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Virus-like Particles of Bovine Papillomavirus Type 4 in Prophylactic and Therapeutic Immunization

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Virus-like particles were produced in insect cells containing either the L1 and L2 capsid proteins of bovine papillomavirus type 4 (BPV-4) or only the L1 protein. Both preparations of VLPs proved to be extremely effective prophylactic vaccines. Thirteen of 15 calves immunised with either L1–L2 VLPs or L1–VLPs were refractory to experimental challenge with high doses of BPV-4 and did not develop papillomas, while 9 of 10 control animals developed multiple oral papillomas. VLPs were not efficient as therapeutic vaccine in calves with established papillomas, although VLP-vaccinated animals appeared to undergo tumour regression more rapidly than nonvaccinated control animals. Antibody responses in VLP-vaccinated calves were associated with prevention of disease but not with regression of papillomas. Thus prophylactic VLP vaccination is effective in preventing disease in this model of mucosal papillomavirus infection. VLPs and native virus share at least some conformational epitopes, as shown by the cross-reactivity of their antibodies.

INTRODUCTION

Papillomaviruses are now recognised as carcinogenic agents in humans and animals (IARC, 1995). In addition to their involvement in cancer, these viruses can cause life-long and debilitating diseases which greatly reduce the quality of life in humans and cause serious agricultural and financial problems in domestic animals. There is therefore a need for papillomavirus vaccines which can prevent or cure viral infection. Vaccination against papillomavirus has a long history (Shope, 1937). The first vaccines were based on crude wart extracts (Shope, 1937; Olson and Skidmore, 1959; Olson *et al.,* 1960) or on purified virus (Jarrett *et al.,* 1990). These vaccines successfully induced protection against experimental virus infection and elicited the production of virus neutralising antibodies in the vaccinated host (Jarrett *et al.,* 1990). However, the difficulties of producing papillomavirus in experimental conditions are well known, and even moderate amounts of virus can only be obtained from some types of papillomas, usually in animals.

More recently viral proteins expressed in bacteria, usually as fusion polypeptides, have been employed as vaccines. These experiments have demonstrated that the structural capsid proteins L1 and L2 can independently protect vaccinated animals from infection with the homologous virus, including bovine papillomavirus-1 (BPV-1), BPV-2, BPV-4, and cottontail rabbit papillomavirus (CRPV) (Pilachinski *et al.,* 1986; Jarrett *et al.,* 1991; Campo *et al.,* 1993a; Christensen *et al.,* 1991; Lin *et al.,* 1992). Vaccination with the early proteins E1, E2, E6, and E7 can accelerate rejection of papillomas induced by CRPV and BPV-4 (Selvakumar *et al.,* 1995a,b; Campo *et al.,* 1993a; Francoise Breitburd, personal communication), as can vaccination with BPV-2 L2 (Jarrett *et al.,* 1991).

Immunisation with the early proteins is accompanied by cellular immune responses (McGarvie *et al.,* 1995; Selvakumar *et al.,* 1995a), deemed responsible for papilloma rejection. The capsid proteins elicit the production of neutralising antibodies (Jarrett *et al.,* 1991; Christensen *et al.,* 1991; Lin *et al.,* 1992; McGarvie *et al.,* 1994; Gaukroger *et al.,* 1996), which in the case of bacterially expressed proteins may be directed against linear epitopes. There is however a large body of evidence that during natural papillomavirus infection neutralising antibodies are produced which are directed against conformational epitopes (Ghim *et al.,* 1991) and that these play an important part in immunity against papillomavirus (Christensen *et al.,* 1994b).

Expression of L1 and L2, or L1 alone, in eukaryotic cells results in the self-assembly of the proteins into virus-like particles (VLPs) (Zhou *et al.,* 1991; Kirnbauer *et al.,* 1992; Hagensee *et al.,* 1993; Kirnbauer *et al.,* 1993; Zhou *et al.,* 1993). VLPs are both structurally (Hagensee

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et al., 1994) and antigenically (Kirnbauer *et al.,* 1992; Rose *et al.,* 1993) similar to virus; they present the conformational epitopes which are necessary for induction of high titre neutralising antibodies (Kirnbauer *et al.,* 1992; Christensen *et al.,* 1994b). Contrary to infectious virus, VLPs do not contain possibly oncogenic papillomavirus DNA, which increases their attractiveness as vaccine candidates (Lowy *et al.,* 1994).

