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Intra-epithelial vaccination with COPV L1 DNA by particle-mediated DNA delivery protects against mucosal challenge with infectious COPV in beagle dogs

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Abstract

Protection against viral challenge with canine oral papillomavirus (COPV) was achieved by immunisation via particle-mediated DNA delivery (PMDD) of a plasmid encoding the COPV L1 gene to cutaneous and oral mucosal sites in beagle dogs. The initial dose of approximately 9µg of DNA was followed by two booster doses at 6 week intervals. A similar approach was used to vaccinate a control group of animals with plasmid DNA encoding the Hepatitis B virus S gene. Following challenge at the oral mucosa with canine oral papillomavirus all animals vaccinated with the COPV L1 gene were protected against disease. However 5 of 6 animals in the control group developed COPV induced papillomas at the oral mucosa. Both cell mediated lymphoproliferative and humoral antibody responses to the DNA vaccine were observed. Our data indicate that PMDD of plasmid DNA can protect against mucosal challenge with papillomavirus.

Human papillomavirus (HPV) infections, especially those affecting the genital mucosae, are associated with significant morbidity and mortality. It is now clear from epidemiological and laboratory studies that infection with specific human papillomavirus (HPV) types, in particular HPV 16 and 18 and their relatives, is the major risk factor for the subsequent development of cervix cancer in women (1). HPV DNA is found in more than 90% of all cervix cancers (13) and some reports suggest that all cervix cancers are HPV associated (28). Cervical cancer is the second commonest cancer in women worldwide; in developing countries it is the commonest female cancer and has a high mortality rate. Immunological intervention by prophylactic vaccination is an attractive prospect and an effective immunisation strategy against these viruses would be a major development in health care for women, especially in the third world, with a significant effect in reducing health care costs in both the developed and undeveloped world.

The extreme species specificity of the papillomaviruses together with the inability to generate usable amounts of infectious genital HPVs by *in vitro* culture has meant that animal models are essential for the development and testing of vaccines and anti-virals against these agents. Three models are of value, the cotton tail rabbit papillomavirus (CRPV), bovine papillomavirus (BPV) and the canine oral papillomavirus (COPV) [for review, see (23)]. In addition to its natural host, the wild cotton tail rabbit, CRPV infects domestic rabbits although the viral life cycle is usually incomplete in this species. CRPV only infects haired skin and, in view of the extreme tissue tropism of the pvs, it is possible that the CRPV/domestic rabbit model may not accurately reflect the immunobiology of the mucosal viruses. BPV-4 is a mucosotropic virus infecting the oropharynx and oesophagus of cattle. However there are difficulties in monitoring the incidence and progression of lesions in these sites and

the specialised husbandry requirements of such large animals precludes their use by most experimenters.

The canine oral papillomavirus (COPV) is good model for mucosotropic pv infection [for review see (18)]. COPV infects domestic dogs and wild canine species on the oropharyngeal and conjunctival mucosae where it induces warts (and very rarely squamous cell carcinomas (3, 26, 29)). The lesions are very similar to those induced by the low risk genital HPVs and contain large amounts of infectious virus. After infection by abrasion of the mucosal surface, oral papillomas appear within 4-8 weeks, before regressing spontaneously a further 4-6 weeks later, after which the animals are immune to challenge with infectious virus (5). The benign lesions rarely cause problems but occasional cases of non-regressing successive crops of warts have been noted (16). The warts contain high levels of virus and therefore are an excellent source of infectious virus for experiments *in vitro*. COPV provides the optimal model for pre-clinical and proof of principle studies for papillomavirus vaccines. The dog, a well characterised laboratory species, can be immunised with the antigen of interest and challenged with high dose of infectious virus on the upper lip, an easily accessible and observable area. The development of lesions is highly reproducible both in terms of wart area and time providing clean readouts for vaccine efficacy. There is a good range of canine immunological reagents and assays of both humoral and cell mediated response can be undertaken, providing fundamental data on the mode of action of the vaccine preparations.

In natural papillomavirus infections in the cow, rabbit and dog antibodies to the major capsid protein L1 are protective (4, 14, 25); prophylactic vaccine development has therefore focussed on this antigen. Prophylactic immunity can be induced in these animal models, with whole virus preparations (2) or virus like particles (VLPs) (6, 12,

25) generated by self assembly of recombinant L1 protein expressed in eukaryotic vectors; denatured L1 does not offer prophylactic protection in the dog (25) and rabbit (14). HPV L1 VLP vaccines are in Phase I/II clinical trials (30) but VLP based vaccines are expensive and, in the long term, may prove unsuitable for use in third world countries. DNA vaccines have features that make them attractive candidates when compared with protein based preparations. Large scale production is simpler; DNA once dried has long term stability over a wide temperature range and recombinant expression vectors encoding the DNA sequence of the antigen are able to induce long term cellular and humoral immunity (7, 19) without the need to purify protein products and produce VLPs. DNA can be delivered intra-muscularly (i.m.) by needle injection (27) or intra-cutaneously (i.c.) (20, 21) using the gene gun for particle-mediated DNA delivery (PMDD). Each route induces both cellular and humoral immunity but PMDD provides a strong cellular and humoral response with 100-1000 fold lower DNA doses than the needle injection route (9, 21). Both routes of vaccination with CRPV L1 DNA, albeit with high DNA inputs, have been shown to induce protective immunity in the rabbit model (8, 24) but proof of principle for cutaneous DNA vaccination against mucosal papillomavirus infections has not been provided to date. In this report we show that immunisation of beagle dogs with low doses of COPV L1 DNA delivered by PMDD to the skin and oral mucosa elicits a modest systemic antibody and T cell lymphoproliferative immune response that completely protects against challenge with high doses of infectious COPV.

MATERIALS AND METHODS

Expression Vectors. The plasmid pCMV-L1 (Glaxo-Wellcome, Stevenage, Herts, UK.) contains the complete COPV L1 open reading frame (ORF) cloned into

vector WRG7077 containing a CMV promoter (PowderJect Vaccines, Madison, Wisconsin, USA.). The L1 gene was derived from pBR322.COPV (DKFZ Referenzzentrum für Humanpathogene Papillomviren, Heidelberg, Germany). Purified pCMV-SPORT-βgal (kind gift from Dr W Peh and Dr J Doorbar, NIMR, Mill Hill, London) contains the β-galactosidase gene under the control of the CMV promoter in a 7.8kb circular DNA plasmid. Plasmid pCMV-HBV-S (PowderJect Vaccines, Madison, WI, USA.) contains the hepatitis B virus surface (HBV-S) antigen under the control of the CMV promoter.

Cassette Preparation. Preparation of cassettes for PMDD was as follows: DNA was precipitated onto 2 μm diameter gold micro-particles (DeGussa Metals Group, South Plainfield, NJ, USA) in the presence of spermidine (Sigma Chemical Co., St Louis, MO, USA) and calcium chloride (Fujisawa, Inc., Melrose Park, IL, USA) as described previously (15). The gold-DNA precipitate was washed and resuspended in 100% ethanol and 0.05 mg/ml polyvinylpyrrolidone (PVP, Sigma) then adsorbed onto the inner surface of Tefzel tubing (TFX Medical Inc., Jaffrey, NH, USA) by centrifugal force using a tube turner device (PowderJect Vaccines, Inc., Madison, WI, USA). The Tefzel tubing was subsequently cut into 1.27 cm length cassettes and stored desiccated at 4°C until use. Each cassette contained theoretical weight of 0.5 mg gold micro-particles coated with a total dose of 0.75 mg plasmid DNA, as assessed by spectrophotometric analysis of DNA eluted from selected cassettes (GeneQuant II, Pharmacia Biotech).

