Immunogenicity of poliovirus B and T cell epitopes presented by hybrid porcine parvovirus particles

Christine Sedlik, Javier Sarraseca, Paloma Rueda, Claude Leclerc and Ignacio Casal*

1 Biologie des Régulations Immunitaires, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France and 2 INGENASA, Hermanos García Noblejas 41, 28037 Madrid, Spain

We have analysed the potential capacity of hybrid porcine parvovirus (PPV) capsids to present foreign epitopes to the immune system. Foreign sequences were introduced into the N and C termini of PPV VP2, which was previously shown to assemble spontaneously into parvovirus-like particles. The integrity of the C terminus was shown to be essential for preserving the structure of the capsid and therefore could not be used for epitope fusion. In contrast, insertion of sequences corresponding to T and B cell poliovirus epitopes in the N terminus did not alter the formation of particles. Moreover, chimeric capsids containing the C3:T epitope were able to induce a T cell response in vivo. However, hybrid particles containing the C3:B epitope fused to the N terminus did not induce any peptide-specific antibody response, suggesting that the inserted B cell epitope was not exposed at the surface of the particles. These results show that the N terminus in PPV empty capsids is not an adequate site for insertion of B cell epitopes, but may be useful for T cell epitope presentation and suggest that the N terminus is located in an internal position.

Virus or yeast proteins which spontaneously assemble into polymeric particulate structures are increasingly used to present foreign antigenic sequences to the immune system. Examples of molecules that have been used for this approach include, among others, hepatitis B virus surface antigen (HBsAg; Valenzuela et al., 1985; Delpeyroux et al., 1986) and core (Clarke et al., 1987; Stahl & Murray, 1989), yeast Ty protein (Adams et al., 1987), rotavirus VP6 protein (Ready & Sabara, 1987) and parvovirus B19 capsids (Brown et al., 1994; Miyamura et al., 1994). A potential use for this approach might be envisaged in the development of peptide and subunit vaccines.

We and others have recently described the synthesis of 'parvovirus-like' particles (VLPs) based on the expression of the major structural protein VP2 of porcine parvovirus (PPV) and canine parvovirus (CPV) in large amounts in insect cells using the baculovirus expression system (Martinez et al., 1992; López de Turiso et al., 1992; Saliki et al., 1992). Parvovirus VLPs, like natural virions, are very stable in extreme environmental conditions and are highly immunogenic. Doses as low as 1-5 µg are able to confer protection to dogs and pigs, and are also immunogenic for other animal species (López de Turiso et al., 1992; Martinez et al., 1992). In this report, we investigated the potential of PPV VLPs to be used as a delivery system for foreign T and B cell epitopes. Besides the applicability to vaccine production, this model of capsid assembly offers the possibility of studying those domains that are essential for the correct folding and protein–protein interactions necessary for the correct capsid formation.

Parvoviruses are small, non-enveloped icosahedral viruses which cause important diseases in man and animals. PPV belongs to the rat parvovirus subgroup of the autonomous parvoviruses genus in the family Parvoviridae (Ranz et al., 1989). The virus capsid contains 60 molecules of VP2. To find those VP2 domains that can be manipulated without altering the formation of capsids is a prerequisite for the use of PPV VLPs as a delivery system. Another requirement is the correct presentation of these epitopes on the surface of the capsids. To identify these regions, we have based our study on two major aspects, the three-dimensional structure of the related CPV virions and antigenicity studies. As a consequence, two positions were chosen for these studies, the N and C termini of the VP2 molecule. The CPV VP2 N terminus contains important neutralizing epitopes (López de Turiso et al., 1991; Langeveld et al., 1993). Unfortunately, although the three-dimensional structure of the CPV virions and empty capsids has been described (Tsao et al., 1991; Agbandje et al., 1993), no spatial position has been assigned to the N terminus of VP2, owing to its disordered nature. There are some indications that in CPV full particles, VP2...
proteins are cleaved into a VP3-like peptide, although a similar treatment of empty capsids does not produce cleavage of VP2 (Clinton & Hayashi, 1976; Tattersall et al., 1977). Therefore, in full capsids there must be partial exposure of the N terminus, which protrudes out of the virus surface through the fivefold axis channel. However, in empty capsids this N terminus may remain inside the capsid (Cortés et al., 1993). It is also remarkable that in parvoviruses the C terminus is not on the virus surface, as is the case for many other icosahedral viruses (Tsao et al., 1991).