Since papillomavirus infections are species restricted, vaccine trials of human papillomavirus (HPV) VLPs cannot be done in animals, and the initial evaluation of the effectiveness of VLP-based vaccines against papillomavirus infection can only be conducted in animal models. VLPs of CRPV were shown to be effective as a prophylactic vaccine against cutaneous infection in domestic rabbits (Breitburd *et al.,* 1995). Since the HPV infections most often associated with human cancer are mucosal, it was important to test the effectiveness of VLP vaccines against animal mucosal papillomaviruses. BPV-4 induces papillomas of the alimentary canal mucosa which can undergo malignant progression (Campo *et al.,* 1993b), thus resembling human mucosal infections. We have generated L1-L2 and L1 VLPs of BPV-4 and have evaluated their efficacy as vaccines in preventing experimental induction of papillomas and in inducing regression of established papillomas.

MATERIALS AND METHODS

Preparation of VLPs

The complete L1 and L2 open reading frames (ORFs) of the BPV-4 genome were amplified by PCR using the recombinant plasmid pBV4 (Campo and Coggins, 1982) as DNA template and oligonucleotide primers incorporating restriction sites as described (Kirnbauer *et al.,* 1993). Thus BPV-4 L1 ORF was amplified and cloned into the *Bgl*II site of pEVmod single expression or of pSynwtVI-double expression baculovirus vectors under the control of the polyhedrin promoter (forward primer, 5′-*Bgl*II-gc ggt aga tct atg tct ttc tgg gtt cca aat tct gc-3′; reverse primer, 5′-*Bgl*II-cc gct aga tct gct cat act gct gca ggt gtt ggc ag-3′). Similarly, the L2 ORF was amplified and cloned into the *Sst*II site of pSynwtVI-, under the control of the promoter pSyn (forward primer, 5′-*Bgl*II-gc ggt ccg cgg aat atg gtt cgt gca gca aga cg-3′; reverse primer, 5′-*Bgl*II-cc gct ccg cgg gca gaa ttt gga acc cag aaa gac-3′). The recombinant plasmids were sequenced across the vector–BPV DNA junctions to ensure correct cloning and then cotransfected with baculovirus DNA (BaculoGold, PharMingen, San Diego, CA) into Sf9 cells. Recombinant baculovirus clones were plaque purified as described (Kirnbauer *et al.,* 1993). L1 and L2 protein expression in Sf9 cells was verified by analysis of protein extracts or purified VLPs by SDS–PAGE and Coomassie staining or by Western blotting. A rabbit antiserum, raised against a bacterially produced BPV-4 L1-βgalactosidase fusion protein (Joan Grindlay, unpublished data), was used to detect L1, and a hyperimmune serum derived from a L2 vaccinated calf (Campo *et al.,* 1993a) was used to detect L2. L1 VLPs and L1–L2 VLPs were produced and purified using sucrose and CsCl gradients as described (Kirnbauer *et al.,* 1993). Self-assembly into VLPs was determined by transmission electron microscopy of purified particle preparations, following negative staining with 1% uranyl acetate (Kirnbauer *et al.,* 1993).

Prophylactic vaccination

Young calves, 8–12 months old, were divided into three groups: group 1 contained 10 calves; group 2 and 3 initially contained 8 calves each, but a calf in group 3 developed a mouth papilloma before the beginning of the experiment and was withdrawn from the trial. Animals in group 1 were not vaccinated; animals in group 2 were vaccinated by intramuscular injection with two doses (V1 and V2) 4 weeks apart of 150 μg L1 VLPs each, and animals in group 3 with two doses of 200 μg of L1–L2 VLPs each. Both antigens were in aluminium gel as adjuvant (Campo *et al.,* 1993a). Two weeks after the second vaccine dose (V2 + 2w), all animals were infected in the palate with a suspension of 10^{11} BPV-4 particles in 10 sites (10^{10} particles per site) as described (Campo *et al.,* 1993a). The calves were examined for number and size of papillomas at approximately 4-week intervals. Blood was taken from each animal before vaccination (p.i.), 1 week and 2 weeks after V2 (V2 + 1w, V2 + 2w; note, V2 + 2w = C, day of challenge), and 2 and 12 weeks after virus challenge (C + 2w, C + 12w). The serum was stored at −70° until testing.

