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A chimeric baculovirus displaying bovine herpesvirus-1 (BHV-1) glycoprotein D on its surface and their immunological properties

A. Peralta · P. Molinari · D. Conte-Grand · G. Calamante · O. Taboga

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Abstract The ability of a recombinant baculovirus containing the ectodomain of the mature sequence of glycoprotein D (gD) fused to the amino-terminus of baculoviral glycoprotein gp64 to display gD on its surface and to serve as an improved immunogen against bovine herpesvirus-1 was tested. The gD–gp64 fusion protein was correctly expressed on the virus particles as revealed by immunomicroscopy assays. Mice immunized with 5×10^8 plaque forming units developed antibodies that specifically reacted in an enzyme-linked immunosorbent assay with recombinant gD and whole bovine herpesvirus-1. These antibodies were able to neutralize bovine herpesvirus-1 in vitro, whereas those elicited by a version of gD expressed in *Escherichia coli* did not. Our data demonstrated that the display on the virion surface of recombinant baculovirus can provide a tool for the development of recombinant vaccines against bovine herpesvirus-1.

Keywords Baculovirus display · BHV-1 vaccines · Viral vectors

A. Peralta · P. Molinari · D. Conte-Grand · G. Calamante · O. Taboga (✉)
Instituto de Biotecnología, CICVyA, INTA,
Castelar CC25 (1712) Buenos Aires, Argentina
e-mail: otaboga@cnia.inta.gov.ar

A. Peralta · P. Molinari · D. Conte-Grand · O. Taboga
Consejo Nacional de Investigaciones
Científicas y Tecnológicas (CONICET),
Rivadavia 1917 (1033) Ciudad Autónoma de Buenos Aires,
Argentina

Introduction

To improve the immunogenic properties of soluble antigens, multimeric, self-assembling proteins have been used as carriers for the delivery of foreign epitopes (Gamvrellis et al. 2004; Noad and Roy 2003; Roy 1996; Tobin et al. 1996). Based on their multimeric nature and due to the presence of T cell epitopes, these carriers elicit humoral and cellular responses. Besides, these macromolecules can be easily purified by simple centrifugation. However, problems associated with toxicity of recombinant products in bacteria, loss of multimerization of core-like particles carrying foreign sequences and restrictions in size of antigenic sites that could be expressed have been reported (Tami et al. 2000).

It has been previously shown that the in-frame fusion of foreign sequences between the signal sequence and ectodomain of the mature sequence of gp64, an outer glycoprotein of *Autographa californica* nuclear polyhedrosis virus (AcNPV), drives the chimeric protein to the surface of the baculovirus and facilitates its purification and concentration (Boublik et al. 1995). Proteins as large as the P1 precursor from foot-and-mouth disease virus (FMDV), a polypeptide of about 74 kDa, could be successfully displayed on the surface of recombinant baculoviruses. This strategy, known as baculovirus display, has been used to develop recombinant vaccines against FMDV (Tami et al. 2000), *Plasmodium berghei* (Yoshida et al. 2003), rubella (Mottershead et al. 1997), and *Theileria parva* p67 antigen (Kaba et al. 2003). Some of them have induced high titers of antigen-specific antibodies.

The baculovirus display system has several potential advantages as a vaccine vehicle: its particulate nature, the intrinsic immunostimulatory effect of many viral proteins, the multimeric presentation of the epitopes, and the proper

conformation of antigens on the virion surface, all of which make them accessible for interactions with cellular components of the immune system.

Bovine herpesvirus-1 (BHV-1), a member of the Alphaherpesvirinae subfamily, causes a set of diseases, including infectious rhinotracheitis, vulvovaginitis, balanoposthitis, conjunctivitis, abortion, and shipping fever in cattle. Currently used BHV-1 vaccines, formulated with either inactivated or modified live virus, present a number of disadvantages. Although inactivated vaccines are usually poor immunogens and may cause clinical disease if insufficiently inactivated, live vaccines may induce immunosuppression (Yates 1982) and complicate the discernment between vaccinated and infected animals. Vaccination with BHV-1 subunits is being explored to solve these problems (Israel et al. 1992; van Drunen Littel-van den Hurk et al. 1993; van Drunen Littel-van den Hurk et al. 1994; Zhu and Letchworth 1996; Zhu et al. 1997).