For immunogenicity studies, two poliovirus epitopes, C3:B and C3:T, were used as a model. C3:B is one of the four neutralization antigenic sites of the poliovirus and is located in positions 93–103 of the VP1 capsid protein (Van der Werf et al., 1983). It has been inserted into different vectors, including HBsAg (Delpeyroux et al., 1986, 1988), diphtheria toxin protein (Phalipon et al., 1989) and membrane and periplasmic Escherichia coli proteins (Charbit et al., 1988; Leclerc et al., 1990). In all these insertions, the epitope was able to elicit neutralizing antibodies, owing to the good accessibility of the epitope at the surface of the hybrid proteins. The C3:T epitope is recognized by poliovirus-specific CD4+ T cells and is located in a position immediately adjacent to the C3:B cell epitope (residues 103–115; Leclerc et al., 1991).

To test the capacity of the PPV VP2 N terminus to be used for epitope insertion, an XhoI site was first introduced into the N terminus by site-directed mutagenesis using PCR. The cloning strategy and DNA manipulations are summarized in Fig. 1. The strategy for N terminus mutagenesis was based on the digestion and replacement of the XhoI–NcoI fragment of pPPV17R by two PCR products, in order to remove a region present in the multiple cloning site of pPPV17R and the VP2 initiation codon. All PCR amplifications were carried out in a total volume of 100 μl with 1 unit of Vent DNA polymerase (New England Biolabs), 10 ng of pPPV17R as a template, 200 μM-dNTPs and 800 ng of each primer. Amplifications involved 25 cycles of denaturation at 93 °C for 1 min, primer annealing at 50 °C for 1 min and extension at 72 °C for 3 min. The PCR products were cloned into XhoI- and NcoI-digested and dephosphorylated pPPV17R. The ligation mixtures were used to transform E. coli DH5 cells. Using this strategy, two new plasmids were obtained: pPPV29, which contains a unique XhoI cloning site adjacent to the original ATG of VP2 and pPPV30, which contains a unique XhoI cloning site but without the initiation codon ATG. To carry out the insertion of C3:B and C3:T poliovirus epitopes into the modified VP2 genes, phosphorylated oligonucleotides were mixed and heated at 70 °C for 15 min before ligation into XhoI-digested, calf intestinal alkaline phosphatase (CIAP)-treated pPPV29 or pPPV30 (Fig. 1). The integrity of the epitope sequences and the correct orientation were confirmed by dideoxynucleotide chain termination sequencing. A total of four constructs were prepared, two for each epitope: pPPV29-C3:B, pPPV29-C3:T, pPPV30-C3:B and pPPV30-C3:T.

Although the three-dimensional model predicted an internal position for the C terminus, the presence of B cell epitopes in this region (Langeveld et al., 1993) suggested to us that it could be used for insertion of foreign epitopes. However, since direct insertions prevented the formation of capsids in B19 (Brown et al., 1994), we chose to delete short fragments of the protein before insertion. To analyse the maximum length of sequence that could be deleted without altering the structure of the capsid, we prepared three different constructs, with progressively longer deletions, by PCR. Three 3’ primers, 5’ GCCTCAACTAGTTGTAGGAA-TATATT 3’, 5’ CATTTAACAGTTCTCATTITTGCTGG 3’ and 5’ GTTTCAACTAGTTATATTCTTAGTTG 3’, corresponding to nucleotides 4449–4475, 4377–4403 and 4323–4349, were synthesized to create deletions of 18, 42 and 60 amino acids, respectively. These primers were used in combination with the 5’ primer 5’ TTCCATGGTGAATAATGGAACAC 3’, extending from position –4 to 23 in the VP2 gene. The SpeI sites used for screening are underlined and the initiation and stop codons introduced in the sequence are shown in bold. The VP2 deletion forms were cloned in Smal-digested CIAP-treated pMTL25 to provide flanking BamHI sites for subcloning in the baculovirus transfer vector pAcYM1 and transformed into E. coli DH5. The recombinant clones were characterized by restriction mapping with SpeI and BamHI and the absence of deleted sequences was confirmed by dideoxynucleotide chain termination sequencing. The resulting plasmids were called pPPV.VP2/ΔC4475, pPPV.VP2/ΔC4403 and pPPV.VP2/ΔC4349; the number indicates the position of the final nucleotide of the deletion.