Therapeutic vaccination

Thirteen calves were infected with BPV-4 in the palate as described above. Six weeks after infection (C + 6w) all calves had well-developed papillomas. They were then divided into two groups of six and seven animals, respectively. Group 1 was the control, while animals in group 2 were inoculated with two doses of 100 μg each of L1–L2 VLPs in incomplete Freund's adjuvant, 2 weeks apart. The calves were examined for number and size of papillomas at approximately 4-week intervals. Blood was taken before virus challenge (p.i.), before vaccination (C + 6w), and after vaccination at 3- to 6-week intervals. Serum samples were stored at −70° until testing.

ELISA

Cattle sera were tested in duplicate 10-fold serial dilutions (10^{-1} to 10^{-6} for VLP-vaccinated animals and 10^{-1} to 10^{-3} for control animals) against L1–L2 VLPs. The same sera were tested in duplicate doubling dilutions (from 1:2 to 1:256) against BPV-4 GST-L2a, GST-L2b, and GST-L2c. GST-L2a, GST-L2b, and GST-L2c correspond to the N-terminus, middle portion, and C-

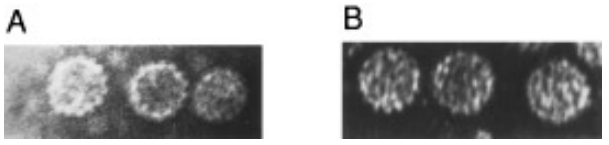

FIG. 1. Electron micrographs of (A) L1–L2 BPV-4 VLPs and (B) BPV-4 virus, both purified by density gradient centrifugation. Magnification is ×150,000.

terminus of the BPV-4 L2 protein expressed in *Escherichia coli* as fusion products of glutathione *S*-transferase (Campo *et al.,* 1993a). After 0.35 μg/well of L1–L2 VLPs or 1 μg/well each of gel-purified GST-L2a, GST-L2b, and GST-L2c were attached to ELISA plates, analyses were carried out as described (Chandrachud *et al.,* 1994, 1995; Kirnbauer *et al.,* 1994). The second anti-bovine IgG antibody was rabbit $F(ab')_2$ conjugated with horseradish peroxidase (Axell) for the analysis of antibodies against VLPs and goat anti-bovine IgG conjugated with alkaline phosphatase (Dynatech) for the analysis of antibodies against the GST-fusion peptides. Appropriate controls of antigen only, antibody only, or conjugate only were carried out for each serum sample. Readings from control wells were negligible; samples were considered positive when their OD readings were >0.1. Titres were defined as the reciprocal of the highest serum dilution giving a positive reading.

Proliferation assays

Fifty millilitres of blood was removed from each animal at appropriate intervals throughout the trial. Blood was separated on Lymphoprep (Nycomed UK) and peripheral blood mononuclear cells (PBM) were removed from the interface and stored in liquid nitrogen. Proliferation assays were performed as described (McGarvie *et al.,* 1995). PBM at 3×10^5 cells per well in RPMI 1640 (Biological Industries) supplemented with 10% heat-inactivated FCS (Advanced Protein Products Ltd.) and 50 μ*M* mercaptoethanol (Sigma) were cultured in medium alone or in the presence of 1 μg GST-L2a, or 5 μg of GST-L2b, GST-L2c, GST, or concanavalin A (Sigma). Cells were cultured at 37° for a total of 72 hr with 1 μCi/well methyl-[^{3}H]thymidine for the last 6 hr. Cells were then harvested with an automatic cell harvester (LKB) and incorporation was counted with a betaplate counter (Wallac). Each assay was performed in triplicate and results were averaged. The results are presented as a stimulation index (SI); SI = cpm of fusion peptide/cpm of GST alone. Given the relatively high SI values in PBM from preimmune animals, only SI $>$ 5 were considered positive. The standard error was less than $\frac{1}{10}$ of the SI for all measurements.

RESULTS

Expression of BPV-4 L1 and L2 in insect cells and production of VLPs

Sf9 insect cells were infected with a single expression baculovirus carrying the BPV-4 L1 ORF or a double expression baculovirus carrying the L1 and L2 ORFs. Western blots of insect cell lysates were analysed with an anti-L1 rabbit antiserum and an anti-L2 bovine antiserum, and the expected bands of 55–60 kDa for L1 and 100 kDa for L2 were detected (data not shown). Self-assembly of L1–L2 or L1 alone into particles was confirmed by electronmicroscopy (Fig. 1A). The particles were morphologically similar to BPV-4 virus (Fig. 1B).

