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Lyssavirus glycoproteins expressing immunologically potent foreign B cell and cytotoxic T lymphocyte epitopes as prototypes for multivalent vaccines

Emmanuel Desmézières,[1] Yves Jacob,[1] Marie-Françoise Saron,[2] Francis Delpeyroux,[3] Noël Tordo[1] and Pierre Perrin[1]

Laboratoire des Lyssavirus[1], Laboratoire de Virologie Expérimentale[2] and Laboratoire d'Epidémiologie Moléculaire des Entérovirus[3], Institut Pasteur 25, rue du Dr Roux, 75724 Paris Cedex 15, France

Truncated and chimeric lyssavirus glycoprotein (G) genes were used to carry and express non-lyssavirus B and T cell epitopes for DNA-based immunization of mice, with the aim of developing a multivalent vaccine prototype. Truncated G (GPVIII) was composed of the C-terminal half (aa 253–503) of the Pasteur rabies virus (PV: genotype 1) G containing antigenic site III and the transmembrane and cytoplasmic domains. The chimeric G (GEBL1-PV) was composed of the N-terminal half (aa 1–250) of the European bat lyssavirus 1 (genotype 5) G containing antigenic site II linked to GPVIII. Antigenic sites II and III are involved in the induction of virus-neutralizing antibodies. The B cell epitope was the C3 neutralization epitope of the poliovirus type 1 capsid VP1 protein. The T cell epitope was the $H2^d$ MHC I-restricted epitope of the nucleoprotein of lymphocytic choriomeningitis virus (LCMV) involved in the induction of both cytotoxic T cell (CTL) production and protection against LCMV. Truncated G carrying foreign epitopes induced weak antibody production against rabies and polio viruses and provided weak protection against LCMV. In contrast, the chimeric plasmid containing various combinations of B and CTL epitopes elicited simultaneous immunological responses against both parental lyssaviruses and poliovirus and provided good protection against LCMV. The level of humoral and cellular immune responses depended on the order of the foreign epitopes inserted. Our results demonstrate that chimeric lyssavirus glycoproteins can be used not only to broaden the spectrum of protection against lyssaviruses, but also to express foreign B and CTL epitopes. The potential usefulness of chimeric lyssavirus glycoproteins for the development of multivalent vaccines against animal diseases and zoonoses, including rabies, is discussed.

Introduction

Vaccines that generate an immune reaction in response to a number of serotypes and to various pathogens would be of great value (European Commission, 1996). Protection against virus diseases is mediated mainly by neutralizing antibodies and/or T lymphocytes [$CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T lymphocytes (CTL)]. Vaccines displaying virus replication generate CTL, whereas inactivated vaccines mainly generate antibodies and Th cells but are not thought to generate CTL. In contrast, administration of naked DNA induces a complete immune response (for review see Donnelly *et al.*, 1997) without the potential risks of pathogenicity associated with live-virus vaccines. These properties, in addition to the versatility of DNA technology, suggest that immunization with plasmids has great potential for the development of multivalent vaccines (Spier, 1997). However, the use of a mixture of plasmids or a single plasmid expressing several antigens may cause interference problems at both the transcriptional and immunological levels (Thomson *et al.*, 1998). A plasmid containing several DNA coding fragments for various epitopes would therefore be a useful alternative.

Rabies viruses and classical vaccine strains are classed as genotype (GT) 1, whereas rabies-related viruses belong to GT 2–7, the most common members being Lagos bat virus (GT 2), Mokola virus (Mok) (GT 3), Duvenhage virus (GT 4), European bat lyssavirus (EBL) 1 (GT 5), EBL2 (GT 6) and Australian bat

Author for correspondence: Pierre Perrin.
Fax +33 1 40 61 32 56. e-mail pperrin@pasteur.fr

lyssavirus (GT 7) (Bourhy *et al.*, 1993; Gould *et al.*, 1998). All of these viruses cause a fatal form of encephalomyelitis and the rabies vaccines currently available only provide protection against GT 1, 6 and 7. These vaccines are not effective against GT 2, 3 or 4, and the extent of protection against GT 5 depends mainly on the vaccine strain (Fekadu *et al.*, 1988; Jallet *et al.*, 1999). Lyssavirus glycoprotein (G) is involved in virus-neutralizing antibody (VNAb) induction and protection. Structurally and immunologically, the G molecule consists of two parts separated by a flexible hinge in the linear region (aa 253–275) (Bahloul *et al.*, 1998; Benmansour *et al.*, 1991; Dietzschold *et al.*, 1990; Jallet *et al.*, 1999): (i) the N-terminal half (the 'site II' region) (aa 1–250) contains antigenic site II; and (ii) the C-terminal half (the 'site III' region) contains antigenic site III and the transmembrane and cytoplasmic domains. This has allowed the construction of several chimeric G genes with various genotypes of lyssavirus and the demonstration that DNA-based immunization with chimeric GEBL1–PV and GMok–PV plasmids [site II region of EBL1 or Mok linked to the site III region of Pasteur rabies virus (PV) (GT 1)] induces protection against challenge with most lyssavirus genotypes (Bahloul *et al.*, 1998; Jallet *et al.*, 1999).