Optimisation of PMDD. Preliminary trials were undertaken on fresh canine cadavers to establish the optimum pressure for delivery of gold particles to the epidermis. Punch biopsies of 6mm were taken immediately after PMDD to cutaneous (caudal ventral abdomen) and mucosal (buccal mucosa of the upper lip) sites at

various pressures. Tissues were embedded in optimal cutting temperature compound (OCT, BDH) and frozen in isopentane (BDH) cooled to its freezing point in liquid nitrogen. Sections were cut on a cryostat, stained routinely with haematoxylin and eosin, and examined by light microscopy. Once the optimal pressure had been established using the cadaver material, the β -galactosidase reporter construct (pCMV-SPORT- β gal) was used *in vivo* on one beagle dog to check that the delivery protocol resulted in protein expression in the target tissues. After PMDD to the oral mucosa 6mm punch biopsies were taken at 2 and 9 days for detection of β -galactosidase activity.

Detection of β -galactosidase Activity. Tissues were embedded in optimal cutting temperature compound (OCT, BDH) and frozen in isopentane (BDH) cooled to its freezing point in liquid nitrogen. Frozen tissue was stored at -70°C ready for cryostat sectioning. $10\ \mu\text{m}$ sections were cut and air dried for at least 30 min. The sections were fixed by immersion in 100% ethanol for 2 min then washed in PBS (3 x 3 min.). The slides were immersed in development solution (70 ml PBS, 10 ml 50mM potassium ferricyanide, 10 ml 50mM potassium ferrocyanide, 4 ml 50mM magnesium chloride, 1 ml 1 % sodium deoxycholate, 2 ml 1% Nonidet P-40. 100 mg x-gal powder was dissolved in 2.5 ml dimethylsulphoxide and added just prior to use) at 37°C for 2 hours. After washing in PBS (3 x 3 min.), the sections were counterstained in Carazzi's haematoxylin (45 seconds) and mounted in aquamount (Gurr). Negative control sections were from normal canine oral mucosa.

Detection of *in vitro* expression. Within several hours of death, tissues from the tongue, ventral abdominal skin and buccal mucosa were collected from dogs euthanased at the Department of Clinical Veterinary Medicine. Tissues were stored on ice. Prior to DNA transfection, the tissues were removed from ice and placed on a

3 cm thick piece of foam for support. PMDD was used at 400 psi to bombard the tissues with pCMV-L1 or pCMV-SPORT-βGal DNA coated gold particles at sites marked by pin prick tattoos of Indian ink. The tissues were then returned to ice and transported to the laboratory in transport medium (a 1:1 mixture of Earle's balanced salt solution and Dulbecco's phosphate-buffered saline). In a laminar flow cabinet, successfully targeted areas of tissue were confirmed by the presence of a superficial gold deposit, and excised by 6 mm punch biopsy. The biopsies were washed in several changes of sterile PBS and placed onto sterile metal grids supported by metal rings at the fluid/air interface of the culture medium (500 ml GMEM, with the following added by filter sterilisation: 50 ml foetal bovine serum, 5 ml kanamycin (10 mg/ml), 125 µl amphotericin (5 mg/ml), 500 µl cholera toxin (10^{-7} M in serum free medium), 250 µl hydrocortisone (1 mg/ml)). The explants were incubated at 37°C, 5% CO₂. Medium was refreshed every other day, and biopsies were harvested at 1, 3 and 5 days after transfection. As negative controls, non-transfected tissues were cultured in a similar manner.

COPV DNA was detected by *in situ* hybridisation and COPV L1 protein was detected by immunohistochemistry as previously described (17) (figure 2).

RNA-RNA *in situ* hybridisation. Digoxigenin labelled riboprobes were prepared as per manufacturers instructions (Roche Diagnostics, Welwyn-Garden-City). Sections on Vectabond-coated slides (Vector laboratories) were dewaxed and rehydrated washed in PBS (2 x 3 min.) and encircled with a silicone pen (DAKO). The tissues were fixed in 4% paraformaldehyde with 5mM MgCl₂ in PBS for 5 min. and re-washed in PBS (2 x 3 min.). After digestion in RNase-free DNase (15 U/ml) in 2 x SSC for 1 hour at 37 °C in a humid chamber, the sections were washed in PBS (2 x 3 min.) and digested in Protease K (50 µg/ml in Protease K buffer (0.02M Tris

pH 7.4, 2mM CaCl₂) for 15 min. at 37 °C. Sections were then washed in PBS (2 x 3 min.) and re-fixed as above for 5 min. After a final wash in PBS (2 x 3 min.), the sections were dehydrated through graded ethanols (30, 50, 70, 90, and 100%, 30 seconds each) and air dried. The section was covered in RNA probe diluted 1:10 or greater in hybridisation buffer (2 x SSC (1 x SSC = 0.15M NaCl, 0.015M sodium citrate), 5% dextran sulphate, 0.2% Marvel (Premier Beverages), 50% deionised formamide), according to the labelling efficiency, coverslipped and sealed with cow gum (Cow Proofings Ltd.). Slides were incubated overnight at 42 °C.

The gum was removed with forceps, and the coverslips soaked off in 2 x SSC, 0.1% SDS. Slides were washed at 55 °C in 2x SSC, 0.1% SDS for 5 min. then incubated in RNAaseA (2 mg/ml in 5xSSC) for 15 min. at 37 °C in a humid chamber. Sections were washed in 0.1 x SSC, 0.04% SDS (2 x 20 min, 65 °C), before soaking in blocking buffer and developing. Sections were counterstained in Carazzi's haematoxylin (30-45 seconds).

Vaccination Protocol. All vaccinations and biopsies were performed under general anaesthesia. Food was withdrawn from the animals by 18.00hrs on the evening prior to anaesthesia. Animals were examined and weighed before premedication with acepromazine maleate (ACP, C-Vet, 2mg/ml) at 0.03mg/kg as a sedative, and buprenorphine hydrochloride (Vetergesic, Animalcare, 0.3mg/ml) at 10µg/kg by intramuscular injection as an analgesic. Anaesthesia was induced by intravenous sodium thiopentane (Thiovet, C-Vet, 2.5%) at approximately 10mg/kg to effect. After endotracheal intubation (cuffed) anaesthesia was maintained to effect with halothane (Fluothane, Mallinckrodt Veterinary) and oxygen (2-3 x respiratory minute volume) using a T-piece with Jackson-Reece modification.

