serum, 1% bovine serum albumin, 1 mM 2-mercaptoethanol, and 1 unit of recombinant erythropoietin per ml (Amgen Biologicals), and incubation at 37°C, 95% humidity, for 6–7 days. Control experiments included assays of preimmune rabbit sera and normal human sera from patients in the convalescent phase of parvovirus infection. Marrow was obtained from volunteers by a protocol approved by the National Heart, Lung, and Blood Institute Institutional Review Board.

RESULTS

Expression of Parvovirus Structural Proteins. Insect cells were infected with recombinant baculoviruses containing, in substitution for the native nuclear polyhedrin protein gene, the gene for either the parvovirus major capsid protein (VP2) or the minor capsid protein (VP1). Immunofluorescence with convalescent phase human antiserum, containing high titers

of antibodies to B19 structural proteins, was used as a first test for parvovirus protein expression. All cells stained specifically with this antiserum, with bright fluorescence observed over cytoplasm and nuclei (Fig. 2*A*); the fluorescent signal was maximal 3–4 days after infection and faded after 1 week of culture, when most cells were no longer viable.

Specific capsid gene expression was determined by immunoblot in cell lysates harvested on day 3 after infection. Bands of the appropriate molecular mass were detected after infection with the VP1-baculovirus (Fig. 2*B*, lane a) or VP2-baculovirus (Fig. 2*B*, lane b) or after coinfection with both recombinant viruses (Fig. 2*B*, lane c). Enough parvovirus structural proteins were produced to be visible after dye staining of polyacrylamide gels of lysates (Fig. 2*C*); parvovirus protein was estimated by densitometry to constitute 2–3% of total cell protein.

Identification of Empty Capsids. When cell lysates were subjected to sequential sedimentation in sucrose and CsCl, banding of parvovirus proteins (determined by immunoblot or immunoprecipitation) was detected at 1.31 g/ml, the density of empty capsids, for cells infected with VP2-baculovirus and cells coinfected with VP1- and VP2-baculoviruses; no parvovirus protein was detected in banded cell lysates from VP1-baculovirus-infected cells (data not shown).

Immune electron microscopy showed typical parvovirus capsids, aggregated by B19 antibody, in samples from cultures coinfected with VP1- and VP2-containing baculoviruses and also in cultures after infection with VP2-baculovirus. No virus particles were seen in lysates of cells infected with VP1-containing baculovirus alone. Direct electron microscopy of harvests from cultures coinfected with VP1- and VP2-bearing baculovirus and from cultures infected with VP2-containing virus only revealed numerous typical parvovirus-like particles not coated with any substance resembling antibody (Fig. 3). A minority of particles was electron dense, the majority was less dense, and some particles were dense in patches. Particles tended to cluster.

Immunoassay. The antigenicity of parvovirus proteins produced in this system was determined using a conventional clinical assay for human antibody to B19 parvovirus. Results for IgG and IgM were qualitatively identical for antigen derived from pooled viremic sera and from baculovirus-infected cell lysates. For the IgG assay, 23 specimens were negative in both immunoassays, 45 were positive in both, and none was discordant. For the IgM assay, 25 specimens were negative in both assays, 43 were positive in both, and none was discordant. Assays based on the two different sources of antigen also gave comparable quantitative results (Fig. 4). Correlation coefficients for P-N absorbance values for serum antigen versus baculovirus antigen were 0.95 for the IgG immunoassays and 0.91 for the IgM assays.