The development of subunit vaccines is focused on three major envelope glycoproteins, designated as gB, gC, and gD. They play key roles in early steps of infection and are major targets for both cellular and humoral immunity. Of the three glycoproteins, gD is proposed as the principal vaccine candidate, as it induces a more consistent and stronger cellular immune response than the others and also because antibodies to gD have the highest neutralizing titers (Hughes et al. 1988; Dubuisson et al. 1992).

Expression of gD in *Escherichia coli* and eukaryotic systems, such as vaccinia, adenovirus, baculovirus, and plants, has been reported (Chase et al. 1990; Kowalski et al. 1993, Perez Filgueira et al. 2003). Previous publications have shown that gD produced in *E. coli* failed to induce protective responses probably due to its unglycosylated nature (van Drunen Littel-van den Hurk et al. 1993).

In this report, recombinant baculoviruses were used to display gD from BHV-1, and the antigenic properties of the fusion protein gD–gp64 exposed on the virion surface were assessed in a mouse model. Our results suggest that recombinant baculoviruses displaying gD may be exploited as promising candidates for the design of subunit vaccines against BHV-1 and provide further evidence about the use of this approach to elicit effective immune responses.

Materials and methods

Cells and viruses

AcNPV was propagated in *Spodoptera frugiperda* Sf9 cells (ATCC) grown at 27°C in TNM–FH medium (Sigma) supplemented with 10% fetal bovine serum (FBS) and antibiotic–antimycotic solution (Gibco). For analysis of the expression of the recombinant proteins in cell extracts, Sf9 cells were infected at a multiplicity of infection (moi) of 10 and harvested 4 days post infection (dpi). For virus stock preparation, Sf9 cells growing in 500-ml spinner flasks were infected at a moi of 0.1 and supernatants collected 5 dpi. Virus titers were determined by plaque assays as previously described (O'Reilly et al. 1992). Small aliquots were concentrated by ultracentrifugation and analysed for the presence of the gD–gp64 fusion protein.

BHV-1 strain LA was propagated in Madin Darby bovine kidney (MDBK) cells grown in Eagle minimal medium (MEM) supplemented with 10% FBS. Confluent monolayers in roller bottles were infected with BHV-1 at a moi of 0.1, and supernatants collected when extensive cytopathogenic effect was observed. Viral suspension was clarified by centrifugation at 3000×*g* for 30 min, diluted to 10^5 tissue-culture infective doses per ml and stored at −70°C. Virus was inactivated by placing it for 1 min at a distance of 11 cm from two General Electric G875 ultraviolet bulbs.

Construction of recombinant bacteria

The sequence encoding the ectodomain of mature gD, lacking the codons for the signal peptide, the membrane anchor, and the cytoplasmic domain, was amplified by polymerase chain reaction (PCR) using the primers TTGCCTACACCCGCGCCGCGGGT (forward) and TCAGGCGTC GGGGGCCGCGGGCGTA (reverse). The 1,025-bp product was cloned into pGEMT Easy vector (Promega) and digested by *Eco*RI (present in the vector to both sides of the insert). The released gD fragment was purified and cloned into pRSETB bacterial vector (Novagen) in frame with a 6-his tag. Integrity and correct insertion of the gD sequence into the pRSETBgD-Ec vector were confirmed by restriction analysis and nucleotide sequencing (data not shown). Recombinant *E. coli* DH5α were obtained by standard transformation (Maniatis et al. 1982). *E. coli* BL21 cells were used for gD-Ec expression.

Construction of recombinant baculoviruses

The same gD-coding sequence expressed in bacteria, lacking the codons for the signal peptide, the membrane anchor, and the cytoplasmic domain, was amplified by PCR using the primers AACCCGGGTTGCCTACACCCGCGCCGC GGGT (forward) and AACTG CAGGGCGTC GGGGGCCGCGGGCGTA (reverse). The 1,032-bp product was digested by XmaI and PstI (underlined sequences) and cloned into polyhedrin-based vector pVLSup1 (Tami et al. 2000) in frame in the MCS at the signal-mature junction of gp64 sequence to originate pVLSupgD. Integrity and correct insertion of the gDc sequence into the pVLSup1 vector were confirmed by restriction analysis and nucleotide sequencing (data not shown). Recombinant baculovirus AcSupgD was

obtained by standard procedures by co-transfection of pVLSupgD and Baculogold AcNPV DNA (Pharmingen) as suggested by manufacturers.