VLPs vaccination prevents development of papillomas

In the control nonvaccinated group 9 of 10 calves developed papillomas 4 weeks after infection and the papillomas were still growing 21 weeks after infection (Fig. 2). On the contrary all the calves (8 of 8) vaccinated with L1 VLPs were protected against challenge with BPV-4 and were still papilloma-free 21 weeks after infection (Fig. 2). Five of the 7 calves vaccinated with L1–L2 VLPs were free of papillomas during the whole course of the experiment; two calves developed one papilloma each 8 weeks after challenge, and 21 weeks after challenge 1 of the 2 calves developed a second papilloma (Fig. 2). The papillomas of both animals were smaller than those in the control animals (not shown). We conclude that vaccination with VLPs protected from viral challenge and prevented papilloma development.

Humoral response to VLPs in VLP-vaccinated and virus-vaccinated calves

Sera from VLP-vaccinated animals and from two animals vaccinated with BPV-4 in an earlier trial (Jarrett *et al.,* 1990) were tested and compared for antibodies to VLPs in ELISA (Fig. 3A). The only sera samples available for the virus-vaccinated animals were preimmune (p.i.), V2 + 2w(C), and C + 5w. None of the animals had antibodies to VLPs before vaccination (p.i.). Anti-VLP antibodies were detectable at V2 + 1w in all the animals vaccinated with L1–L2 VLPs or with L1 VLPs. At V2 + 2w(C),

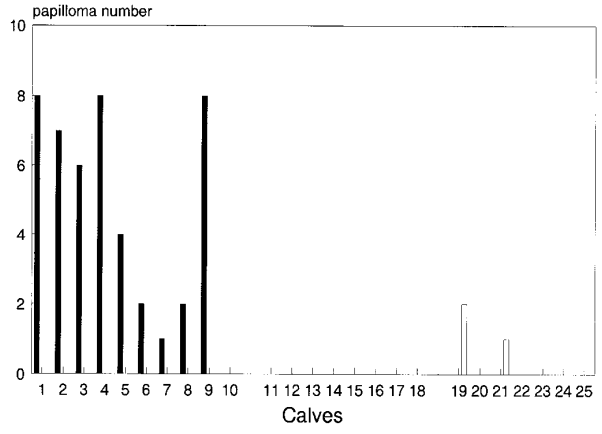

FIG. 2. Numbers of palate papillomas in control calves (1–10) and in calves prophylactically vaccinated with L1 VLPs (11–18) and L1–L2 VLPs (19–25), at 21 weeks after experimental challenge with BPV-4.