Production of Neutralizing Antisera. Rabbits were inoculated with empty capsids composed either of VP1 and VP2 or of VP2 alone, and sera were tested for ability to neutralize virus activity in progenitor assays of CFU-E. None of the animals inoculated with low doses of antigen (20 μg per injection) produced neutralizing antisera. However, neutral-

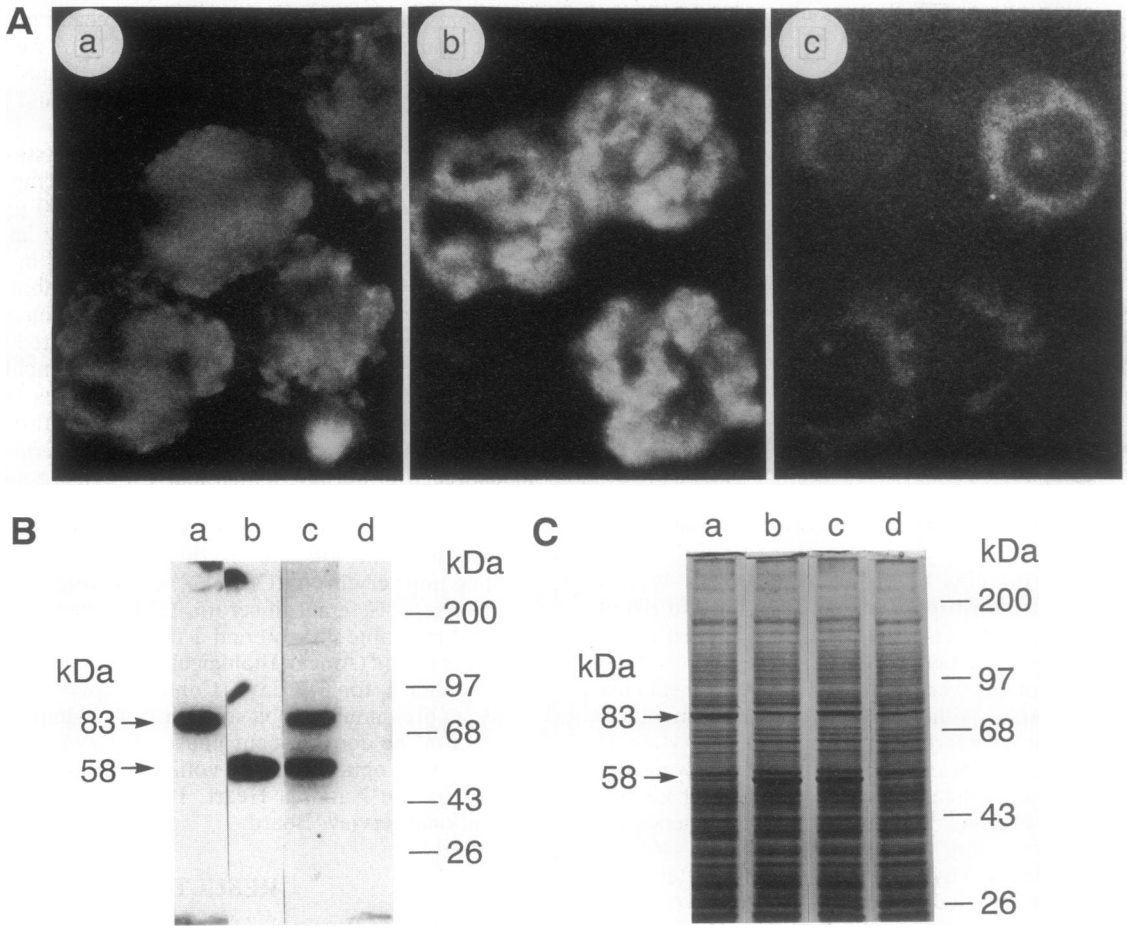

FIG. 2. Expression of B19 parvovirus proteins in insect cells infected with recombinant baculoviruses. (*A*) Immunofluorescence of Sf9 cells infected with VP1- (a), VP2- (b), and wild-type baculoviruses (c) after staining with convalescent phase antiserum to B19 parvovirus. (×1100.) (*B*) Immunoblot of lysates from cells infected with VP1- (lane a), VP2- (lane b), VP1- plus VP2- (lane c), and wild-type baculoviruses (lane d) after development with convalescent phase antiserum followed by ^{125}I-labeled protein A. (*C*) Coomassie blue dye-stained polyacrylamide gel of Sf9 cell lysates after infection with VP1- (lane a), VP2- (lane b), VP1- plus VP2- (lane c), and wild-type baculoviruses (lane d).