In the present paper, we investigate the effectiveness of lyssavirus G further as a carrier for foreign antigens in multivalent vaccine prototypes. DNA coding fragments for epitopes mimicking minimal antigens that induce both humoral and cellular immune responses were fused to those encoding the truncated PV G (site III region: GPVIII) or inserted into the hinge region of the full-length chimeric EBL1–PV G. The inserted sequences corresponded to various combinations of two well-characterized epitopes: the C3 B cell epitope of the poliovirus type 1 capsid VP1 protein and the CTL epitope of the lymphocytic choriomeningitis virus (LCMV) nucleoprotein. The C3 poliovirus epitope induces the synthesis of VNAb (Delpeyroux *et al.*, 1990) and the LCMV epitope is involved in both the induction of CTL and protection against LCMV challenge in $H2^d$ mice (Aichele *et al.*, 1990). Combination of these foreign epitopes with the truncated G induced weak immunological responses in mice, whereas their insertion into the chimeric G induced humoral and cellular immune responses against both the parental lyssaviruses and poliovirus and gave partial protection against LCMV. These results have implications for the development of multivalent vaccines by using fragments of genes encoding antigens involved in protection against various animal diseases, including zoonoses and lyssavirus encephalomyelitis.

Methods

■ **Mice, cells, viruses and antigens.** Seven-week-old female $H\text{-}2^d$ BALB/c mice were purchased from the 'Centre d'Elevage et de Recherche' Janvier (Legenest St Isle, France).

BHK-21 cells were grown in Eagle's minimal essential medium (MEM) containing 5% foetal bovine serum (FBS) and 5% newborn calf serum (Perrin, 1996). Neuroblastoma cells (Neuro-2a) were grown in MEM containing 8% FBS. The interleukin-2 (IL-2)-dependent CTL line (CTLL) was cultured as described previously (Perrin *et al.*, 1988).

Fixed rabies PV-Paris/BHK-21 (Pasteur virus), EBL1b and LCMV strains were maintained by serial passage in BHK-21 cells as described previously (Perrin, 1996; Perrin *et al.*, 1996; Saron *et al.*, 1997). EBL1b (strain 8916FRA) was derived from a French bat isolate (Amengual *et al.*, 1997). The LCMV strain Arm/53b was kindly provided by M. Oldstone and M. McChesney (Scripps Clinic, La Jolla, CA, USA).

β-Propiolactone-inactivated, purified lyssaviruses (IPLV) were prepared as described elsewhere (Perrin, 1996).

■ **Insertion of DNA encoding foreign B and T cell epitopes into truncated or chimeric G genes.** Previously described truncated (GPVIII) and chimeric (GEBL1-PV) lyssavirus G genes (Jallet *et al.*, 1999) were introduced into the eukaryotic expression vector pCIneo (Promega), which was then propagated and amplified in *Escherichia coli* strain DH5*α* by standard protocols (Maniatis *et al.*, 1982).

DNA fragments encoding foreign B and/or $CD8^+$ T cell epitopes were inserted into the *Eco*RI restriction site (located at position 253) of the truncated or chimeric lyssavirus G gene in the area encoding the hinge region (aa 253–275). The B cell epitope (B) corresponded to fragment C3 (aa 93–103: DNPASTTNKDK) of the poliovirus VP1 protein. The T cell epitope (t) corresponded to aa 119–127 (PQASGVYMG) of the LCMV nucleoprotein. A larger form of this epitope (T), corresponding to aa 117–132 (ER–PQASGVYMG–NLTAQ), was also inserted. The sequence encoding the LCMV t epitope was used in the p(B-t)$_2$-GPVIII and pGEBL1-(B-t)$_2$-PV chimeras, whereas the sequence encoding the larger, T, epitope was used in pGEBL1-(T)-PV, pGEBL1-(T-B)-PV and pGEBL1-(B-T)-PV. The different plasmids used (see Fig. 1) were obtained as follows.

The plasmids p(B-t)$_2$-GPVIII and pGEBL1-(B-t)$_2$-PV were generated by a two-step insertion of a synthetic adaptor into the unique *Eco*RI restriction sites of pGPVIII and pGEBL1-PV, respectively. This adaptor was produced by using 200 pmol of each of the following primers: B-tp1 (5′ AATTCAGATAACCCGGCGTCGACCACTAACAAGGATAAG-CTGTTCGCAGTGCCTCAGGCCTCTGGTGTGTATATGGGT 3′) and B-tp2 (5′ AATTACCCATATACACACCAGAGGCCTGAGGC-ACTGCGAACAGCTTATCCTTGTTAGTGGTCGACGCCGGGT-TATCTG 3′).

For pGEBL1-(B)-PV and pGEBL1-(T)-PV, the synthetic adaptors used for the insertion were, respectively, for B: Bp3 (5′ AATTTGGAT-AACCCGGCGTCGACCACTAACAA 3′) and Bp4 (5′ AATTCTTA-TCCTTGTTAGTGGTCGACGCCGGG 3′), and for T: Tp5 (5′ AATT-TGGAGAGACCTCAGGCCTCTGGTGTGTATATGGGTAATCTT-ACGGCCCAG 3′) and CTLp6 (5′ AATTCTGGGCCGTAAGATT-ACCCATATACACACCAGAGGCCTGAGGTCTCTCCA 3′).