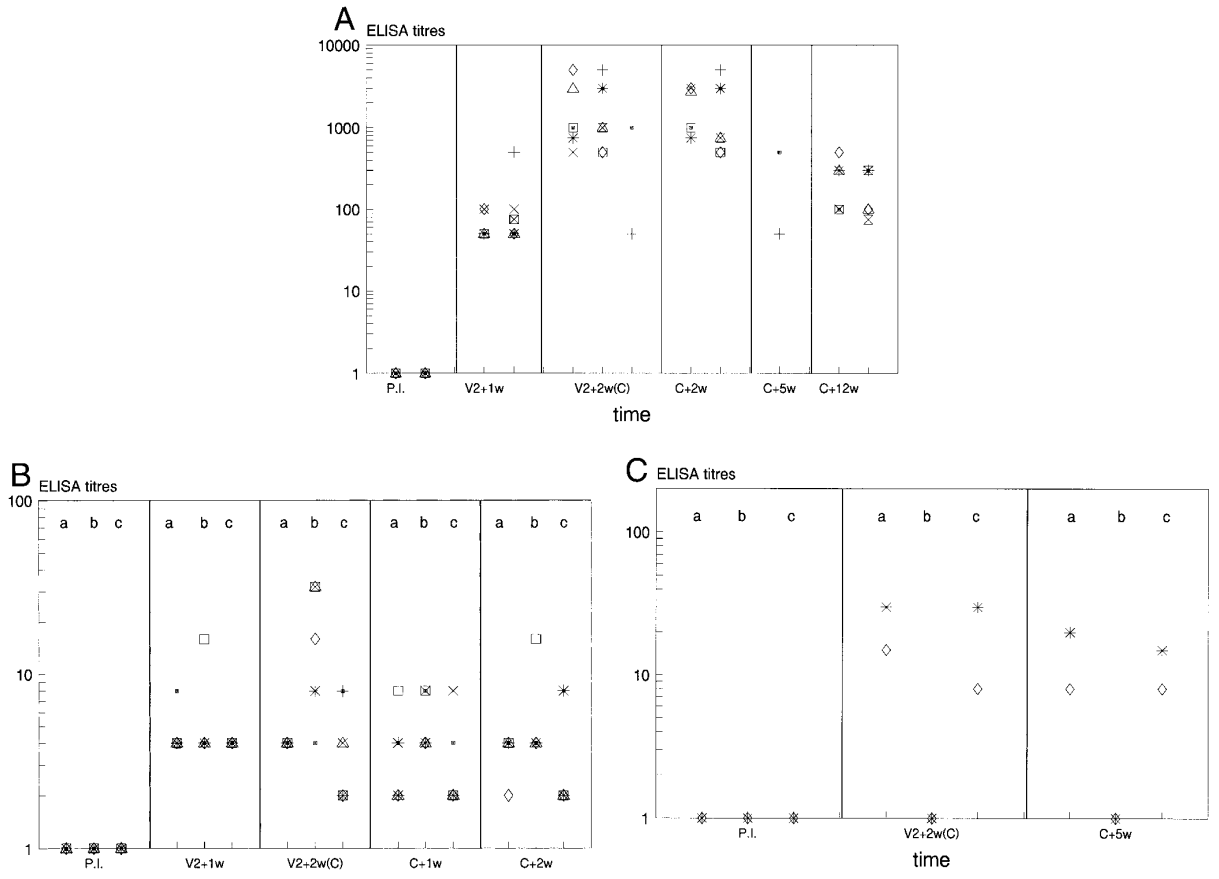

FIG. 3. (A) Humoral immune response to BPV-4 VLPs in VLP-vaccinated calves and virus-vaccinated calves. Each symbol represents an individual animal. The first column at each time point represents titres of anti-VLP antibody in calves vaccinated with L1–L2 VLPs, and the second column, titres of anti-VLP antibody in calves vaccinated with L1 VLPs. The third column at V2 + 2w(C) and the single column at C + 5w represent the titres of anti-VLP antibodies in two virus-vaccinated calves. The antigen on the ELISA plates was L1–L2 VLPs. (B) Antibody titres to L2 in VLP-vaccinated calves and (C) in virus-vaccinated calves. At each time point, the first column represents titres to L2a (the N-terminus of L2), the second column titres to L2b (its internal portion), and the third column titres to L2c (its C-terminus). Each symbol represents an individual animal. The data shown are representative of two independent measurements.

the antibody titre had increased more than one log in both groups of VLP-vaccinated animals; titres did not appreciably change after BPV-4 infection (C + 2w). At C + 12w, antibody titres had started to decline. Of the two virus-vaccinated calves, one had a relatively high antibody titre in the VLP ELISA both before and after challenge, comparable to the titre in the VLP-vaccinated animals, while the other reacted poorly. These results indicated that at least some antigenic determinants are shared between BPV-4 virions and BPV-4 VLPs. Sera from nonvaccinated infected control animals did not react in the VLP ELISA (data not shown), indicating that BPV-4 is poorly immunogenic when experimentally delivered in the palate.

Humoral response to L2 peptides in VLP-vaccinated and virus-vaccinated calves

Vaccination with bacterially expressed L2 protein of BPV-4 elicits the production of virus neutralising antibodies and prevents infection (McGarvie *et al.,* 1994; Gaukroger *et al.,* 1996). In L2 vaccinated animals the antibody response is directed to the N- and C-termini, L2a and L2c, respectively, with little or no response to the middle portion, L2b (Chandrachud *et al.,* 1995). We monitored the antibody response to the three portions of L2 in both L1–L2 VLP- and virus-vaccinated animals. Sera from VLP-vaccinated calves reacted poorly with L2a and L2c, but a weak response to L2b was observed, particularly at V2 + 2w (Fig. 3B). In contrast, sera from the two virus-vaccinated calves responded modestly to L2a and L2c but not at all to L2b (Fig. 3C).