Six beagles were vaccinated with the pCMV-L1 plasmid at 12 sites (total dose 9µg DNA); three on each side of the buccal mucosa and three on each side of the caudal ventral abdominal midline. Boosting vaccinations were repeated in an identical manner after 6 weeks. After 12 weeks a second boost was given at six sites, three on each side of the buccal mucosa. At the end of the immunisation schedule each dog had received a total of 27µg DNA. The control group comprised of six beagle dogs and all were vaccinated with the HBV S plasmid in an identical manner to the L1 vaccinees i.e. one primary immunisation and two boosts, a total of 27µg HBV-S DNA. All vaccinations were performed using the PowderJect XR1 device at a delivery pressure of 400psi.

Serum Collection from Beagles. Blood samples were withdrawn aseptically from the cephalic vein under gentle manual restraint or under general anaesthesia if collection coincided with vaccination or challenge. The samples were centrifuged (IEC Sentra-7) at 2,500g for 10 minutes, the serum aspirated, aliquoted and frozen at -70°C for use in ELISA studies later.

ELISA. Flexible PVC microplates were coated with 100ng per well of purified COPV particles or HBV S protein suspended in carbonate buffer, pH 9.6, at 4°C overnight. The plates were washed with PBS/0.05% Tween 20 and blotted dry prior to the addition of 100 µl per well of PBS/Tween containing 2% w/v skimmed milk powder (ELISA diluent) and incubation at 37 °C for 30 min. Following further washing, 50µl aliquots of individual serum dilutions, from a doubling series made for each dog serum sample, were added to duplicate wells of the coated plates. Negative controls received diluent alone. The plates were re-incubated at 37°C for 1.5 hours, washed again, and peroxidase-conjugated, rabbit anti-dog IgG (1/500, ICN Immunobiologicals) was added to all wells. Following incubation for a further 1.5

hours and a final wash, the plates were developed using an o-phenylene diamine/peroxide substrate in urea buffer (Sigmafast). Colour development was stopped with 20% sulphuric acid and absorbance levels were read at 490nm using a Molecular Devices plate reader.

Isolation of Peripheral Blood Monocytes (PBMC). 10 ml of canine blood was collected into a universal tube containing 100 µl of 100 mg/ml EDTA and diluted with an equal volume of PBS/EDTA (5.38 ml 0.5M Disodium EDTA in 500 ml 1x PBS). 5 ml of diluted blood was layered on top of 5 ml Ficol-Paque (Pharmacia), then centrifuged in a Sorvall (RC-3B) for 45 min at room temperature, 1,500 g, with the brake turned off. Cells from the interface were collected using a 1.0 ml Gilson pipette. To this an equal volume of 2% RPMI (RPMI-1640 (Sigma) 25 mM HEPES and NaHCO₃, 0.3g L-Glutamine, Penicillin/Streptomycin, 10⁻⁵ M 2-Mercaptoethanol (Sigma), 2% Foetal Calf Serum (FCS) (Harlan)) was added and the cells pelleted at 1,500 g for 5 min, re-suspended in 2% RPMI then centrifuged again. Cells were then re-suspended with 5ml 10% RPMI (The same as 2% RPMI but with 10% FCS) and counted (with Tryphan blue 1:1).

Lymphoproliferative Assay. Samples were arranged in triplicate, in a 96-well round bottom tissue culture plate (Falcon). All antigens were suspended in 100µl 10% RPMI/well. The antigens used were Concanavalin A to a final concentration of 2.5µg/ml as a positive proliferating agent control, purified COPV particles at 10⁻³ to 10⁻⁸ dilutions of 0.45µg L1 protein/µl stock, and negative control wells containing 100µl of medium only. 1x10⁵ PBMC/well suspended in 100 µl 10% RPMI were added to the plate containing the antigen. The plates were then incubated for 5 days at 37°C, 5% CO₂. ³H-Thymidine at 1µCi/well was added on the 5th day and the plates incubated for a further 6 hours. The cells were then harvested (LKB Wallac 1295-001

cell harvester) onto filtermats (Helis Bio) and read using LKB Wallac 1205 beta plate liquid scintillation counter. The standard index (S.I.) was then calculated by taking the mean cpm of the triplicate samples (after addition of antigen) and dividing this figure by the mean cpm of the negative control samples (media only).

Viral Challenge. Under general anaesthesia the mucosa of the upper lip was lightly scarified over an area approximately 5mm x 5mm, using the tip of a scalpel blade, until a light ooze of blood occurred. A 10 μ l aliquot of purified COPV (approximately 45ng of COPV L1 protein) from a clinical case of oral papillomatosis (17) was applied to each site by pipette and allowed to absorb for a few minutes. 10 sites were challenged in each dog, 5 on each side of the upper lip. The sites of challenge did not co-localise with the sites of immunisation. COPV virus was isolated and purified as described previously (17).

RESULTS

β -Galactosidase Expression Study. In order to determine the optimum pressure for delivery of gold particles into oral and cutaneous sites in the dog, a fresh canine cadaver was used to test different pressures for gold delivery. Cryostat sections from biopsies taken immediately after the procedure showed that a pressure of 400 psi delivered gold particles into the epidermis, reaching the basal layer (data not shown). This pressure was used subsequently in the *in vivo* work. To establish whether the delivery technique and construct expression system were effective *in vivo*, a β -galactosidase reporter construct (pCMV-SPORT- β gal) was delivered by PMDD into canine oral mucosa. β -galactosidase activity was detected in small amounts in the superficial layers of the oral mucosal epithelium in biopsies taken two days later, but not in those taken nine days later or in negative control samples. Gold

particles were seen in the epidermis at two days, but not nine days, although occasional gold particles remained in the underlying dermis at nine days (figure 1).

DNA Vaccination and Viral Challenge. 12 beagle dogs were vaccinated as described earlier. Delivery of DNA coated gold particles using the PowderJect XR1 device was associated with the development of localised inflammation and haemorrhage, due to ballistic damage of the cutaneous blood vessels by the gold particles and/or helium exhaust blast, together perhaps with physically-induced (non-specific) degranulation of cutaneous mast cells. These reactions were rapid in onset, typically being noticeable within a few minutes of vaccine delivery. These reactions were restricted to the sites of vaccination, and comprised focal, reddened, oedematous, slightly raised swellings. Some sites developed serocellular oozing and crusting after 1 to 2 days. Biopsy from the dog used in the β -galactosidase study at two days after vaccination confirmed the presence of a prominent cellular inflammation response, including numerous neutrophils. All sites healed completely with no lasting adverse effects.

All animals were boosted with the DNA vaccine at 6 and 12 weeks after and challenged at 15 weeks after primary vaccination with approximately 45ng of purified COPV particles per site as described above. Weekly blood sampling occurred through out the experiment. After viral challenge the sites were examined and photographed at weekly intervals. None of the six dogs vaccinated with the COPV L1 encoding plasmid developed papillomas at any of the challenge sites, 60 sites in total. Of the six control dogs five developed oral papillomatosis (figure 3). Three of these developed prominent warts at each of the ten oral sites challenged (figure 4): two dogs developed smaller warts at the majority of sites. No observable warts were detected on the one remaining animal in the control group, although ELISA data indicated that

the animal seroconverted at the same time as the other members of the challenge group and achieved comparable anti-L1 antibody titres indicating that it had received a viral challenge and suggesting that it had undergone a sub clinical infection (figure 5).