Analysis of gD-Ec expression and purification

Twenty-five ml of transformed *E. coli* BL21 fresh cultures were grown at 37°C in Luria-Bertani medium containing 100 μg ml^{-1} ampicillin until OD_{600}=0.4, and the expression of gD-Ec was induced by addition of isopropyl-beta-D-thiogalactopyranoside at a final concentration of 1 mM. Samples of 500 μl were pelleted and disrupted in 2× loading buffer, boiled for 5 min, and loaded onto 10% sodium dodecyl sulfate (SDS) polyacrylamide gels.

To purify gD-Ec, pellets from induced cultures were disrupted in 6 M Guanidinium buffer and supernatants were passed through Probond columns (Invitrogen) as suggested by manufacturers. As the affinity chromatography did not render a homogeneous product, gD-Ec present in eluted fractions was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution to avoid the detection of antibodies directed to bacterial proteins.

Analysis of gD–gp64 expression

Sf9 infected cells ($1–2\times10^6$) growing in 25-cm^2 flasks were collected 4 dpi and disrupted in 200 μl of loading buffer, boiled for 5 min, and 10 μl loaded onto 10% SDS polyacrylamide gels. After electrophoresis, proteins were electrotransferred to nitrocellulose filters (MSI) and probed with a monoclonal antibody (Mab) to gD or Mab AcV5 to AcNPV gp64 (kindly provided by Dr. Gary Blissard). Presence of recombinant protein was revealed with anti-mouse alkaline phosphatase conjugated and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP).

For analysis of viral expression, 4 ml of 4-dpi clarified supernatants were concentrated by ultracentrifugation onto 25% sucrose cushion at 131,000×*g* for 1.5 h. The viral pellet was resuspended in 60 μl of PBS pH 6.2 and 15 μl disrupted in 2× loading buffer, boiled for 5 min, and loaded onto 10% SDS polyacrylamide gels.

For the detection of gD–gp64 multimers, baculovirus pellets were resuspended in 1× loading buffer containing different amounts of 2-mercaptoethanol (2-ME) from 0 to 6×10^{-1} mM, incubated for 30 min at 37°C, and loaded onto 10% SDS polyacrylamide gels.

Immunogold labeling

Supernatants of infected cells were harvested 5 days postinfection and clarified by centrifugation at 2,000 rpm. Clarified supernatants containing recombinant baculoviruses were loaded onto a 25% sucrose cushion (25% *w*/*w* sucrose, 10 mM ethylenediamine tetraacetic acid, 5 mM NaCl) and pelleted for 80 min at 131,000×*g*. Pellets were resuspended in PBS pH 6.2. Formvar-coated grids were floated on a drop of concentrated virus suspension for 120 min. After that, grids were floated on a drop of 0.1% bovine serum albumin (BSA) and exposed to an anti-gD Mab (1:30 dilution in 0.1% BSA) overnight at 4°C. Grids were washed five times in PBS for 5 min and exposed to an anti-mouse immunoglobulin G (IgG) conjugated with 10.3-nm gold particles (1:30 dilution in 0.1% BSA) for 90 min at room temperature. Grids were washed a further five times with PBS and three times with water for 5 min, stained with phosphotungstic acid for 2 min and observed with a Jeol 1200 EX II electron microscope operating at 85 kV.

Animals, inoculations, and serum sampling

Male BALB/c mice (6–8 weeks old) were used for vaccination. Animals received, by the intraperitoneal route, three doses of an oil-based vaccine formulated with incomplete Freund adjuvant (IFA) at days 1, 16, and 45 for each immunogen. Mice received 0.2 ml of this vaccine containing a final amount of 5×10^8 plaque forming units (pfu) of AcSupgD. A negative control group was immunized with 5×10^8 pfu of Acgp64, a baculovirus over-expressing gp64 from an extra copy of this gene, and positive control groups were immunized with 1 μg of recombinant gD produced in *E. coli* (gD-Ec) or with $10^{5.5}$ tissue culture lethal doses 50% of inactivated BHV-1 vaccine.

Animals were bled at different times postvaccination and were all the time maintained with free access to sterile food and water.