Plasmids pGEBL1-(B-T)-PV and pGEBL1-(T-B)-PV were constructed by inserting DNA fragments encoding the T and B epitopes into pGEBL1-(B)-PV and pGEBL1-(T)-PV, respectively, under the same conditions.

The identity of each construct was confirmed by automatic sequencing using a dye termination reaction on an ABI 377 sequencer (Perkin-Elmer).

■ **Transient expression experiments.** The transient expression of G and related foreign antigens from plasmids was tested by transfecting Neuro-2a cells by using FuGENE 6 transfection reagent, according to the manufacturer's instructions (Boehringer Mannheim). Each well of a Nunc Labtek cell culture chamber (Life Technologies) was inoculated with 3×10^4 cells (in MEM, 10% FBS) and incubated for 24 h at 37 °C in a humidified atmosphere containing 7·5% CO_2. The plate was washed with MEM without FBS and the wells were filled with 100 µl transfection solution (0·5 µg plasmid, 1·5 µl FuGENE 6 transfection reagent and

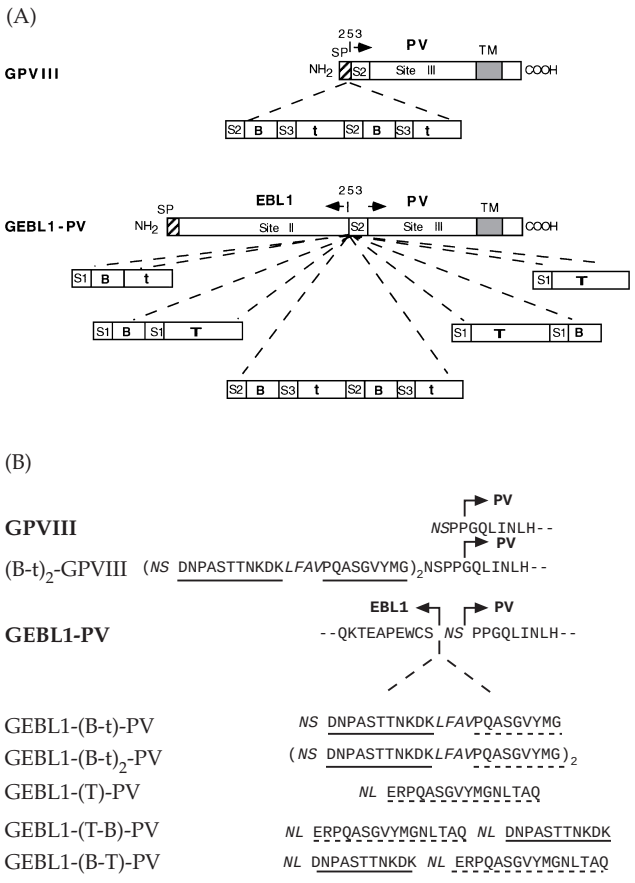

Fig. 1. Schematic representation of lyssavirus G proteins carrying foreign B and T cell epitopes (monomers and polymers): the C3 poliovirus epitope inducing VNAb-producing B cells (B) and the LCMV nucleoprotein epitope (T or t) inducing $CD8^+$ cells in H-2^d mice. Foreign epitopes were fused with truncated G (GPVIII) or inserted into the chimeric lyssavirus G (GEBL1-PV). (A) Diagram of epitope fusions or insertions. SP, Signal peptide; TM, transmembrane domain; S1, S2 and S3, peptides NL, NS and LFAV used as spacers. (B) Diagram of the fused or inserted amino acid sequences. The spacers are in italics and the B and CTL epitopes are underlined with full or dotted lines, respectively.

98·5 µl serum-free medium). The plate was incubated for 18 h at 37 °C in the presence of 7·5% CO_2. MEM containing 5% FBS (200 µl) was then added to each well and the plate was incubated for 24 h at 37 °C in the presence of 7·5% CO_2. Transient expression was then assessed by indirect immunofluorescence.

■ **Immunofluorescence microscopy.** Two mouse MAbs directed against rabies PV G (D1 MAb) or poliovirus type 1 capsid VP1 protein (C3 MAb) were used for antigen staining. The D1 MAb recognizes site III of native but not SDS-treated G and the C3 MAb recognizes the 93–103 region of the poliovirus type 1 capsid VP1 protein. A neutralizing polyclonal antibody (PAb) from rabbit directed against native poliovirus type 1 was also used.

Transient expression was assessed after fixing of cells in 3% paraformaldehyde (Sigma) (20 min incubation at room temperature) without permeabilization. Fixed cells were incubated for 1 h at room temperature with MAb or PAb. They were then washed with PBS and incubated for 1 h at room temperature with goat anti-mouse or anti-rabbit FITC-conjugated secondary antibody (Nordic Immunology Labs). Cells were washed with PBS, mounted in Mowiol (Sigma) and examined with a Leica inverted fluorescence microscope.

■ **Injection of plasmids into mice.** BALB/c mice were anaesthetized with pentobarbital (30 mg/kg). Fifty µg plasmid (diluted in PBS) was injected into each anterior tibialis muscle. For studies of protection against LCMV, mice received 3·5 µg cardiotoxin (Latoxan A. P.) in each leg 4 days before immunization to degenerate the muscle.