Induction of Humoral Antibody Response. Sera from all 12 Beagles was analysed for antibody response to whole COPV virus or HBV S antigen. All six beagles vaccinated with HBV S DNA were taken as controls and were numbered 1 to 6. The six beagles vaccinated with COPV L1 were numbered 7 to 12. All animals from the L1 group (numbers 7-12) showed a specific anti COPV L1 antibody response to vaccination (figure 6). This antibody response against COPV was associated with protection from the subsequent viral challenge. The control group (numbers 1-6) however, showed no significant COPV L1 antibody response to COPV prior to viral challenge (figure 5). This lack of anti-COPV antibody was associated with a susceptibility to subsequent viral challenge, as illustrated by papilloma formation at the challenge sites.

Animals 9, 10, 11 and 12 in the L1 vaccine group all showed a similar response; a specific anti-L1 antibody response was elicited by the vaccinations. The circulating antibody levels induced by the vaccine were then reduced after the animals were challenged, remaining stable at this lower level. Animal number 7 showed a moderate level antibody response to vaccination, which was maintained for approximately one month after challenge. Thereafter, the antibody titre rose to a maximum by 8 weeks post challenge and was maintained for at least a further month. The final animal dog 8 showed only a low anti-L1 antibody response to virus, this low response was maintained throughout the experiment did not alter on challenge and the animal was protected (figure 6).

In the control group (animals 1-6) a specific anti-COPV L1 antibody response was elicited post viral challenge. The antibody titre increased from 4 weeks post infection to a maximum at 8 weeks post infection, this titre then decreased and was maintained at the lower level for the duration of the experiment. In animals 6, 3, and 5 florid papilloma formation matched the antibody titre with maximum wart size and number at 8 weeks post challenge. Animals 4 and 2 had warts of shorter duration and size but showed a similar antibody response and titre. The final animal, number 1, did not have any observed papillomas but the onset, duration and extent of the anti-L1 antibody response was similar to that of the other members of the group (figure 5). It seems likely that this animal either had a sub clinical infection or a papilloma of short duration. All of those animals in the control group tested for an antibody response against HBV S antigen showed a significant response after vaccination (figure 7).

***In Vitro* proliferative response of PBMC.** All PBMC showed a proliferative response to Con A as a positive control antigen, with an S.I. of up to 300. All six beagles vaccinated with HBV S DNA were taken as controls. These animals showed no significant proliferative response to COPV prior to challenge (figure 8). Following challenge all control animals showed a specific proliferative response to COPV with an average S.I. of 3.7, which was statistically significant. Four of the six beagles vaccinated with COPV L1 DNA showed a specific cellular proliferation in response to COPV with an S.I. of 2.2 to 3.4 after vaccination, the remaining 2 animals (1 and 2) showed only a minimal proliferative response before challenge (figure 8). The average S.I. for the L1 group was a significant proliferation response of 1.9. After challenge all six animals had a response to COPV with an average S.I. of 2.2.

DISCUSSION

The work described above shows that COPV L1 specific, humoral, prophylactic immunity can be elicited by intra cutaneous and mucosal immunisation with the COPV L1 gene on a CMV driven plasmid. The vaccination protocol used resulted in complete protection for all animals at 60 of 60 sites challenged with purified COPV. A total dose of less than 30 μ g of DNA in total, delivered in 3 doses of 9 μ g was sufficient to protect individuals against viral challenge. This vaccine dose elicited not only anti L1 IgG antibodies recognising COPV virions but also a specific T lymphoproliferative response to COPV virions.

In the experiments described in this report, sera from all animals were analysed against whole virus rather than COPV VLPs. This provided a cleaner readout in ELISA and ensured that the antibody response detected was directed predominantly against conformational epitopes found on native virions. The anti-L1 titres generated were modest, a phenomenon observed also with COPV L1 VLP vaccines (25). The weak anti COPV response observed with the DNA vaccine does not appear to be a reflection of PMDD since the control animals vaccinated with HBV S encoded in an identical vector and vaccinated by identical routes generated high titres of anti HBV S antibody. It is possible that this reflects the response to secreted HBV-S protein as opposed to intra-cellular COPV L1 protein. Despite the modest L1 serum antibody titres all animals were protected against mucosal challenge with high doses of COPV. The control HBV S vaccinated beagles showed no significant antibody response to COPV prior to challenge illustrating the specificity of the response in the L1 vaccinees. Following challenge the 6 control animals showed the expected response to infection with anti L1 IgG titres increasing until 8 weeks post challenge, then decreasing slowly and plateauing at a stable value. This ELISA data

illustrates the reproducibility of the challenge in this outbred population. Three animals were highly susceptible to infection developing florid papillomas 5-6 weeks at all sites post challenge. The papillomas reached 10-12mm in size and persisted for 5-6 weeks before regressing completely. Two animals had smaller papillomas of shorter duration appearing 6 weeks post challenge and one animal had no visible papillomas. However that individual had the expected anti L1 IgG response, seroconverting at the same time as the other members of control group and reaching comparable titres. It is possible that this animal had a small papilloma of short duration missed in the weekly examination of the oral cavity or alternatively it underwent a subclinical infection. Vaccinees were progeny from the same sire/dam matings as the control group and all vaccinees were protected from viral challenge, no warts being detected in any animal.

Effective prophylactic vaccination with “live” (5) or formalin inactivated (2, 10) crude COPV wart extract has been shown previously and vaccination with COPV L1 VLPs has been shown to be highly effective (10, 25). It is known that this prophylactic immunity is mediated by serum immunoglobulin and that these are neutralising antibodies recognising conformational determinants (11, 25). DNA vaccines encoding COPV antigens have not been previously described. However a CRPV L1 DNA vaccine delivered i.m has been shown to induce protection in the rabbit (8) although the vaccine dose used was 100 times greater (1mg per dose) than that employed in the present study. It is known, however, that PMDD induces a strong cellular and humoral response with 100-1000 fold lower doses of DNA when compared with i.m. injection (9, 22). Protection in the rabbit was achieved with intracutaneous vaccination of CRPV L1 DNA using a dose of 90 µg in total (24) still 10 times greater than that employed in the present study.

DNA vaccines have many advantages when compared with protein or attenuated viral vaccines. They are highly stable, easily administered and inexpensive to produce in large quantities. After purification, super-coiled plasmids can be lyophilised for storage at room temperature for long periods whereas proteins are considerably less thermostable. The manipulation of DNA vaccines is relatively straightforward: they can encode multiple antigens and potentially one could immunise against several papillomavirus types in one poly valent vaccine, a strategy which may well be necessary for an effective prophylactic vaccine against high risk genital HPV types. Long term persistence of antigen expression from polynucleotide vaccines allows an enduring T cell response as well as establishment of immunological memory (20). The data presented in this report are encouraging for the development of DNA vaccines for papillomavirus infections. The results support the use of L1 as the target antigen for prophylactic immunisation and illustrate the viability of this mode of vaccination in a mucosal papillomavirus infection that closely mirrors human mucosal papillomavirus infections.