Cellular response to L2 peptides in VLP-vaccinated calves

Given the differences in antibody response to the three fragments of L2 between virus- and VLP-vaccinated calves, the cellular response to the L2 peptides was investigated in the latter. PBM from five calves vaccinated with L1–L2 VLPs were assayed for their ability to respond to the three fragments of L2 in proliferation

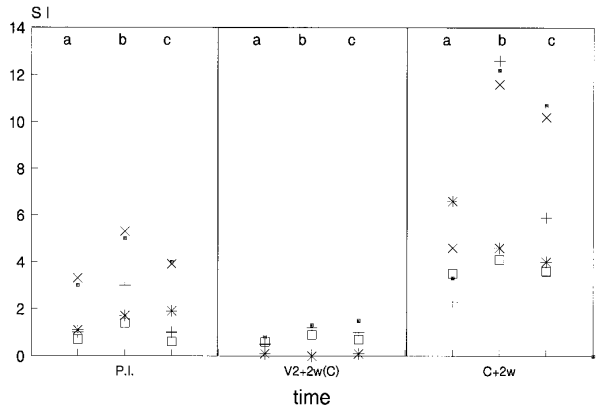

FIG. 4. PBM proliferation *in vitro* to L2. Proliferation is expressed as stimulation index (SI). Columns as in Figs. 3B and 3C.

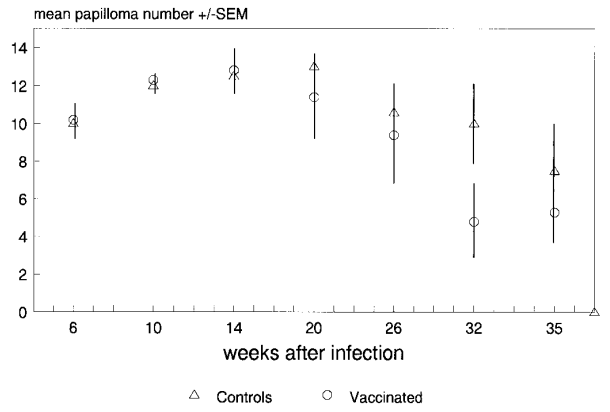

FIG. 5. Mean number of papillomas (± SEM) in control calves and in calves inoculated with L1–L2 VLPs 6 and 8 weeks after virus challenge.

assays up to C + 2w (Fig. 4). There was little or no response to the three L2 fragments at p.i., V2 + 2w(C), and C + 2 days (C + 2d not shown). At C + 2w PBM from two of the five animals responded to both L2b and L2c, PBM from an additional animal responded to L2b only, and PBM from a fourth animal responded modestly to L2a (Fig. 4). PBM from five infected control calves did not respond to any of the three L2 peptides, with the exception of PBM from two calves which responded to L2b at C + 2w (data not shown). A comparison with virus-vaccinated calves could not be carried out, as no PBM from these animals were available.

Therapeutic vaccination with VLPs.

Vaccination of calves with BPV-2 L2 induced regression of skin warts (Jarrett *et al.,* 1991); regression of skin warts or alimentary canal papillomas was also occasionally observed when papilloma-bearing calves were vaccinated with BPV-2 or BPV-4 virus, respectively (WFH Jarrett, unpublished observations). To determine if BPV-4 VLPs induced papilloma rejection, calves were infected with BPV-4 as described. Six weeks after viral challenge (C + 6w) all animals had developed papillomas; six calves were kept as control and seven calves were immunised with two doses of VLPs 2 weeks apart. The calves were monitored approximately every 4 weeks until Week 35 after challenge (Fig. 5). At C + 6w, the number of papillomas was similar in the two groups and increased slightly in both groups up to C + 14w. At C + 20w one of the vaccinated calves had undergone rejection and was papilloma-free. At C + 26w the number of papillomas was decreasing in the two groups, but total rejection was observed in two calves in the vaccinated group only. Tumour regression proceeded during the next 9 weeks: one animal in the control group and three in the vaccinated group were papilloma-free at C + 32w; at C + 35w two control calves and four vaccinated calves had undergone complete rejection. Although regression started earlier in the vaccinated group and more animals were tumour-free, the differences between the two groups were small and not statistically significant (Fischer's exact test, $P = 0.16$).

Humoral response to VLPs in calves therapeutically vaccinated with VLPs.