All the recombinant clones containing the modified VP2 forms were BamHI-digested and subcloned into the baculovirus transfer vector pAcYM1 (Matsuura et al., 1987). For the N terminus, the pAcYM1-derived recombinant transfer vectors were designated pAcPPV29-C3:B, pAcPPV30-C3:B, pAcPPV29-C3:T and pAcPPV30-C3:T. The correct orientation and the sequences between the translation initiation codons and the transcription initiation sites was determined by sequence analysis. The open reading frame of the hybrid genes was situated 25 bp downstream of the polyhedrin promoter. These 25 bp proceed from the pMTL24 multiple cloning site.

Recombinant baculoviruses were obtained from co-transfections involving 500 ng of Bsu36I-linearized
Fig. 1. Construction of PPV VP2 N-terminal insertion vector and baculovirus transfer vectors. Scheme employed to mutate the N terminus of the PPV VP2 gene and to insert the foreign epitopes. The original vector pPPV17R was used to mutate the N terminus and to introduce the XhoI sites. The sequence of the 5' oligonucleotide for VP2 mutagenesis is shown together with the sequence of the 3' oligonucleotide used in the PCR mutagenesis. The 3' primer corresponded to positions 3370-3400 (on the coding strand, according to the sequence of Ranz et al., 1989) containing the internal NcoI site. The sequence of the oligonucleotides encoding neutralization epitope C3:B and the T cell epitope C3:T are shown together with the restriction sites HindIII and Nhel used for the screening of the recombinant constructs. The modified VP2 genes containing the poliovirus epitopes insertions were cloned into the baculovirus transfer vector pAcYM1.

AcRP23-lacZ virus DNA (Possee & Howard, 1987) and the individual transfer vectors (2 μg) using the lipofectin (Gibco BRL) technique as described by Felgner et al. (1987). The recombinant viruses were selected by their lacZ-negative phenotypes and plaque-purified before preparation of high-titre virus stocks for each recom-
Fig. 2. Analysis and subcellular localization of fusion proteins expressed in insect cells. (a) Coomassie blue-stained SDS-polyacrylamide gels of infected insect cells harvested at 72 h post-infection; PPV, purified PPV VP2 particles; M, molecular mass markers; CE, crude extract from infected cells; ST, supernatant from lysed cells. (b) Western blot analysis of recombinant proteins. Bound antibodies were detected as described in the text.

binant virus. For the N terminus, four recombinant baculoviruses AcPPV29.C3:B, AcPPV30.C3:B, AcPPV29.C3:T and AcPPV30.C3:T were generated. Spodoptera frugiperda Sf9 cells were infected at a m.o.i. of 1 p.f.u./cell with a recombinant baculovirus expressing one of the hybrid VP2 PPV proteins or were mock-infected. Cells were collected at 72 h post-infection (p.i.), washed with PBS, lysed with 25 mM-NaHCO₃ pH 9.5 at a density of 5 x 10⁶ cells per ml and mixed with equal volumes of SDS-sample buffer. Samples were boiled for 5 min before loading onto 9% SDS–polyacrylamide gels. For immunoblotting analyses, proteins were transferred to a nitrocellulose membrane using a semi-dry system (Bio Rad). The blots were probed with an anti-PPV polyclonal serum (a gift from Dr K. Dalsgaard, State Veterinary Institute for Virus Research, Lindholm, Denmark) to confirm the presence of the VP2 gene product, with an anti-C3:B monoclonal antibody (kindly given by Dr R. Crainic, Institute Pasteur, Paris, France) and with polyclonal anti-C3:T mouse serum to check the expression of these epitopes. Bound antibodies were detected using an alkaline phosphatase-conjugated IgG (Sigma) and nitroblue tetrazolium (Gibco BRL) as substrate. The highest level of expression was observed for the recombinant AcPPV30.C3:B. Lower expression was detected in the lanes corresponding to AcPPV29.C3:T and AcPPV30.C3:T. No detectable expression was observed for the recombinant AcPPV29.C3:B. Therefore, we selected the recombinant baculoviruses AcPPV30.C3:B and AcPPV29.C3:T for further production of chimeric particles (Fig. 2 a, b). The size of the chimeric proteins was slightly higher than the size of the wild-type VP2 due to the peptide insertions of 11 (C3:B) and 13 (C3:T) amino acids. The chimeric proteins gave strong positive reactions with the antibodies specific for VP2 PPV and for the two inserted epitopes. VP2–C3:T preparations were more unstable than the original VP2 PPV, indicated by the presence of several extra bands in the immunoblot. The level of expression of AcPPV30.C3:B was similar to that obtained with wild-type VP2, around 10 µg/10⁶ cells, whereas the expression of the chimeric VP2 containing C3:T was 5–10 times lower.