■ **IL-2 release assay.** To test the activity of Th cells induced by plasmids, mouse splenocytes were stimulated *in vitro* in order to activate IL-2-producing cells. Spleens were removed from naive or plasmid-injected mice 14 days after injection. Splenocytes (1 ml aliquots, each containing 6×10^6 cells) were stimulated with 0·5 µg lyssavirus antigen (IPLV-PV or IPLV-EBL1) or 5 µg concanavalin A (ConA; Miles) (Joffret *et al.*, 1991). The IL-2 produced in the supernatant of splenocyte cultures was titrated by bioassay in CTLL, as described previously (Perrin *et al.*, 1988). CTLL cell proliferation was determined in triplicate, based on the uptake of [^{3}H]thymidine (New England Nuclear). Under these conditions, the cells stimulated were mostly $CD4^+$ Th cells, which produced IL-2 (Joffret *et al.*, 1991).

■ **Antibody assays.** For serum antibody assay, blood was collected on various days by retroorbital puncture under anaesthesia. Anti-rabies virus IgG was titrated by ELISA with microplates coated with purified rabies virus G (Perrin *et al.*, 1985, 1986) and rabbit anti-mouse IgG isotype conjugated with peroxidase as the secondary antibody (Nordic Immunology Labs). The titre corresponded to the reciprocal dilution of the serum sample giving an absorbance twice that of 1:20-diluted serum from PBS-injected mice. Lyssavirus-neutralizing antibodies were titrated by the rapid fluorescent focus inhibition test (RFFIT) (Smith *et al.*, 1996), with modifications described previously (Perrin *et al.*, 1986), using infected (PV or EBL1) cells. Anti-PV neutralizing antibody titres are expressed in IU/ml using the 2nd International Standard (Statens Seruminstitut, Copenhagen, Denmark) as the reference. For the determination of anti-EBL1 neutralizing antibody titres, we assumed that the serum dilution causing 50% inhibition of the fluorescent focus rate had the same VNAb titre as the reference assayed against PV.

Production of antibodies against poliovirus type 1 was assayed by ELISA as described previously (Delpeyroux *et al.*, 1990) using microplates coated with a synthetic peptide. This peptide consisted of a trimer of aa 93–103 of VP1 and rabbit anti-mouse IgG isotype conjugated with peroxidase as the secondary antibody (Nordic Immunology Laboratories). The titre corresponded to the reciprocal dilution of the serum sample giving an absorbance twice that of 1:20-diluted serum from PBS-injected mice.

■ **Protection test.** Two groups of 10 mice with degenerated muscle were injected with either plasmid or PBS. They were then challenged intracerebrally (i.c.) on day 21 with $10^{1·5}$ p.f.u. LCMV diluted in 30 µl. Mice were killed 21 days after the challenge to test virus clearance in kidneys by RT–PCR (Martins *et al.*, 1995).

Results

Transient expression of lyssavirus G and a foreign B epitope

Plasmids containing foreign epitopes encoding sequences fused to the truncated (pGPVIII) or chimeric (pGEBL1-PV) lyssavirus G genes were tested for their ability to transiently transfect Neuro-2a cells. The truncated plasmids exhibited indirect immunofluorescence staining (in the cytoplasm) only

Table 1. Detection of lyssavirus G and poliovirus epitopes in Neuro-2a cells by indirect immunofluorescence

Cells were transiently transfected with chimeric pGEBL1-PV plasmids carrying various combinations of foreign B and CTL epitopes. 0, No fluorescence; +, ++, +++, increasing levels of immunofluorescence.

Plasmid	Indirect immunofluorescence with: Anti-PV MAb (D1)	Anti-PV PAb	Anti-poliovirus MAb (C3)
pCIneo	0	0	0
pGEBL1-PV	++	++	0
pGEBL1-(B-t)-PV	+	+	+
pGEBL1-(B-t)$_2$-PV	++	++	++
pGEBL1-(T)-PV	+++	+++	0
pGEBL1-(T-B)-PV	++	++	++
pGEBL1-(B-T)-PV	0	0	0 (++*)

* After permeabilization.

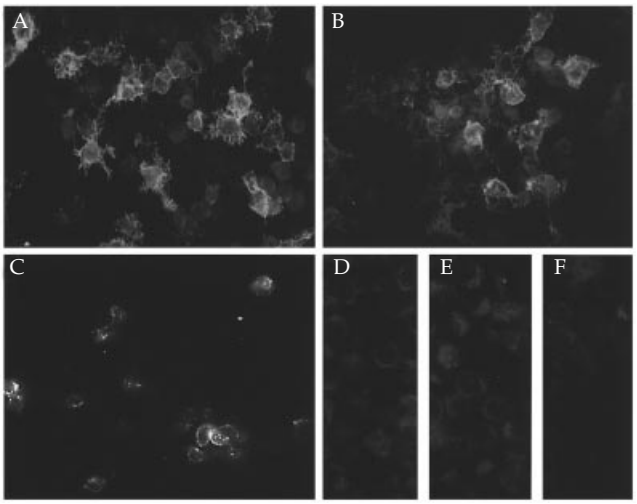

Fig. 2. Indirect immunofluorescence microscopy of antigens expressed in Neuro-2a cells transfected with pGEBL1-(B-t)$_2$-PV (A–C) or pCIneo (D–F). Cells were stained with anti-rabies virus MAb D1 (A, D), anti-poliovirus MAb C3 (B, E) or anti-poliovirus type 1 PAb (C, F).