REFERENCES

1. **Anonymous.** 1995. Human Papillomaviruses, vol. 64. World Health Organization International Agency for Research on Cancer, Lyon, France.
2. **Bell, J. A., J. P. Sundberg, S. J. Ghim, J. Newsome, A. B. Jenson, and R. Schlegel.** 1994. A formalin-inactivated vaccine protects against mucosal papillomavirus infection: a canine model. *Pathobiology.* 1994;. **62**:194-8.
3. **Bregman, C. L., R. S. Hirth, J. P. Sundberg, and E. F. Christensen.** 1987. Cutaneous neoplasms in dogs associated with canine oral papillomavirus vaccine. *Vet-Pathol.* 1987 Nov;. **24**:477-87.
4. **Campo, M. S.** 1997. Vaccination against papillomavirus in cattle. *Clin-Dermatol.* 1997 Mar-Apr;. **15**:275-83.
5. **Chambers, V. C., and C. A. Evans.** 1959. Canine oral papillomatosis. I. Virus assay and observations on the various stages of the experimental infection. *Cancer Research.* **19**:1188-1195.
6. **Christensen, N. D., C. A. Reed, N. M. Cladel, R. Han, and J. W. Kreider.** 1996. Immunization with viruslike particles induces long-term protection of

- rabbits against challenge with cottontail rabbit papillomavirus. *J-Virol.* **70**:960-5.
7. **Ciernik, I. F., J. A. Berzofsky, and D. P. Carbone.** 1996. Induction of cytotoxic T lymphocytes and antitumor immunity with DNA vaccines expressing single T cell epitopes. *J-Immunol.* 1996 Apr 1; **156**:2369-75.
 8. **Donnelly, J. J., D. Martinez, K. U. Jansen, R. W. Ellis, D. L. Montgomery, and M. A. Liu.** 1996. Protection against papillomavirus with a polynucleotide vaccine. *J-Infect-Dis.* 1996 Feb; **173**:314-20.
 9. **Falo, L. D., Jr.** 1999. Targeting the skin for genetic immunization. *Proc-Assoc-Am-Physicians.* 1999 May-Jun; **111**:211-9.
 10. **Ghim, S., J. Newsome, A. B. Jenson, R. Schlegel, J. Suzich, J. Tamura, W. White, F. Hill, P. Warrener, J. A. Bell, and J. Sundberg.** 1995. Formalin-inactivated oral papilloma extracts and recombinant L1 vaccines protect completely against mucosal papillomavirus infection: a canine model., p. 375-379, *Vaccines 95.* Cold Spring Harbour Laboratory Press.
 11. **Ghim, S. J., J. Newsome, J. Bell, J. A. Suzich, J. P. Sundberg, R. Schlegel, and A. B. Jenson.** 1997. Spontaneously regressing oral papillomas induce systemic antibodies that neutralise canine oral papillomavirus., p. 322. 16th International Papillomavirus Conference, University of Sienna, Italy.
 12. **Kirnbauer, R., L. M. Chandrachud, B. W. O'Neil, E. R. Wagner, G. J. Grindlay, A. Armstrong, G. M. McGarvie, J. T. Schiller, D. R. Lowy, and M. S. Campo.** 1996. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. *Viol.* **219**:37-44.
 13. **Liaw, K. L., A. G. Glass, M. M. Manos, C. E. Greer, D. R. Scott, M. Sherman, R. D. Burk, R. J. Kurman, S. Wacholder, B. B. Rush, D. M. Cadell, P. Lawler, D. Tabor, and M. Schiffman.** 1999. Detection of human papillomavirus DNA in cytologically normal women and subsequent cervical squamous intraepithelial lesions. *J-Natl-Cancer-Inst.* 1999 Jun 2; **91**:954-60.
 14. **Lin, Y. L., L. A. Borenstein, R. Ahmed, and F. O. Wettstein.** 1993. Cottontail rabbit papillomavirus L1 protein-based vaccines: protection is achieved only with a full-length, nondenatured product. *J-Virol.* 1993 Jul; **67**:4154-62.
 15. **Macklin, M. D., and e. al.** 1998. Immunization of pigs with a particle-mediated DNA-vaccine to influenza A virus protects against challenge with homologous virus. *J Virol.* **72**:1491-1496.
 16. **Nicholls, P. K., B. A. Klaunberg, R. A. Moore, E. B. Santos, N. R. Parry, G. W. Gough, and M. A. Stanley.** 1999. Naturally Occurring, Nonregressing Canine Oral Papillomavirus Infection: Host Immunity, Virus Characterization, and Experimental Infection. *Virology.* **265**:365-374.
 17. **Nicholls, P. K., R. A. Moore, N. Parry, B. Klaunberg, and M. A. Stanley.** 1999. **Naturally-occurring, non-regressing canine oral papillomavirus infection: host immunity, virus characterisation and experimental infection.** *Virology* (In Press).
 18. **Nicholls, P. K., and M. A. Stanley.** 1999. Canine papillomavirus--A centenary review. *J-Comp-Pathol.* **120**:219-233.
 19. **Pardoll, D. M., and A. M. Beckerleg.** 1995. Exposing the immunology of naked DNA vaccines. *Immunity.* **3**:165-9.
 20. **Pertmer, T. M., M. D. Eisenbraun, D. McCabe, S. K. Prayaga, D. H. Fuller, and J. R. Haynes.** 1995. Gene gun-based nucleic acid immunization:

- elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine*. **13**:1427-30.
21. **Raz, E., D. A. Carson, S. E. Parker, T. B. Parr, A. M. Abai, G. Aichinger, S. H. Gromkowski, M. Singh, D. Lew, M. A. Yankauckas, and et al.** 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc-Natl-Acad-Sci-U-S-A*. **91**:9519-23.
 22. **Robinson, H. L., and C. A. Torres.** 1997. DNA vaccines. *Semin-Immunol*. 1997 Oct.; **9**:271-83.
 23. **Stanley, M. A., P. J. Masterson, and P. K. Nicholls.** 1997. In vitro and animal models for antiviral therapy in papillomavirus infections. *Antiviral Chemistry and Chemotherapy*. **8**:381-400.
 24. **Sundaram, P., R. E. Tigelaar, and J. L. Brandsma.** 1997. Intracutaneous vaccination of rabbits with the cottontail rabbit papillomavirus (CRPV) L1 gene protects against virus challenge [published erratum appears in *Vaccine* 1998 Apr;16(6):655]. *Vaccine*. **15**:664-71.
 25. **Suzich, J. A., S. J. Ghim, F. J. Palmer Hill, W. I. White, J. K. Tamura, J. A. Bell, J. A. Newsome, A. B. Jenson, and R. Schlegel.** 1995. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. *Proc-Natl-Acad-Sci-U-S-A*. 1995 Dec 5;. **92**:11553-7.
 26. **Teifke, J. P., C. V. Lohr, and H. Shirasawa.** 1998. Detection of canine oral papillomavirus-DNA in canine oral squamous cell carcinomas and p53 overexpressing skin papillomas of the dog using the polymerase chain reaction and non-radioactive in situ hybridization. *Vet-Microbiol*. 1998 Feb 28;. **60**:119-30.
 27. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, and et al.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein [see comments]. *Science*. 1993 Mar 19;. **259**:1745-9.
 28. **Walboomers, J. M., J. M.V., M. M.M., B. F.X., K. J.A., S. K.V., S. P.J., P. J., M. C.J., and M. N.** 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology*. **189**:12-19.
 29. **Watrach, A. M., E. Small, and M. T. Case.** 1970. Canine papilloma: progression of oral papilloma to carcinoma. *Journal of the National Cancer Institute*. **45**:915-920.
 30. **WHO.** 1999. The current status of development of prophylactic vaccines against human papillomavirus infection. Technical Report. WHO Department of Vaccines and Other Biologicals.