Analysis of antibody response to gD polypeptide

ELISA tests

Serum samples from mice inoculated with AcSupgD were tested by enzyme-linked immunosorbent assay (ELISA). gD-Ec was adsorbed to Immulon II plates (Dynatech) at a concentration of 0.02 μg/well in buffer carbonate. Plates were blocked with PBS–Tween 20 (PBST) 1% gelatin (blocking buffer) and subsequently incubated for 1 h at 37°C with mice serum samples and, then, a secondary anti-mouse-Ig (G+M)-specific HRP-conjugated antibody. Primary and secondary antibodies were diluted with the same blocking buffer. The reaction was developed by addition of *o*-phenylenediamine-H_2O_2 in citrate buffer pH 5 and read 3 min later at 490 nm in an MR 500 Microplate Reader (Dynatech). Titers were expressed as $\log_{10}$ of the reciprocal of the highest serum

dilution, which gives OD readings at least twofold higher than those of control animals.

Western blot assays

Cell lysates from MDBK cells infected with BHV-1 were ultracentrifuged 110 min at 80,000×*g*, and then, the resuspended pellet was loaded onto to 30–50% discontinued sucrose cushion and ultracentrifuged 90 min at 100,000×*g*. The band containing the virus was collected, disrupted in 200 μl of loading buffer, boiled for 5 min, and 10 μl loaded onto 10% SDS polyacrylamide gels. After electrophoresis, proteins were electrotransferred to nitrocellulose filters (MSI), blocked overnight with PBST 5% skim milk, and probed with sera from vaccinated mice in 1:200 dilutions for 1 h at 37°C. Blots were washed and then incubated with an AP-labeled anti-mouse IgG goat antiserum (Dakkopats) for 1 h at 37°C. After extensive washing, the reaction was developed by the addition of the NBT/BCIP substrate.

Seroneutralization

Neutralizing antibodies in sera were measured by a standard plaque reduction assay. Briefly, 100 pfu of BHV-1 were diluted 1:2 with twofold serial dilutions of mice sera collected at day 60 and incubated for 1 h at 37°C. Dilutions were added to monolayers of MDBK cells and 72 h postinfection; the number of remaining infectious virions was determined by fixing and staining of monolayers with crystal violet.

Competition assay

MDBK monolayers grown in 60-mm dishes containing 1×10^6 cells were pre-incubated for 20 min at room temperature with serial dilutions of AcSupgD and Acgp64 (from 9.6×10^6 to 2.4×10^6 pfu) in TNM–FH in a final volume of 400 μl. Control dishes were pre-incubated with TNM–FH alone. Then, dishes were incubated with 60 pfu of BHV-1 for 30 min at 37°C. After two washes with MEM-D medium, 4 ml of an overlay (MEM-D supplemented with 10% FBS plus 0.7% low melting point agarose, Sigma) were added and dishes incubated at 37°C for 72 h. Monolayers were fixed with 10% formaldehyde and stained with crystal violet.

Results

Expression of gD–gp64 fusion protein

The gD gene fragment coding for amino acids 19 to 360, lacking its signal peptide and the hydrophobic C-terminal domain, was inserted into pVLsup vector to express the gD–gp64 fusion protein on the virion surface. As a control, a baculovirus overexpressing gp64 from a second copy inserted in polyhedrin locus was used.

Once the expression of the recombinant proteins in cell extracts infected with AcSupgD and Acgp64 was confirmed (data not shown), budded viruses were purified from sucrose cushions, separated in a 10% SDS-PAGE and transferred to a nitrocellulose membrane to determine whether the fusion gD–gp64 was efficiently incorporated into the viral particle. When blots were analysed with specific Mab to gD, viral extracts exhibited a major reactive band of about Mr 130,000, which is in the range of the expected molecular weight for this fusion and no bands were detected in AcSupgp64. The same band was revealed by Mab AcV5 directed against gp64. An additional band was detected at Mr 64,000, which represents the wt copy of gp64 and is overexpressed in AcSupgp64 (Fig. 1a and b).