For purification of the VP2 particles and immunization studies, cytoplasmic extracts from bicarbonate-lysed cells were precipitated with 25% ammonium sulphate. The resultant pellet, containing most of the VP2 protein, was resuspended in PBS and filtered through a Sephadex G50-80 spin column to desalt it. To analyse their particulate structure, these preparations were subjected to isopicnic ultracentrifugation in CsCl gradients for 24 h at 48000 r.p.m. in a SW55.5 rotor (Kontron). Both types of fusion proteins formed an opalescent band at a density of 1.33 g/cm³, which indicates the presence of VLPs. These particles could be stored at 4 °C for at least 6 months without alterations in their properties (data not shown). A large number of particles was observed by electron microscopy analysis of both types of fusion proteins expressing C3:B and C3:T epitopes (Fig. 3). However, the morphology of the two types of particles was quite different. PPV C3:B particles (Fig. 3 a) showed a morphology very similar to the original capsids and virions. In contrast, PPV C3:T (Fig. 3 b) particles had a less defined shape and were denser and less hexagonal than the particles expressing C3:B. Since both preparations were subjected to the same treatment, it is possible that the specific sequence of the T cell epitope may interfere with the correct assembly of the capsids. The chimeric particles retained their ability to haemagglutinate guinea pig red blood cells. Nevertheless, the haemagglutination (HA) titres per µg of C3:T particles were 10–50 times lower than those obtained with the C3:B particles or with the normal VP2 particles. These data confirmed that chimeric VP2 proteins containing poliovirus epitopes formed particles. The formation of particles containing C3:B was, however, more efficient than particles containing C3:T.

In the case of the C-terminal deletion mutants, the same procedures were followed to obtain three different recombinant baculoviruses, AcVP2/ΔC4475, AcVP2/ΔC4403 and AcVP2/ΔC4349. The recombinant C-terminal-truncated forms of VP2 were analysed following similar procedures. In each case, a different protein band corresponding to the truncated form was observed by Coomassie blue staining (data not shown). The levels of expression were high and the stability of the proteins adequate. However, none of these proteins assembled into particles. Even the smaller deletion (18 amino acids) did not allow particle formation, although
some aggregation was observed. The unusual internal position of the C terminus in parvovirus capsids could be required for preserving the structure of the capsid. Therefore, in contrast to the N terminus, the C-terminal region was shown to be essential for the correct formation of empty capsids and therefore could not be used for insertion of foreign epitopes. Thus, we used the N-terminal insertion particles for further experiments.

The capacity of recombinant PPV capsids expressing poliovirus C3:B and T cell epitopes to induce T cell responses and/or antibody production against the inserted peptides was then analysed in BALB/c mice (H-2\textsuperscript{a}), which have been previously shown to develop good immune responses against these epitopes (Leclerc \textit{et al.}, 1991). In a first set of experiments, BALB/c mice were subcutaneously injected either with 10 \( \mu \)g of PPV-C3:T or PPV-C3:B (as a negative control) in complete Freund's adjuvant (CFA); a control group received 10 \( \mu \)g of the synthetic C3:T peptide in CFA. Two weeks later, lymph node cells were removed and a single cell suspension was prepared in RPMI 1640 (Seromed)
supplemented with 0.5% normal mouse serum, 1.5% FCS, 2 mM-glutamine, 50 μM-2-mercaptoethanol and antibiotics. The cells (8 x 10^5) were incubated with different doses of C3:T peptide in flat-bottomed microculture plates (Costar) at a final volume of 0.2 ml. The results of this experiment (Fig. 4 a) clearly demonstrated that subcutaneous immunization with hybrid PPV-C3:T in CFA induced a good peptide-specific proliferative response. This response was clearly specific since lymph node cells from mice injected with PPV-C3:B (Fig. 4 a) or with empty PPV (data not shown) did not respond to stimulation in vitro by the C3:T peptide.