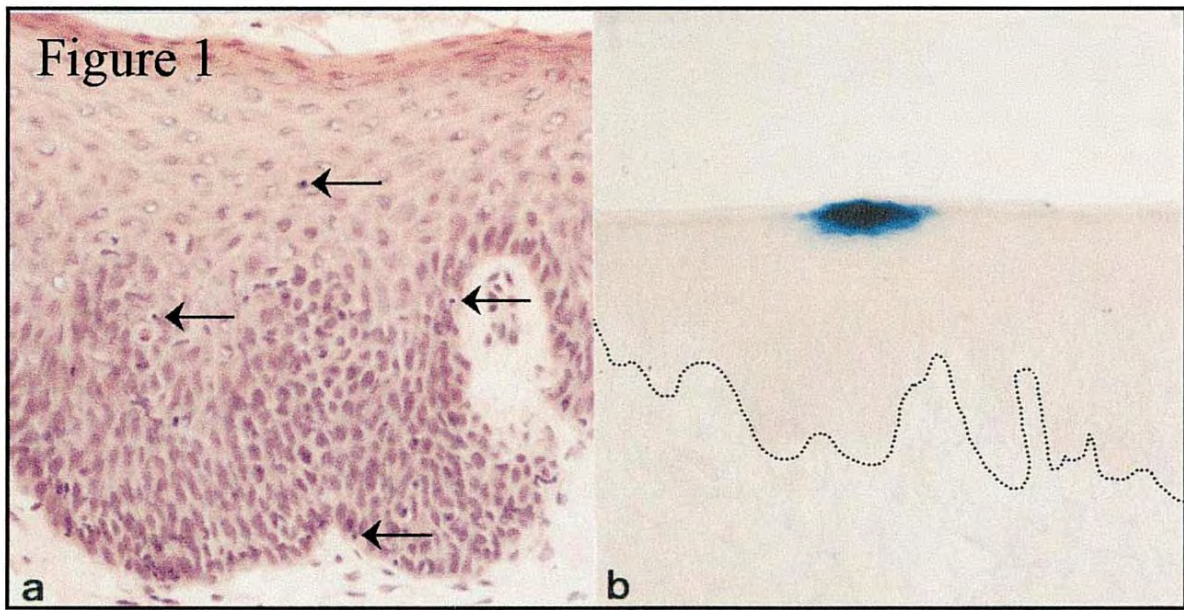


Fig. 1. In vivo trial of reporter b-gal construct 2 days after PMDD. Gold particles are seen 2 days after PMDD (a), expression at this stage is confined to superficial epithelium (b). The basal layer is denoted with a dotted line. Magnification: a: $\times 400$ b: $\times 200$.

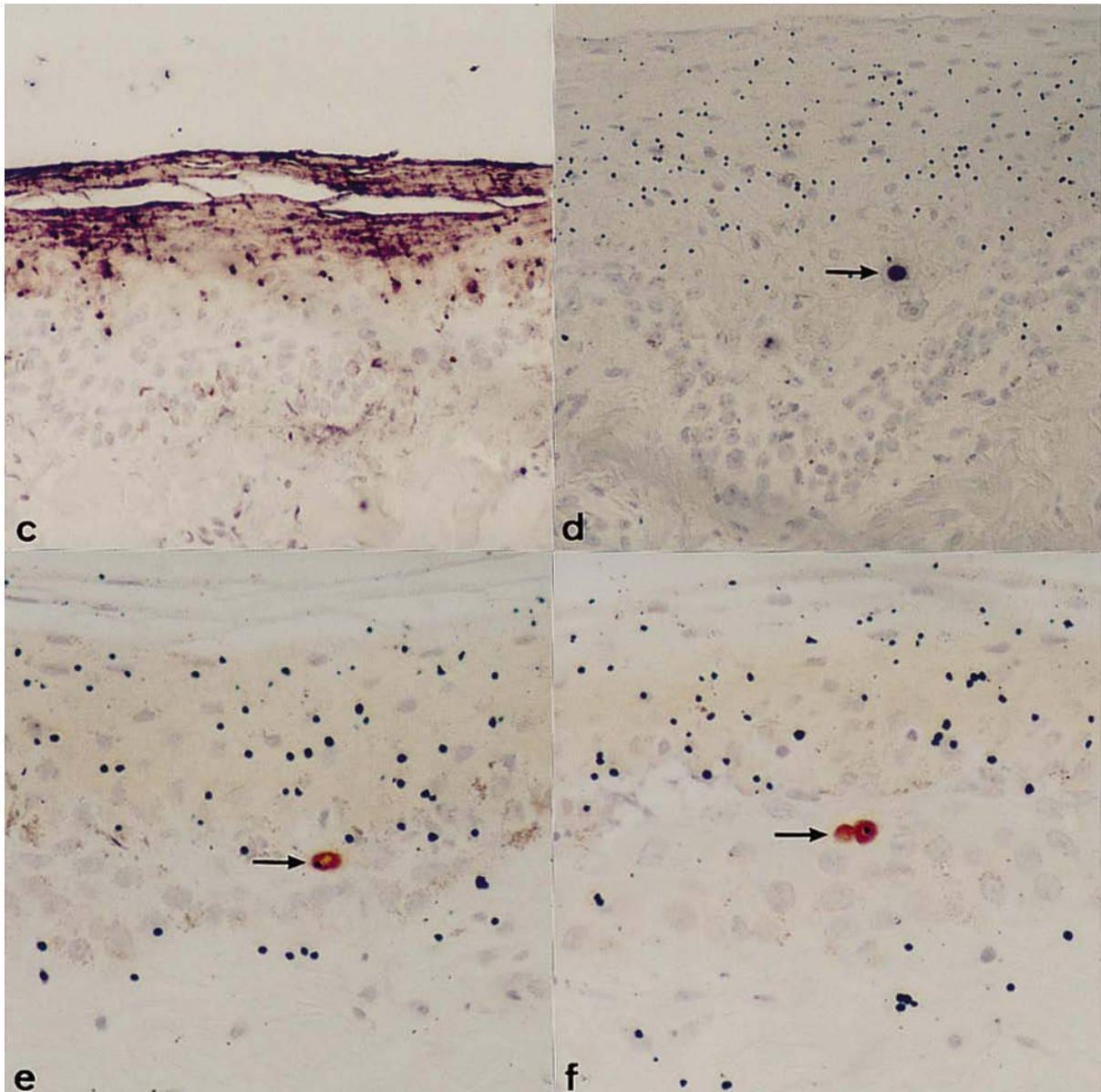


Figure 2: *In vitro* expression of COPV L1 construct after PMDD at 400 psi. Detection of COPV DNA by DNA *in situ* showed streaks of DNA shed from gold particles in the upper epithelium at day 1 (A). Detection of L1 RNA transcripts by RNA *in situ* hybridisation revealed occasional positive cells from day 1 (B). Immunohistochemistry for L1 protein using CAMVIR 1 showed a similar pattern of occasional positive cells, often associated with a gold particle (C and D). A and B x200, C and D x400.