Electron microscopy

The detection of gD–gp64 fusion protein in supernatants of Sf9 cells infected with AcSupgD was compatible with the incorporation of gD–gp64 into the budded baculovirus (Boublik et al. 1995). To further confirm the association of gD–gp64 with viral particles, purified baculoviruses were examined by immunogold labeling. The presence of gold particles at the end of the virion is consistent with the

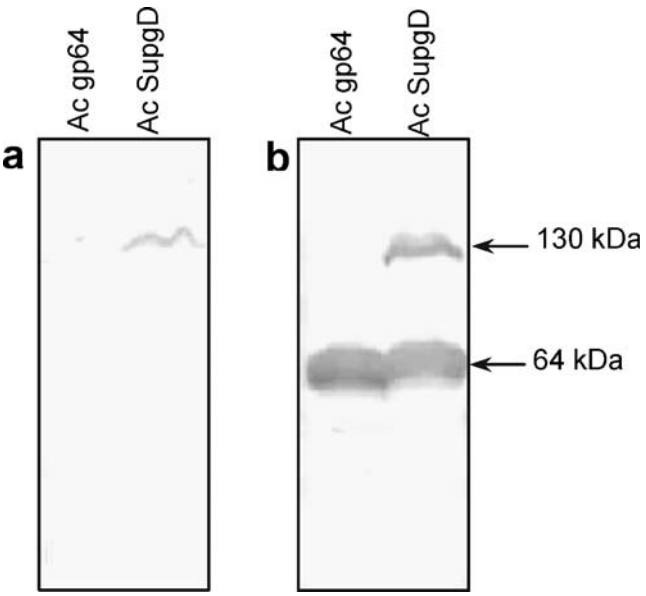

Fig. 1 Expression of recombinant fusion proteins in baculovirus virions. Supernatants of Sf9 cells infected either with AcSupgD or Acgp64 were harvested 5 dpi, ultracentrifuged, resuspended in loading buffer, and subjected to electrophoresis on 0.1% SDS–10% polyacrylamide. Western blots were revealed with a monoclonal antibody to gD (**a**) or a monoclonal antibody to gp64 (**b**). Protein sizes are indicated with *arrows*

previously reported localization of gp64 and recombinant proteins fused to gp64 in the peplomers of baculovirus (Fig. 2). Labeling of AcSupgp64 incubated with the Mab to gD showed no decoration of the peplomer with gold particles (data not shown). These results suggest that the gD–gp64 fusion protein is incorporated with a similar pattern to that of wild-type gp64.

Oligomerization of the gD–gp64 fusion protein

To investigate the oligomerization of the recombinant protein on the baculovirus surface, SDS-PAGE under partially reduced conditions were carried out. Figure 3 shows that the gD–gp64 fusion protein was detected as multiple bands under nonreducing and partially reducing conditions ranging from 0 to 0.6 mM 2-ME. The results suggest that a high proportion of the recombinant gD–gp64 is present as homo- and hetero-oligomers on the surface of recombinant baculoviruses.

Binding of AcSupgD to MDBK cells

gD is essential for penetration of alphaherpesvirus into cells. A phenomenon termed "interference" has been postulated to be the result of competition for a limited number of gD receptors on the cell surface, a fact which affects penetration but not viral binding or post-penetration steps (Dasika and Letchworth 1999; Campadelli-Fiume et al. 1988).

To investigate whether gD on the surface of recombinant virions retained the ability to interact with cellular receptors, MDBK cells were preincubated with dilutions of AcSupgD or Acgp64 and, then, infected with a fixed number of BHV-1. The results showed that AcSupgD diminished the number of plaques in a dose-dependent manner, suggesting that a significant part of the native gD conformation was retained in the recombinant baculovirus AcSupgD (Fig. 4). Although significantly lower, Acgp64 showed some degree of inhibition, related with the ability of baculoviruses to bind mammalian cells.

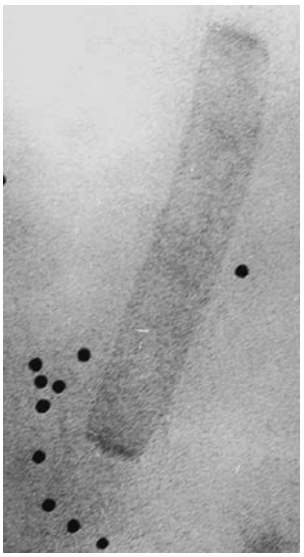

Fig. 2 Immunogold labeling of recombinant AcSupgD baculovirus. Viruses purified through a sucrose cushion were adsorbed to Formvar-coated grids, and the presence and localization of gD–gp64 fusion protein on the surface of the virion was detected with an anti-gD monoclonal antibody and an anti-mouse IgG-gold conjugate. The figure is representative of all fields examined. *Bar* 50 nm