after permeabilization, as reported previously for pGPVIII (Jallet *et al.*, 1999). With the exception of pGEBL1-(B-T)-PV, transfection with the chimeric plasmids resulted in the expression of poliovirus- and lyssavirus-related antigens at the cell membrane of non-permeabilized cells, similar to that seen in response to transfection with native pGEBL1-PV (Table 1). Fig. 2 illustrates the results obtained after transfection with the chimeric pGEBL1-(B-t)$_2$-PV. The rabies virus G site III region was recognized by PV D1 MAb (Fig. 2A) and the poliovirus insert was recognized by the C3 MAb (Fig. 2B) and by the anti-poliovirus type 1 PAb (Fig. 2C). No staining was observed with the same antibodies in cells transfected with

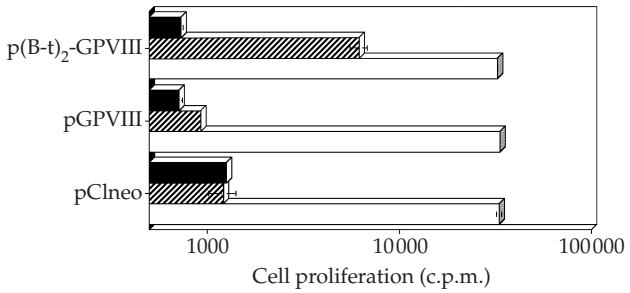

Fig. 3. Induction of IL-2-producing cells by pGPVIII encoding poliovirus and LCMV epitopes. BALB/c mice (two animals for each plasmid) were injected with plasmids intramuscularly (50 μg into each anterior tibialis muscle). Spleens were removed 14 days later and splenocytes were stimulated *in vitro* by cell culture medium (solid bars), specifically by IPLV-PV (hatched bars) or polyclonally by ConA (open bars). The amount of IL-2 released was assayed in triplicate by using CTLL and cell proliferation is expressed as c.p.m.

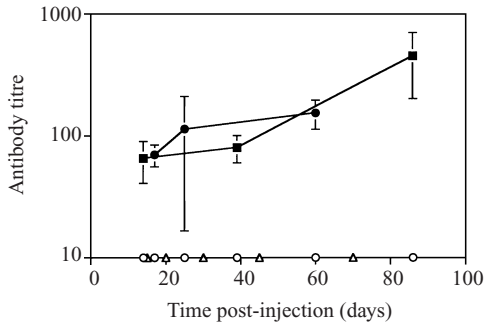

Fig. 4. Kinetic study of antibody production against poliovirus peptide and rabies virus induced by p(B-t)$_2$-GPVIII or pGPVIII in BALB/c mice. Mice were injected with 40 μg p(B-t)$_2$-GPVIII (■, ●), pGPVIII (○) or pCIneo (△). After puncture by the retroorbital route on various occasions, sera were assayed by ELISA to determine the antibody production against poliovirus peptide (■) or rabies virus (●, △, ○). Titres are reciprocal dilutions, as described in Methods.

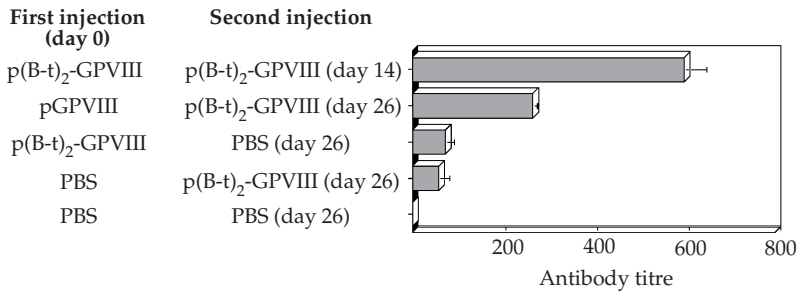

Fig. 5. Effect of priming on the poliovirus C3 peptide antibody production induced by p(B-t)$_2$-GPVIII. Five groups of three mice each were given PBS (two groups), p(B-t)$_2$-GPVIII (two groups) or pGPVIII on day 0. One group injected with p(B-t)$_2$-GPVIII did not receive a second injection, whereas the second group received a second injection of p(B-t)$_2$-GPVIII on day 14. The group injected with pGPVIII on day 0 received a second injection of p(B-t)$_2$-GPVIII on day 26. All animals were tested for anti-peptide antibody by ELISA on day 39. Titres are reciprocal dilutions, as described in Methods.

pCIneo (Fig. 2D). This clearly shows that the chimeric G expressed either the native poliovirus B cell epitope alone or in association with the LCMV CD8$^+$ T cell epitope at the cell surface and that synthesis of lyssavirus G protein was maintained.

Immunogenicity of a foreign B cell epitope carried by truncated G

No IL-2 was produced by splenocytes stimulated *in vitro* by IPLV-PV 14 days after injection (Fig. 3): injection of the truncated pGPVIII plasmid into mice did not induce IL-2-producing cells. In contrast, a significant amount of IL-2 production was detected following p(B-t)$_2$-GPVIII injection and *in vitro* stimulation with IPLV-PV (Fig. 3). This shows that fusion of the foreign epitopes to GPVIII significantly increases the production of Th cells that are directed to the site III region of the rabies virus G.