The calves vaccinated therapeutically with L1–L2 VLPs were analysed for their immune response to the antigen. The presence of serum antibodies to VLPs was determined by ELISA in sera taken before challenge (p.i.), at C + 6w (before vaccination), and at 3- to 6-week intervals thereafter (Fig. 6). Although all vaccinated and control calves had papillomas by C + 6w, none of them had a measurable antibody response to VLPs, with the exception of a single vaccinated animal with a low antibody titre. However, all vaccinated calves had sero-converted by C + 10w; all the control animals remained negative (not shown), confirming the poor immunogenicity of both BPV-4 inoculum and BPV-4 progeny. VLP titres were still high in the vaccinated animals at C + 14w, but the number and size of papillomas was not affected (Fig.

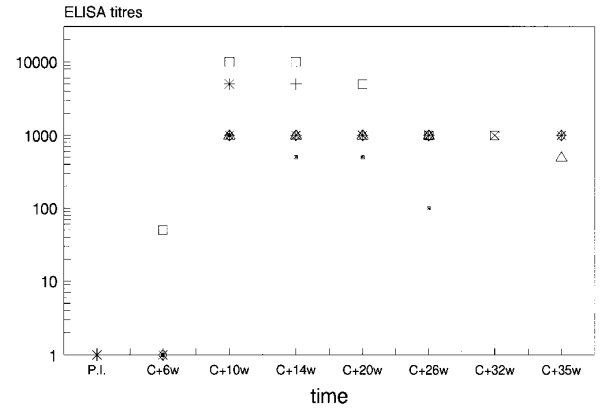

FIG. 6. Humoral immune response to BPV-4 VLPs in calves vaccinated therapeutically with VLPs. Each symbol represents an individual animal.

5). Thereafter in most animals titres did not appreciably change, even during the rejection period, and there was no correlation between titres and regression.

DISCUSSION

In this study, we have explored the vaccination potential of L1 and L1–L2 VLPs in the prevention and treatment of mucosal infection by BPV-4. VLP vaccination efficiently prevented the development of experimentally induced papillomas, while the effect of VLPs on established papillomas was equivocal.

Vaccination with native BPV-4 prevents development of papillomas of the alimentary canal mucosa (Jarrett *et al.,* 1990), leading to the prediction that vaccination with BPV-4 VLPs would be equally effective. Although it has recently been shown that vaccination with CRPV VLPs prevents cutaneous papillomas in rabbits (Breitburd *et al.,* 1995), the efficacy of vaccination with VLPs against a mucosal papillomavirus had not been established. Given the clinical relevance of mucosal HPV infection, the need for an appropriate vaccine and the possibly critical differences between mucosal and cutaneous immunity (Roche and Crum, 1991), it was important to validate the VLP approach in a mucosal papillomavirus system.

The successful prophylaxis described here shows that VLP vaccination can provide a high level of protection also against mucosal papillomavirus infections. This conclusion is supported by a recent prophylactic vaccine trial with VLPs of canine oral papillomavirus (COPV), in which protection of dogs from experimental oral challenge by COPV was observed (Suzich *et al.,* 1995).

Both L1–L2 VLPs and L1 VLPs were effective as prophylactive vaccines. None of the animals vaccinated with L1 VLPs developed papillomas, confirming the conclusions from the CRPV study (Breitburd *et al.,* 1995) that the presence of L2 in VLPs is not necessary for prevention of disease. Vaccination with L2 alone however can prevent papilloma development both in the BPV-4 and CRPV systems (Christensen *et al.,* 1991; Lin *et al.,* 1992; Campo *et al.,* 1993a), and it thus appears that immunity from disease can be elicited by at least two different viral antigens. Two of the animals vaccinated with L1–L2 VLPs did develop one or two small papillomas, respectively. We do not consider this to imply that L1–L2 VLPs are less effective than L1 VLPs. In our experience, a few vaccinated animals, independently of the nature of the vaccine or of the adjuvant, may develop a negligible number of papillomas which often regress while still in the first stages of growth. Whether this implies that prevention of disease is not always due to prevention of infection remains to be established.