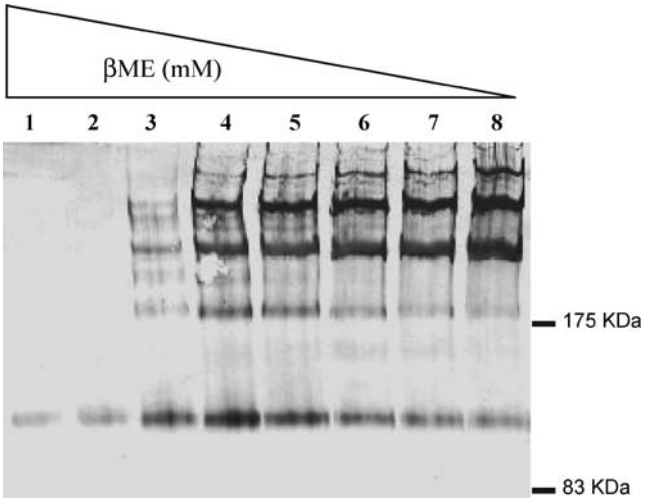

Fig. 3 Oligomerization of gD–gp64 fusion protein. AsSupgD virions were processed for Western blot under partially reducing conditions (6×10^{-1}; 3×10^{-1}; 1.5×10^{-1}; 6×10^{-2}; 3×10^{-2}; 1.5×10^{-2}; 6×10^{-3}; 0 mM; from line 1 to 8). The different oligomers were revealed using a monoclonal antibody to gD. Protein markers are indicated with *bars*

Induction of an anti-BHV-1 response in mice immunized with recombinant baculoviruses AcSupgD

The immunogenicity of virions displaying gD protein was evaluated in a mouse model. Adult mice were immunized intraperitoneally with recombinant budded virions AcSupgD emulsified in IFA. As a negative control, the same quantity of Acgp64 was used. Serum samples were extracted at different times postinoculation to measure the reactivity against BHV-1 antigens. The recombinant gD-Ec was utilized as antigen in an indirect ELISA to test the

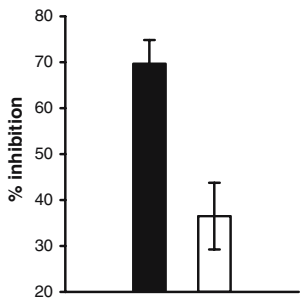

Fig. 4 Binding of AcSupgD to MDBK cells. MDBK cells were pre-incubated with 107 pfu of AcSupgD (*black bar*) or Acgp64 (*white bar*). Inhibition was measured as $100-(100\times N/T)$, where N is the number of plaques of BHV-1 in monolayers pre-incubated with baculoviruses, T is the number of plaques of BHV-1 in monolayers pre-incubated with TNM–FH medium. *Bars* represent the mean value of triplicates

ability of AcSupgD to induce specific antibodies through time. Results demonstrated that after vaccination, mice immunized with gD-Ec and mice immunized with AcSupgD exhibited the highest antibody titers (Fig. 5a and Table 1). Both titers were higher than those elicited by a BHV-1 inactivated vaccine up to 90 dpi. Western blot experiments based on purified BHV-1 were carried out to demonstrate the reactivity of the elicited antibodies to the native gD. The results showed that both recombinant immunogens elicited a humoral immune response specific to native gD, indistinguishable from that elicited by inactivated whole viruses (Fig. 5b). In the latter case, the sera also revealed extra bands, other than native gD, due to reactivity to other structural proteins.

Ability of antibodies elicited by recombinant vaccines to neutralize BHV-1

To evaluate the ability of antibodies elicited by recombinant gD-Ec or recombinant baculovirus AcSupgD to neutralize the infectivity of BHV-1 in vitro, serial dilutions of pools of sera from mice vaccinated with both recombinant vaccines were confronted to a fixed quantity of BHV-1 particles. As negative and positive controls, pools of sera from mice vaccinated with AcSupgp64 or BHV-1 inactivated vaccine were used, respectively.