Similarly, although pGPVIII did not induce anti-rabies virus antibody production, p(B-t)$_2$-GPVIII induced the synthesis of significant levels of antibodies directed against both rabies virus G and poliovirus peptide (Fig. 4). However, VNAb directed against PV were not observed (data not shown). Thus, the fusion of foreign epitopes to the truncated G not only significantly increased the production of antibodies directed against the site III region of PV G, but also led to antibody production against the poliovirus B cell epitope.

The induction of antibodies directed against the poliovirus C3 peptide by p(B-t)$_2$-GPVIII was also tested after priming with either pGPVIII or p(B-t)$_2$-GPVIII itself (Fig. 5). When p(B-t)$_2$-GPVIII was injected without priming and tested 13 days [PBS injected on day 0, p(B-t)$_2$-GPVIII injected on day 26] or 39 days later [p(B-t)$_2$-GPVIII injected on day 0, PBS injected on day 26], the anti-peptide antibody titres were 65 and 80, respectively. However, when priming was performed with pGPVIII [pGPVIII injected on day 0, p(B-t)$_2$-GPVIII injected on day 26] or with p(B-t)$_2$-GPVIII [p(B-t)$_2$-GPVIII injected on day 0, p(B-t)$_2$-GPVIII injected on day 14] the titres were 280 and 600, respectively. This clearly demonstrates that both types of priming increased the production of antibodies directed against the poliovirus peptide.

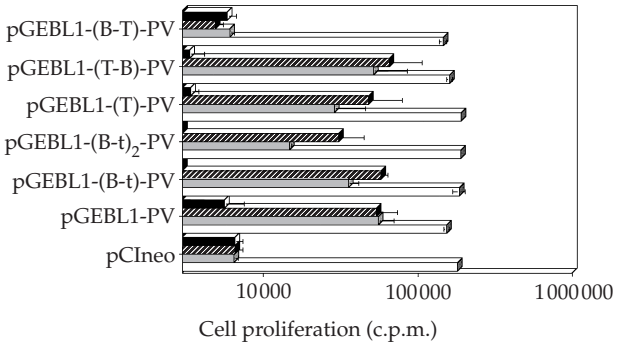

Fig. 6. Induction of IL-2-producing cells by pGEBL1-PV encoding poliovirus and LCMV epitopes. BALB/c mice (two animals for each plasmid) were injected with 40 μg of various plasmids. Experimental conditions for immunization and IL-2 assay were the same as those reported in Fig. 3. Splenocytes were stimulated *in vitro* by cell culture medium (solid bars), specifically by inactivated purified lyssaviruses (IPLV-PV, hatched bars; IPLV-EBL, shaded bars) or polyclonally by ConA (open bars).

In summary, the fusion of the foreign B and CD8$^+$ T cell epitopes with the truncated G induced the production of both Th cells and antibodies directed against rabies and polioviruses.

Immunogenicity of the foreign B cell epitope carried by the chimeric G

IL-2-producing cells that had been induced by the chimeric pGEBL1-PV plasmid carrying various combinations of the two foreign epitopes were tested after mice splenocyte stimulation *in vitro* with inactivated and purified PV or EBL1 (Fig. 6). With the exception of pGEBL1-(B-T)-PV and pCIneo, which were ineffective, all plasmids induced IL-2 production at a level similar to that induced by the pGEBL1-PV plasmid. The insertion of 49 foreign amino acid residues in the chimeric G [pGEBL1-(B-t)$_2$-PV] did not decrease IL-2 production significantly. A positional effect was observed for pGEBL1-(B-T)-PV and pGEBL1-(T-B)-PV; the insertion of the B epitope

Table 2. Antibody production induced by chimeric pGEBL1-PV plasmids carrying various combinations of foreign B and CTL epitopes

BALB/c mice (three for each plasmid) were injected with 50 μg of various plasmids in each tibialis muscle. Sera were assayed on day 21 by the RFFIT method for PV or EBL1 VNAb (titre in IU/ml) and for poliovirus anti-peptide antibody by ELISA (reciprocal dilution). Results are expressed as mean titres and standard deviations are given in parentheses.

Plasmid	PV	EBL1	Poliovirus peptide
pCIneo	0 (0)	0 (0)	0 (0)
pGEBL1-PV	5·9 (2·1)	21·9 (1·8)	0 (0)
pGEBL1-(B-t)-PV	0·4 (0·5)	0·2 (0·08)	250 (25)
pGEBL1-$(B\text{-}t)_2$-PV	0·9 (0·05)	1·1 (0·07)	1100 (200)
pGEBL1-(T)-PV	2·6 (0·2)	5·2 (0·2)	0 (0)
pGEBL1-(T-B)-PV	1·8 (0·2)	8·0 (1·0)	1510 (490)
pGEBL1-(B-T)-PV	0·06 (0·06)	0·6 (0·07)	810 (210)

immediately behind the EBL1 sequence reduced the induction of Th cells stimulated by lyssavirus G. This effect was less pronounced with pGEBL1-$(B\text{-}t)_2$-PV. Therefore, it seems that, when the B cell epitope does not follow the EBL1 sequence, the chimeric G can carry foreign B and $CD8^+$ T cell epitopes with no negative effect on its ability to induce IL-2-producing cells.