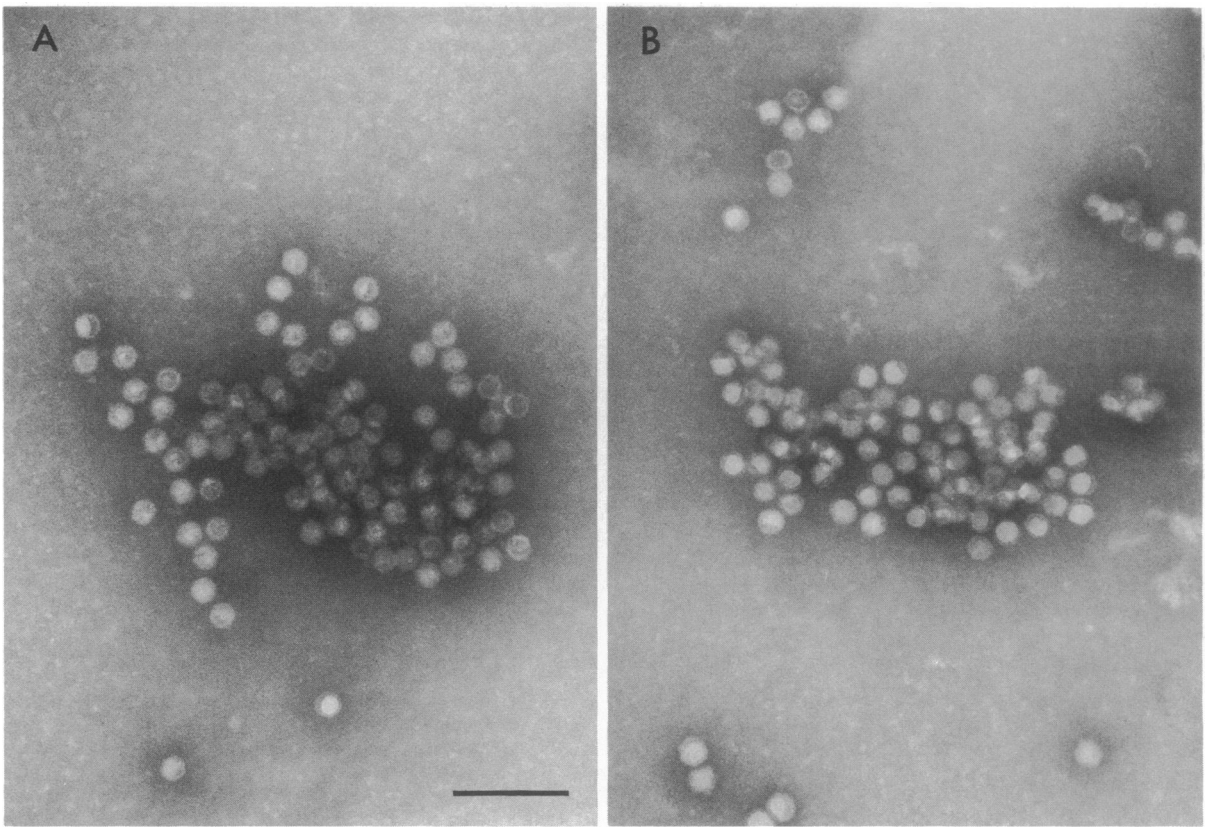

FIG. 3. Direct electron microscopy of capsids. After infection with either VP1- plus VP2- (*A*) or VP2- (*B*) baculoviruses cell lysates were subjected to equilibrium density gradient sedimentation and examined by transmission electron microscopy after negative staining. (×154,000; bar = 100 nm.)