Table 1 shows that, although gD-Ec elicited the higher antibody titers as measured by ELISA tests, these antibodies did not neutralize the infectivity of BHV-1 in cell cultures. On the contrary, sera from mice vaccinated with AcSupgD were capable of neutralizing BHV-1 in vitro to levels similar to those showed by the inactivated vaccine, suggesting that a significant part of the native gD conformation was retained in the recombinant fusion protein displayed on the surface of baculoviruses. The proper conformation of gD on the virion surfaces was also supported by competition assays with whole BHV-1 to the cellular receptors. Figure 4 shows that AcSupgD specifically competed for the binding to cell receptors, whereas a lower binding was observed when Acgp64 was used.

Discussion

In this report, we describe the construction of recombinant baculoviruses expressing a fusion protein between gD from BHV-1 and a second copy of gp64 from AcNPV. This chimeric protein was successfully displayed on the surface of the budded baculovirus, and the pattern of incorporation to the budded virions seemed to proceed in a similar way to that of the wild-type gp64. It has been demonstrated that incorporation of fusions of some proteins to gp64 into the membrane of recombinant budded virions occurs by an active incorporation as an integral component of the assembling from gp64 (Boublik et al. 1995; Tami et al. 2000). Our results suggest that gD–gp64 fusion protein incorporates to the virion surface following a similar pattern of oligomerization. This oligomerization could also contribute to a higher immunogenicity of gD as occurs with other multimeric antigens (Francis and Clarke 1989). The presence of gD–gp64 fusion on the budded baculovirus did not alter viral infectivity, as recombinant virus routinely exhibited high titers, similar to those obtained with wild-type AcNPV.

It has been previously demonstrated that gD induces immunity resulting in significant reductions in viral

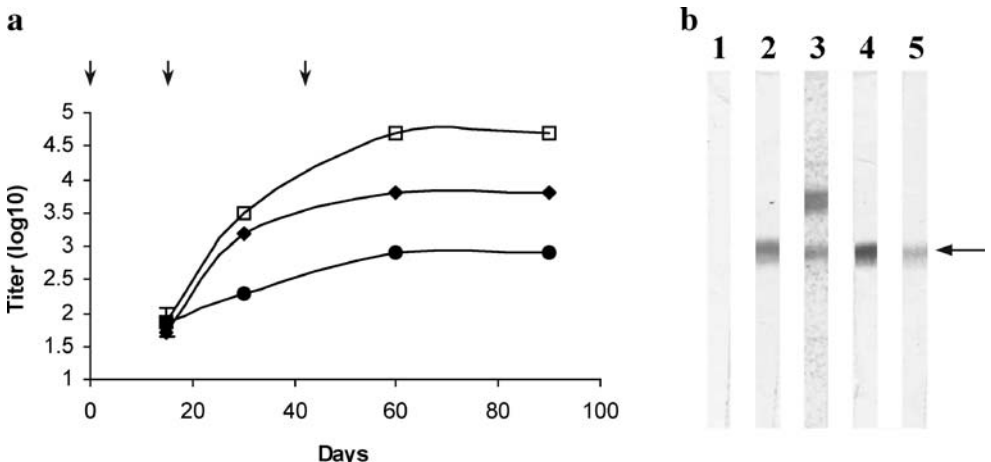

Fig. 5 Detection of anti-gD antibodies in mice intraperitoneally inoculated with recombinant baculovirus AcSupgD in ELISA and Western blot assays. **a** Shows mean titer values and standard deviations obtained from individual sera at days 15, 30, 60, and 90 post-first-inoculation in a dilution of 1:200. *circle* BHV-1 inactivated vaccine, *diamond* AcSupgD animals immunized and (*open square*) gD-Ec protein. *Arrows* indicate days of vaccination. **b** Shows Western blots of strips in which purified BHV-1 was blotted. Strips were revealed with pools of sera from the different groups taken at day 90, Acgp64 (*lane 1*), AcSupgD (*lane 2*), BHV-1 inactivated vaccine (*lane 3*), gD-Ec (*lane 4*), Mab anti-gD (*lane 5*). *Arrow* indicates the position of gD

Table 1 Vaccination results of mice

	Mice immunized with				
	AcSupgD	BHV-1 inact.	Acgp64	gD–Ec	Medium
ELISA titer[a]	3.8	2.9	–	4.1	ND
Neutralizing titer[b]	1.84	2	<1	<1	<1