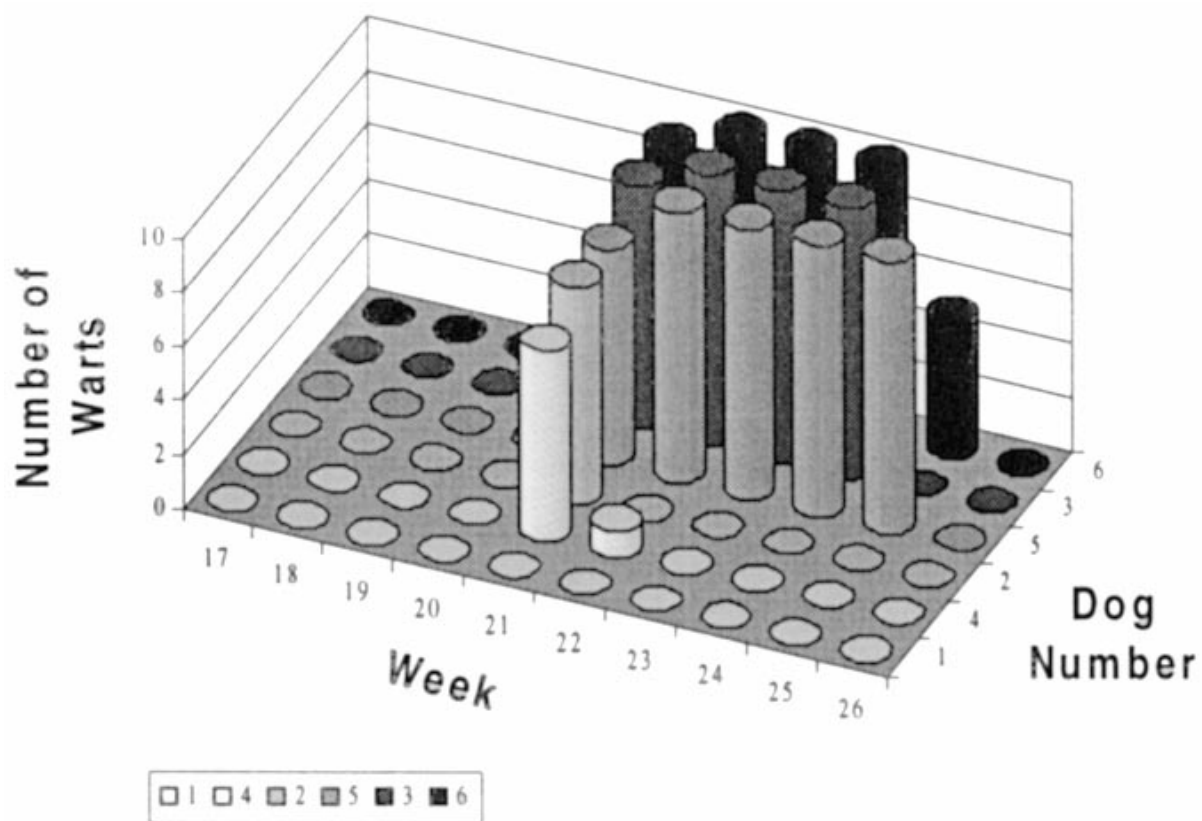


Fig. 3. Appearance of COPV induced papillomas. The number of papillomas and their duration is presented.



(A)



(B)



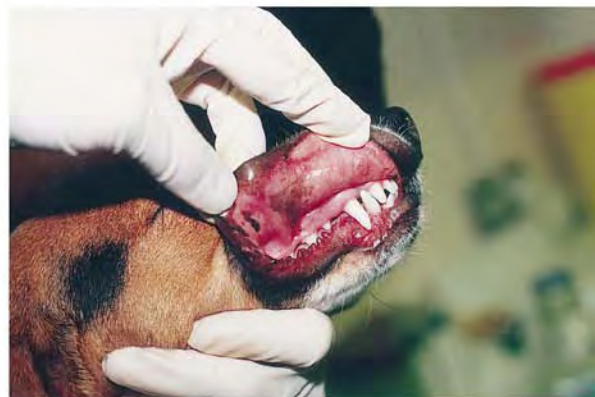
(C)



(D)



(E)



(F)

Fig. 4. Chronological record of papilloma life cycle from first appearance 5 weeks post challenge (A) through mature wart 8 weeks post challenge (D) to full regression 10 weeks post challenge (F).

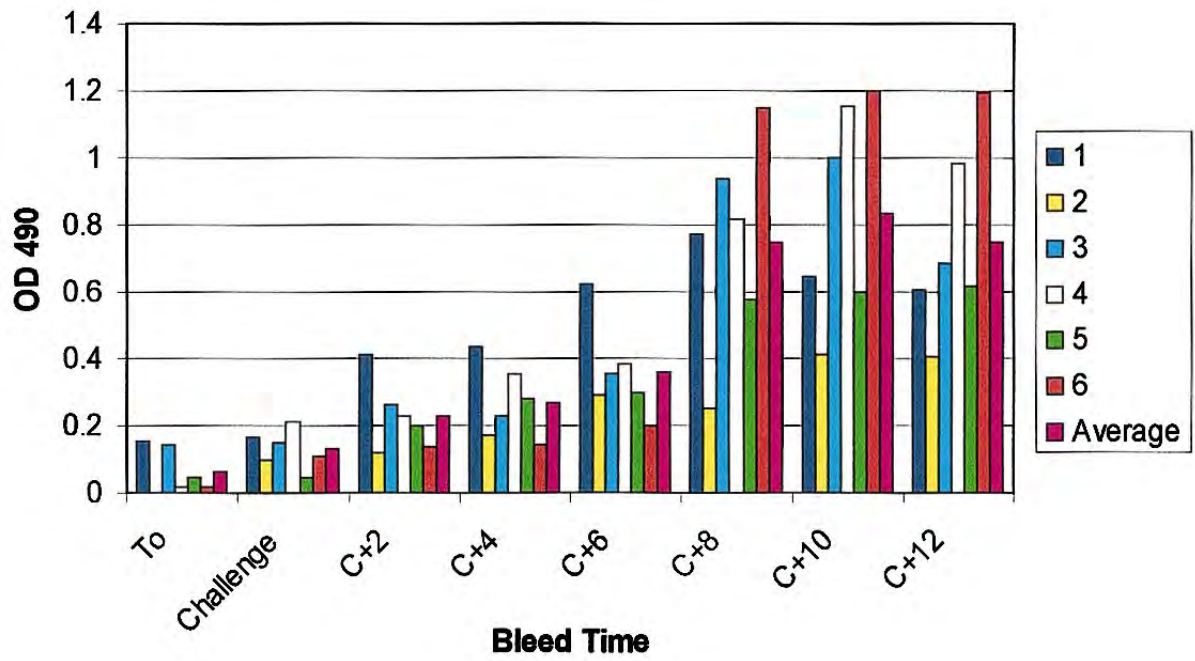


Fig. 5. Graph illustrating serum IgG response to COPV in control HBV vaccinated animals.