izing antibodies were produced in three of three animals immunized with larger quantities (200 μg per injection) of empty capsids, composed of VP1 and VP2; these antigens were obtained by coinfection of insect cells with the two individual recombinant viruses (Fig. 5*A*). The titers of neutralizing activity in two animals were comparable to those observed in convalescent phase human sera (Fig. 5*B*). In contrast, none of the sera from the three animals inoculated with capsids composed of VP2 alone contained neutralizing activity (Fig. 5*A*), although sera from all six animals immunized with 200 μg of antigen contained precipitating antibodies [determined by Ouchterlony analysis (data not shown)].

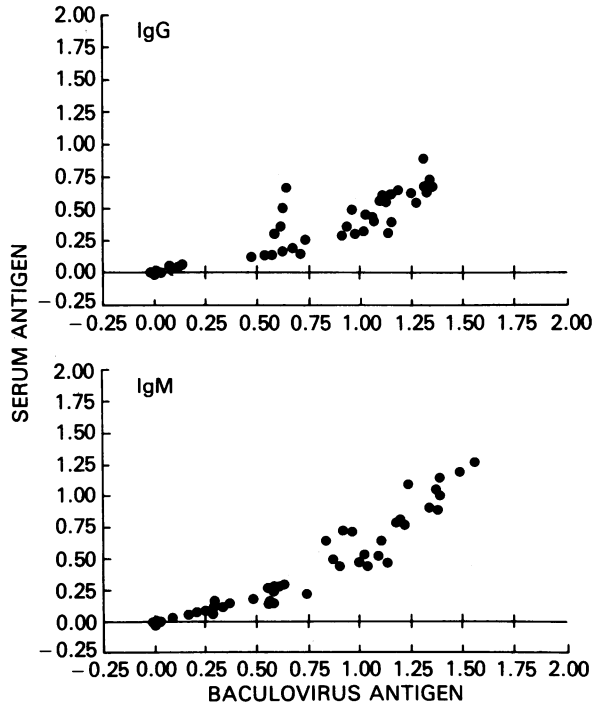

FIG. 4. Capture immunoassay comparing antigen derived from sera of infected patients with Sf9 cell lysate after *in vitro* infection with VP2-baculorviruses.

DISCUSSION

Empty viral capsids are produced in natural infection of animals and in tissue culture cells infected with parvoviruses (17), and capsid formation is believed to precede DNA insertion and virion assembly (18). Empty parvovirus capsid formation has also been accomplished experimentally with temperature-sensitive mutants (19) and chimeras (20) of rodent parvoviruses and by transfection of a mammalian cell line with human parvovirus structural protein genes (21). Expression of novel proteins in baculovirus can be easily repeated once the recombinant viruses have been constructed; in addition, the quantity of recombinant protein expressed and the ease of cell culture make the baculovirus system more efficient in labor and materials than mammalian cell culture. Although B19 parvovirus proteins have been expressed in bacteria (22, 23) and a cyclic peptide based on the virus's sequence has been synthesized (24), assays based on these products have been imperfect, usually due to inability to detect IgM antibody or a general lack of specificity or sensitivity. We show here that baculovirus-generated B19 parvovirus capsid proteins completely substitute for intact virions in conventional immunoassays for antivirus IgG and IgM. Similar results have been reported