Sera were sampled 21 days after the last booster, pooled and ELISA, and seroneutralizing titers were determined.
Each value represents the average of at least two determinations.
ND Not determined
[a] Endpoint titers were calculated by reference to the mean value plus two standard deviations of Acgp64.
[b] Endpoint titers are expressed as the log10 of serum dilution which caused 50% plaque number reduction.

replication and shedding (van Drunen Littel-van den Hurk et al. 1993, 1994) and monoclonal antibodies to gD neutralize the virus in vitro and inhibit virus adsorption and penetration (Hughes et al. 1988; van Drunen Littel-van den Hurk et al. 1994; Dubuisson et al. 1992). Lack of glycosylation and improper conformational folding has been associated to low neutralizing antibody responses of the recombinant gD protein (van Drunen Littel-van den Hurk et al. 1993).

We constructed recombinant baculovirus using the baculo-display approach because there are similar post-translational modifications and protein processing between insects and mammals. As previously suggested, virus-like particles based on auto-assembling proteins usually fails to incorporate large or complex sequences (Tami et al. 2000). The baculovirus display combines the advantages of multimeric presentation with the ability to display full-length sequences with glycosylation patterns and conformational constrains. One of the main problems for the production of proteins in heterologous systems is to obtain a product with the proper conformation, a factor that can drastically affect its biological functionality. It has been previously shown that soluble gD or UV-inactivated herpes simplex virus-1 (HSV-1) bind a limited number of cell membrane sites and block HSV-1 infection (Johnson and Ligas 1988; Johnson et al. 1990). In agreement with this, in the present work, AcSupgD was able to block the penetration of BHV-1 virions in MDBK cells as suggested by the observed reduction in the plaque number (70% of inhibition). Although statistically lower, Acgp64 showed 40% of inhibition, related to the ability of baculovirus to bind mammalian cells rather than a nonspecific interaction (Boyce and Bucher 1996).

On the other hand, to test the antigenic properties of the baculovirus-displayed gD, we performed immunological experiments in an animal model. The humoral immune response measured by ELISA tests was tenfold higher than that registered for mice vaccinated with the inactivated BHV-1, indicating that the gD displayed on virions was very immunogenic in mice and retained B cell epitopes that were also present in the viral particle.

Although the antibody titers of sera from mice vaccinated with the soluble protein expressed in bacteria were higher than those elicited in mice vaccinated with AcSupgD, only this group showed a marked ability to neutralize BHV-1 in vitro. This property, together with the ability of AcSupgD to inhibit BHV-1 penetration to MDBK cells, strongly suggests that gD retained structural properties of the authentic gD.

The expression of antigens involved in protective immune responses through the baculovirus display system offers many advantages over the approach of recombinant subunit vaccines based on soluble proteins: first of all, the ability of insect cells to process and modify heterologous proteins as the mammalian systems; secondly, the particulated nature and the presence of numerous identical copies of the antigen attached to the carrier; finally, the ease to purify a complex macromolecule like a baculovirus. However, in addition to the above-mentioned advantages, baculoviruses used as carriers can display large antigens as compared to short epitopes inserted in virus-like and core-like particles. Previous results indicate that gp64 enveloped glycoprotein mediates the internalization of viral DNA via membrane fusion and endosomal maturation, releasing the viral genome into toll-like receptor 9-expressing cellular compartment, inducing a strong innate immune response (Abe et al. 2005). Furthermore, the frequency of CpG motifs in the AcNPV genome is similar to that of bacterial DNA and significantly higher than that of mammalian DNA (Abe et al. 2003, 2005). Considering that baculovirus are incapable of replicating in mammalian hosts, mammals do not have a preexisting immune response to this kind of vectors. Unlike mammalian live vectors, baculovirus displaying foreign antigens are subunit vaccines and could elicit immunity against the foreign antigen even in presence of a preexisting immune response against the vector. The absence of replication also provides high levels of safety. Besides, baculovirus can easily be propagated to a large scale at very high titers by using lepidopteran larvae, making the costs of production almost negligible, an important consideration for vaccine development in third-world countries and, specifically, in veterinary vaccines.

Further investigation will improve the efficacy of this presentation system, combining the property of displaying antigens on their surface with the ability of baculovirus to transduce genes involved in the immunomodulation of the immune system into mammalian cells.

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A. P. and P. M. contributed equally to this work.

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