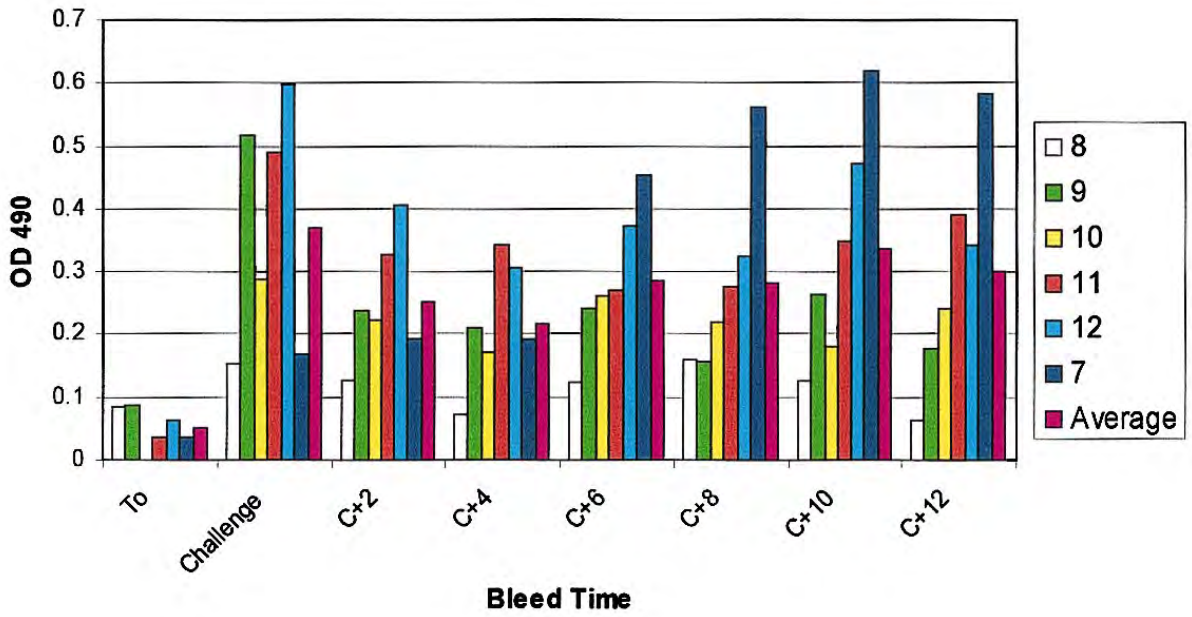


Fig. 6. Graph illustrating serum IgG response to COPV in COPV L1 vaccinated animals. Data represented here is for sera diluted 1 in 320. To represents sera taken on Day 0, C_2 represents 2 weeks post challenge, C_4 represents 4 weeks post challenge etc.

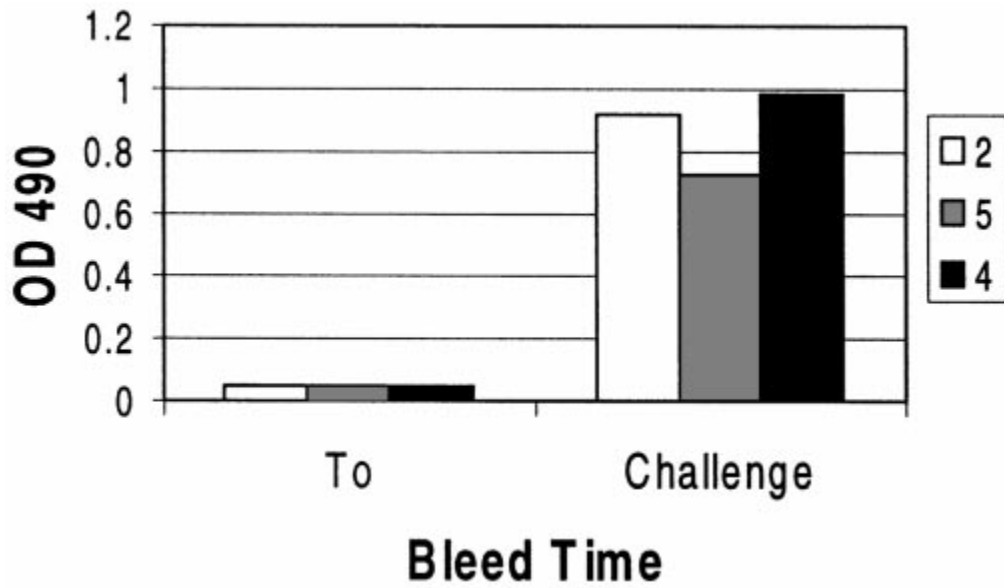


Fig. 7. Graph illustrating IgG response to HBV-S antigen in HBV vaccinated animals. Data represented here is for sera diluted 1 in 320. To represents sera taken at Day 0 and Challenge represents sera taken on the day of challenge.

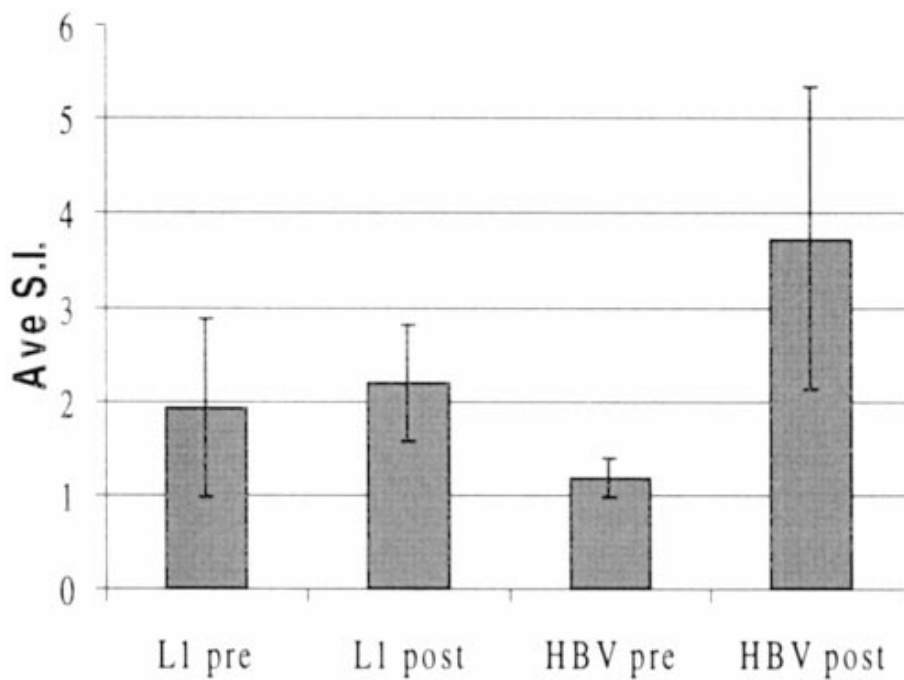


Fig. 8. Graph showing animals' average lymphoproliferative response to COPV pre challenge following vaccination and post challenge with native virus. The COPV L1 vaccinated group and the control HBV vaccinated group are both shown.