In a second set of experiments, BALB/c mice were injected intraperitoneally (i.p.) with PPV-C3:T or PPV-C3:B in alum and their spleen cells were stimulated in vitro with the C3:T peptide (Fig. 4b). Under these conditions, PPV-C3:T with alum was able to stimulate an in vivo proliferative response of spleen cells to the peptide. In contrast, PPV-C3:B control particles or empty PPV (data not shown) did not induce such a peptide-specific proliferative response.

Altogether, these results demonstrated that the recombinant PPV capsids expressing the poliovirus T cell epitope were efficiently processed by antigen-presenting cells and therefore stimulated in vivo T cell responses against the foreign inserted T cell epitope.

We next investigated the ability of PPV recombinant capsids expressing the C3:B epitope to induce antibody responses against this inserted peptide. These experiments were performed in BALB/c mice, which were previously shown to produce anti-C3:B antibodies after immunization with the synthetic peptide C3:TB in CFA

(Leclerc et al., 1991). Mice were injected i.p. with antigen alone or with either CFA or 1 mg of alum. Mice were bled at different times after immunization and sera were individually tested for anti-C3:TB or anti-PPV antibodies by ELISA assay, as previously described (Leclerc et al., 1991). As shown in Fig. 5(a), after one single injection of C3:TB peptide in CFA, BALB/c mice developed high anti-peptide antibody titres, confirming our previous observations (Leclerc et al., 1991). In contrast, mice immunized i.p. with hybrid PPV-C3:B particles did not develop anti-C3:B antibody responses even after three injections of the recombinant particles. Even a fourth injection of these particles in CFA was not able to raise anti-peptide antibody response (Fig. 5a). However, these chimeric particles were able to induce high anti-PPV antibody responses (Fig. 5b), which excludes the possibility that the lack of anti-C3:B antibody responses was due to poor immunogenicity of the recombinant particles. Similar results were obtained in DBA/1 mice (data not shown). These findings are in partial contradiction with those obtained by Brown et al. (1994) with parvovirus B19, who claimed that the N terminus was immunogenic. This discrepancy could be due to the known differences in the immunogenicity of the VP1–VP2 junction regions of B19 (Saikawa et al., 1993) and the three-dimensional structure of B19 and the rat parvovirus group VP2 particles (Agbandje et al., 1994). Although the C3:B epitope is expressed into PPV particles, its failure to induce antibody responses could be due to a lack of exposure at the surface of the recombinant particles. This hypothesis could be supported by some of our previous observations using
immunogold labelling of the N terminus of CPV procapsids (Cortés et al., 1993) and because dogs immunized with CPV VP2 particles did not develop antibodies against the N-terminal region (J. Langeveld, personal communication). These results, therefore, strongly suggest that the VP2 N terminus is not exposed on the surface of PPV or CPV empty capsids.

In conclusion, the results described here show that foreign epitopes can be inserted in the N terminus of PPV particles without disrupting the formation of capsids. In this region, the epitopes were correctly expressed and the chimeric proteins were able to form particulate structures, as shown by electron microscopy and HA assays. However, the present study has clearly established that neither the N nor the C termini of PPV VP2 particles can be used to insert foreign B cell epitopes. Other alternatives will be investigated in the future, such as insertion into the four external loops of the VP2 protein. In contrast to B cell responses, the induction of CD4+ T cell responses does not require the expression of the epitope at the surface of the particles. This discrepancy explains why N-terminal T cell epitope insertion, presumably internal, retained the immunodominance of the T cell response. Such strategy would potentially be of great interest in the control of some diseases where the antibody response is deleterious for the host, as in a number of human and animal viruses (Porterfield, 1986).

This work was carried out as a collaborative project between Immunologia y Genética Aplicada S.A. (INGENASA) and Institut Pasteur in a BIOTECH project (EEC Biotechnology B102-CT92-0290) funded by the EU. We thank Kristian Dalsgaard and A. Bettn (SVIVR, Lindholm, Denmark) for technical assistance with the electron microscope, E. Cortés for help in the production of the recombinant particles and Teresa de Magistris for a critical review of the manuscript.

References


(Received 27 January 1995; Accepted 20 April 1995)