Vaccination with L1–L2 VLPs of calves with established infection did not appear to induce papilloma regression although regression started earlier in vaccinated calves than in control animals. The difference between the two groups, although suggestive of a therapeutic effect, is too small to be statistically significant. These results are similar to those obtained with BPV-2 or BPV-4 virus vaccines, when regression of skin warts or alimentary canal papillomas respectively was observed in some, but not all, animals (WFH Jarrett, unpublished observations). Larger groups of animals, such as those used for the investigation of E7 as a therapeutic vaccine (Campo *et al.,* 1993a), would be needed to substantiate any therapeutic effect of VLP vaccination. Vaccination with VLPs, whether given before or after viral challenge, induced a vigorous antibody immune response to the antigen (cf. Figs. 3A and 6), showing that VLPs are efficient at presenting epitopes to the humoral effector arm of the immune system in the natural host of a papillomavirus. However, although anti-VLP (or anti-virus) antibodies are presumably neutralising and therefore responsible for protection from disease (Breitburd *et al.,* 1995; Suzich *et al.,* 1995), the same antibodies do not appear to have a role, or not a clearly definable one, in papilloma regression.

The epitopes presented by VLPs are shared, at least partially, with native virus, as antisera from virus-vaccinated calves recognise VLPs. Similar results have previously been observed with other papillomavirus types and their respective VLPs (Kirnbauer *et al.,* 1994; Christensen *et al.,* 1994a, b; Suzich *et al.,* 1995).

While intramuscular vaccination with VLPs or virus elicited a humoral immune response, intraoral inoculation of challenge virus failed to do so, and no detectable response to virus or VLPs was observed in nonvaccinated control animals, even when they were bearing papillomas producing progeny virus. In the course of our studies on vaccination and immunity against BPV, we have repeatedly observed this lack of antibody response to challenge or progeny virus during the first stages of infection. A response to capsid proteins or to the early protein E7 has been observed in some animals at later stages of infection and appears to be associated with natural rejection (Chandrachud *et al.,* 1994; Knowles *et al.,* in preparation). The reasons for the failure of the immune system to recognise incoming virus, even when injected intradermally in heavy doses, are not clear. Two possible factors are the lack of adjuvant in the inoculum and the administration of a single dose. The absence of a response to progeny virus until much later in infection may be explained by the fact that papillomas are restricted to the epithelium and therefore somewhat shielded from immunological recognition.

We have shown that vaccination with BPV-4 L2 or its N-terminus prevents induction of papillomas (Campo *et al.,* 1993a; Chandrachud *et al.,* 1995). Therefore we examined the antibody response to three fragments of L2 in VLP- and virus-vaccinated animals, to see to what extent L2 is presented by native virus or VLPs and whether the

response is comparable in the two groups of vaccinated animals. Nonvaccinated animals had no detectable anti-L2 antibodies, confirming previous results (McGarvie *et al.,* 1994; Chandrachud *et al.,* 1995; Knowles *et al.,* in preparation). In contrast, a response to L2, albeit weak, could be detected in vaccinated calves. Titres were generally low, but comparable between VLP- and virus-vaccinated calves (cf. Figs. 3B and 3C). Therefore L2, although a good immunogen when administered intramuscularly as a subunit vaccine, is not well presented by vaccine virus or vaccine VLPs. Indeed, in VLP-vaccinated calves the cellular response to L2 is detected only after virus challenge, similar to what was already observed in L2-vaccinated animals (Chandrachud *et al.,* 1995).

A qualitative difference in the antibody response to L2 was observed between VLP- and virus-vaccinated animals. While virus-vaccinated animals, like L2-vaccinated animals (Chandrachud *et al.,* 1995), mount an antibody response to the N-terminus and C-terminus of the protein, but not to its middle portion, in the VLP-vaccinated calves both the humoral and the cellular response was primarily directed to the middle portion of L2 (cf. Fig. 3B with Figs. 3C, and 4 of this paper with Fig. 4 of Chandrachud *et al.,* 1995).

It is not known whether these divergent results reflect real differences in the L2 epitopes presented by VLPs, native virus or bacterially derived fusion proteins, or minor structural variations among virus isolates and VLP preparations. It has recently been reported that virions and VLPs of BPV-1 consistently differ in their presentation of surface epitopes (Ghim *et al.,* 1996). Whether this is the case also for BPV-4 virions and VLPs would require analysis of several different preparations of both. Antibodies to the middle portion of L2 in VLP-vaccinated animals are unlikely to contribute to protection from virus challenge, as virus neutralising anti-L2 antibodies are directed against the N-terminus of the protein (Chandrachud *et al.,* 1995; Gaukroger *et al.,* 1996). Protection in L1–L2 VLP-vaccinated animals is therefore likely to be mediated by anti-L1 antibodies, as confirmed by the clinical efficacy of L1 VLPs.

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