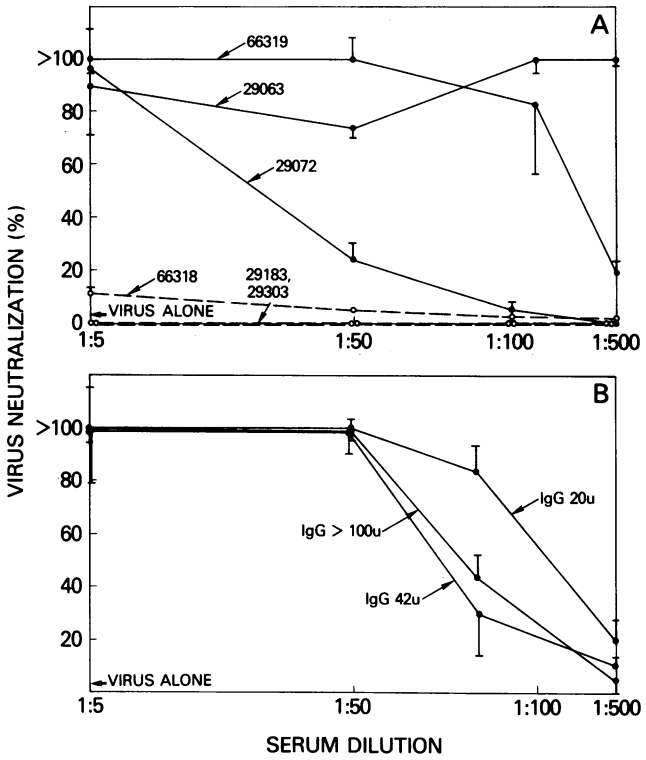

FIG. 5. Neutralization of B19 parvovirus infectivity by rabbit antisera to empty capsids. (*A*) Sera from six rabbits immunized with partially purified capsids composed of VP1 plus VP2 (solid circles and lines) or VP2 alone (dashed lines, open circles) were tested for their ability to abrogate the cytotoxic effect of B19 parvovirus in CFU-E assays of normal bone marrow. (*B*) For comparison, serum from a normal individual, obtained at different times after an acute infection and containing varying quantities of antiparvovirus IgG determined by capture immunoassay, was tested simultaneously. In this experiment, CFU-E-derived colony formation from normal bone marrow numbered 50 per 10^5 cells. Addition of virus alone resulted in 1 per 10^5 (indicated by arrow). Fifty or more colonies per plate was made equivalent to 100% neutralization. The experiment illustrated is one of two with similar results.

recently using a different baculovirus system by Brown and coworkers (25): their recombinant protein functioned like intact virions in an immunofluorescent assay and in immunoblot analysis of sera (however, they did not demonstrate capsid formation).

Neutralizing antibodies to human parvovirus were made in rabbits inoculated with empty capsids after coinfection with recombinant baculoviruses that expressed VP1 and VP2. The baculovirus capsids should be a suitable reagent for human vaccine trials, not only because they are easy to produce and purify but also because, in contrast to 3-11-5 cell lysates, they lack the simian virus 40 enhancer-promoter element and are synthesized in untransformed cells. The ratio of VP1 to VP2 in empty capsids is higher after coinfection of insect cells than in empty capsids produced by 3-11-5 cells. Immunization with capsids that contained only VP2 did not result in neutralizing antibody, although precipitating antibodies to capsid antigen were present in the sera of all immunized animals. Possibly of great importance, the proportion of VP1 may be increased by altering the multiplicity of infection of the respective baculovirus species (unpublished data), which may augment the ability of the host to produce neutralizing antibody. Other nonparvovirus epitopes might be substituted for this sequence and presented to the host immune system in the context of a stable, noninfectious particle.

That capsids, morphologically indistinguishable from natural capsids, could assemble from VP2 protein alone was unexpected. In computer modeling, VP1 has been proposed as an internal protein that stabilized the capsid structure (26). Our data suggest that VP1 is not required for capsid assembly; in fact, VP1 alone was incapable of forming a capsid structure. Only VP1-containing capsids allowed production of neutralizing antibodies in rabbits. Human convalescent phase antisera and pooled immunoglobulin also recognize mainly VP1 rather than VP2 (27). These data are consistent with VP1's presence on the surface of the capsid structure, where it might function as the ligand for the cellular receptor for the virus. Alternatively, VP1 might stabilize the capsid structure or alter the conformation of VP2 epitopes.

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