The insertion of foreign epitopes into pGEBL1-PV was also studied to assess the induction of antibodies against poliovirus peptide and its effect on VNAb directed against PV and EBL1 (Table 2). All plasmids containing the B cell epitope induced the production of antibodies against poliovirus. Again, if the B cell epitope immediately followed the EBL1 sequence [pGEBL1-(B-t)-PV and pGEBL1-(B-T)-PV], the insertion of foreign epitopes decreased anti-lyssavirus VNAb production, whereas it remained at an acceptable level with pGEBL1-$(B\text{-}t)_2$-PV. In contrast, high levels of VNAb were observed in animals injected with pGEBL1-(T)-PV or pGEBL1-(T-B)-PV. It can be also observed that, if the B cell epitope did not follow the EBL1 sequence immediately, a parallelism was observed between Th cell activation (IL-2 production) and antibody production (Table 2 and Fig. 6). In contrast, if the B cell epitope followed the EBL1 sequence immediately, IL-2-producing cells were induced [with pEBL1-(B-t)-PV] or were not induced (with other plasmids).

Thus, the chimeric G carried and expressed *in vivo* both poliovirus and lyssavirus neutralizing B cell epitopes. However, the presence of the poliovirus B cell epitope located immediately behind the EBL1 sequence [except for the $(B\text{-}t)_2$ insertion] reduced the immunogenicity of both the site II and site III regions of the chimeric lyssavirus G.

Protection against a lethal challenge of LCMV

As the CTL epitope is involved in protection against LCMV (Battegay *et al.*, 1992), several truncated and chimeric G genes encoding it were tested for their protective activity in mice. While pCIneo, pGEBL1-(T)-PV and pGEBL1-(B-T)-PV provided no protection, p$(B\text{-}t)_2$-GPVIII and pGEBL1-$(B\text{-}t)_2$-PV provided 20 and 13% protection, respectively, against a lethal challenge with LCMV performed on day 21 [p$(B\text{-}t)_2$-GPVIII] or 42 [EBL1-p$(B\text{-}t)_2$-GPVIII]. However, the chimeric pGEBL1-(T-B)-PV provided 70% protection. Under these conditions, the surviving animals eliminated the virus completely 21 days after infection (data not shown) when monitored by RT–PCR (Park *et al.*, 1997). Therefore, for some constructs, the chimeric G allowed correct presentation of the LCMV-protective CTL sequences.

Discussion

A trial was recently reported in which infants were immunized with a combined vaccine (diphtheria, tetanus, whole cell pertussis and inactivated poliomyelitis) and a rabies vaccine (Lang *et al.*, 1997). For veterinary use, combined vaccines including a vaccine against rabies have also been used for the immunization of dogs (leptospirosis and feline distemper, hepatitis and parvoviruses), cats (feline panleukopenia, calici- and parvoviruses) and cattle (foot-and-mouth disease virus) (Pastoret *et al.*, 1997). Thus, a multivalent vaccine conferring protection against several pathogens, including lyssaviruses, would have obvious advantages.

In this paper, we investigated the development of multivalent DNA vaccine prototypes. We have shown recently (Bahloul *et al.*, 1998; Jallet *et al.*, 1999) that (i) plasmids encoding lyssavirus G induce both humoral and cell-mediated immune responses including CTL; and (ii) chimeras of different lyssavirus genotypes can broaden the spectrum of protection against lyssaviruses. In the present work, a truncated rabies virus G (PVIII) and a chimeric lyssavirus G (EBL1-PV) were used to carry and express well-characterized non-lyssavirus B and $CD8^+$ T cell epitopes: the C3 epitope of poliovirus type 1 VP1 protein (involved in VNAb induction) and the $CD8^+$ T

cell epitope of LCMV nucleoprotein (involved in protection of the $H2^d$ mouse).

The truncated gene encoding the dimer of foreign epitopes, p(B-t)$_2$-GPVII, induced *in vivo* anti-poliovirus peptide antibody production, indicating that the B cell epitope was expressed. Anti-rabies virus antibodies were also detected 14 days after injection of the plasmid into mice, while 21 days were required to observe a weak induction with pGPVIII. This induction only occurred if IL-2 was injected at the same time (Jallet *et al*., 1999). However, anti-rabies virus antibodies were not neutralizing and the titres against rabies and polioviruses remained low. The protection induced against LCMV was weak. Nevertheless, it appears that the fusion of the foreign (B-t)$_2$ epitopes was clearly beneficial to the immunological properties of GPVIII, and vice versa. Further evidence for this was also provided by the significant increase of IL-2-producing cells that was observed after injection of mice with p(B-t)$_2$-GPVIII and following splenocyte stimulation *in vitro* by IPLV-PV. Finally, PVIII alone was able to prime (B-t)$_2$-GPVIII, inducing a significant increase in anti-poliovirus peptide antibody production. Thus, it appears that the Th cells that were induced by GPVIII alone, although in limited amounts, provided a helper effect to antibody-producing B cells directed against the foreign B epitope. Taken together, these results indicate that the truncated G is immunologically potent and could be used to carry larger antigenic fragments.

The chimeric gene encoding foreign epitopes was found to be efficient in inducing antibody and Th cell production and in providing protection against LCMV challenge. However, inserted sequences sometimes had a negative effect on the biological and immunological response of the pGEBL1-PV chimera: (i) pGEBL1-(B-T)-PV was expressed in the cell cytoplasm and induced poliovirus antibody production but was unable to induce Th cells and antibodies against lyssaviruses, or to provide protection against LCMV; (ii) pGEBL1-(B-t)-PV was expressed at the cell membrane, but induced weak production of antibody and provided minimal protection, whereas Th cell production was maintained; and (iii) the other combinations allowed expression at the cell membrane, induced the production of antibodies and Th cells and provided protection against LCMV [except pGEBL1-(T)-PV]. Thus, structural constraints appear to be important in the chimera. Insertion of any type of foreign epitope reduced VNAb production against both PV and EBL1 relative to that of the parent construct, pGEBL1-PV. Nevertheless, for at least two constructs [pGEBL1-(T-B)-PV and pGEBL1-(T)-PV], the VNAb titres were maintained at a protective level. Indeed, we have shown previously that, when VNAb titres in mice sera are above 1·5 IU/ml after DNA-based immunization, all animals survive an i.c. challenge (Jallet *et al*., 1999). In addition, levels of antibodies against the poliovirus peptide measured after induction with pGEBL1-(T-B)-PV were similar to those obtained by a single injection of the same epitope in hybrid hepatitis B surface antigen particles (Delpeyroux *et al*., 1990).

The protection against LCMV provided by pGEBL1-(T-B)-PV was high, showing that the efficacy of the chimera was similar to that of the gene encoding the full LCMV nucleoprotein (Martins *et al*., 1995; Yokoyama *et al*., 1995) or to that induced by recombinant bacteria (Goossens *et al*., 1995) or bacterial toxin carrying the same epitope (Saron *et al*., 1997). These results demonstrate clearly that foreign B and T cell epitopes can be expressed in an immunologically potent form and correctly presented by both MHC II and MHC I molecules.

The insertion of B-t dimer epitopes into the chimera [pGEBL1-(B-t)$_2$-PV] seemed to have a different effect than fusion with the truncated glycoprotein. Indeed, the VNAb titres against both PV and EBL1 decreased, whereas the ability to induce IL-2-producing cells and poliovirus antibodies were only marginally affected. This decrease could be due to the size of the insert (49 amino acids). Moreover, two types of LCMV CTL epitopes were inserted: the sequence coding for PQASGVYMG, corresponding to the epitope presented by $H2^d$ MHC I (t), and the sequence ER–PQASGVYMG–NLTAQ, corresponding to the larger CTL epitope (T). The expression of the short epitope protected 13–20% of the animals [p(B-t)$_2$-GPVIII and pGEBL1-(B-t)$_2$-PV], whereas the expression of the larger epitope protected 70% of the animals [pGEBL1-(T-B)-PV]. Thus epitopes t and T provided protection against LCMV challenge when carried either by the truncated or the chimeric G molecule. However, neither pGEBL1-(T)-PV nor pGEBL1-(B-T)-PV provided protection. Consequently, the inability of these constructs to provide protection might be due to factors other than the flanking amino acids being in direct contact with the short CTL epitope (Bergmann *et al*., 1996), because the larger epitope was effective in pGEBL1-(T-B)-PV. The nature and position of both the epitope(s) and the flanking regions, as well as the length of the inserted sequence, could be alternative destabilizing factors.

The present work with truncated or chimeric glycoproteins suggests opportunities both to broaden the spectrum of protection against lyssaviruses and to express foreign B and T cell epitopes efficiently. In addition, it may be possible to insert into the chimeric G various epitopes presented by different MHC alleles, in order to obtain CTL production in animals from different genetic backgrounds or in humans (An & Whitton, 1997; Hanke *et al*., 1998), since (i) at least 49 amino acids residues can be inserted with satisfying immunological responses; (ii) the effective delivery of multiple CTL epitopes involved in protection against many viruses, tumours, intracellular bacteria and parasites has been reported (Suhrbier, 1997; Thomson *et al*., 1998); and (iii) degenerate CTL epitopes can be recognized by multiple MHC I molecules, which reduces the number of epitopes that need to be used (Doolan *et al*., 1997). Moreover, the truncated or chimeric lyssavirus G is more potent than an 'artificial polytope' (multiple contiguous CTL epitopes linked to a linear B cell epitope) (Thomson *et al*., 1998). Indeed, Thomson *et al*. (1998) reported that no antibody against a B cell epitope was induced by this polytope, due to

the lack of help from $CD4^+$ T cells. In both types of lyssavirus G, memory $CD4^+$ T cells were induced, providing a complete immune response against foreign epitopes. Moreover, DNA-based immunization with a homogeneous, full-length rabies virus G has been shown recently to be effective in non-human primates (Lodmell *et al*., 1998). Therefore, multiple B and T cell epitopes could presumably be inserted and expressed with a view to human and/or veterinary use.

The truncated and chimeric lyssavirus G carrying antigen fragments may be a new prototype for the development of multivalent vaccines against various zoonoses including rabies. DNA-based immunization was used in these experiments but other systems able to induce CTL might also be effective, such as recombinant vaccinia virus, which has been used widely in Europe for oral vaccination of foxes (Pastoret *et al*., 1997).

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