Running Title: Canine oral papilloma regression

Regression of canine oral papillomas is associated with infiltration of CD4+ and CD8+ lymphocytes

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Summary

Canine oral papillomavirus (COPV) infection is used in vaccine development against mucosal papillomaviruses. The predictable, spontaneous regression of the virally-induced papillomas makes this an attractive system for analysis of the cellular immune events associated with wart clearance. A panel of monoclonal antibodies against canine immune cells was used to determine the timing and phenotype of immune cell infiltration throughout the wart life cycle in experimental infections. Influx of lymphocytes coincided with the onset of wart regression. Both CD4+ and CD8+ cells were present early on in large numbers, with CD4+ cells being the most numerous. Lymphocytes were concentrated at the dermo-epidermal interface, and keratinocyte apoptosis was prominent at the onset of regression. These findings, along with the results of immunohistochemistry using TCRαβ, TCRγδ, CD1a, CD1c, CD11a, CD11b, CD11c, CD18, CD21and CD49d-specific monoclonal antibodies, were compared with similar work in the human, ox and rabbit models. There were important differences between these systems. Unlike bovine papillomavirus lesions, those of COPV did not have a significant gamma/delta T-cell infiltrate. Furthermore, COPV lesions had numerous CD4+ cells, unlike regressing cottontail rabbit papillomavirus lesions. The lymphocyte infiltrate in the dog was similar to that described in human papillomavirus lesions. The results demonstrate that CD4+ and CD8+ cell influx marks the onset of wart regression, and indicate that COPV is valid as a model system for studies of papillomavirus immunity.

Introduction

Papillomaviruses have been associated with numerous benign lesions including warts of the skin, oral cavity, larynx and anogenital region (Shah & Howley, 1996). Some of the lesions regress spontaneously but others prove refractory to treatment (Beutner & Ferenczy, 1997). The impact of benign papillomavirus infections is increased in people immunosuppressed either therapeutically or from HIV infection (Palefsky *et al*., 1998). In addition to the benign or low-risk human papillomavirus (HPV) infections of the genital tract (e.g. HPV-6 and -11), there are papillomavirus types associated with a high risk of progression to malignancy (e.g. HPV-16, -18, -31). The association between these high-risk papillomaviruses and the development of cervical carcinoma is strong enough for HPV-16 and HPV-18 to be defined as carcinogens (Anonymous, 1996) and it now seems possible that all cases of cervical cancer may be associated with HPV (Walboomers *et al*., 1999). The high frequency of papillomavirus infections, the severity of the lesions, and the inadequacy of current therapies have led to intense efforts to understand the biology and immunity associated with these viruses. Because of the species and tissue specificity of papillomaviruses, and their requirement for differentiating epithelia in order to complete the life cycle (Stanley, 1994a, Stanley, 1994b), only recently have methods been developed for their propagation *in vitro* (Frattini *et al*., 1996, Meyers *et al*., 1992, White *et al*., 1998). Despite this, there remains a need for *in vivo* studies of papillomavirus biology and host immunity, indeed animal models have been described recently as the "gold standard" by which *in vitro* models should be assessed (Chow & Broker, 1997). In human papillomavirus infections, the impossibility of knowing when an infection began and when a wart is about to regress makes examination of the events in regressing lesions difficult. Clinical biopsies of isolated regressing warts provide some information on the cellular immune response, but represent only single

snapshots of regression and so are difficult to interpret. Whilst it is possible to compare groups of warts in advanced regression with stable or progressing warts, studies such as these do not provide a complete chronological picture of wart regression. Animal models of mucosal papillomatosis, such as the canine and bovine models (reviewed in Campo, 1997, Nicholls & Stanley, 1999, Nicholls & Stanley, 2000) provide an opportunity to obtain a chronological series of biopsies spanning the entire wart life cycle from infection through to resolution. Canine oral papillomavirus (COPV) is of particular interest because experimentally-induced papillomas develop after only 4 to 8 weeks, and the resulting mucosal papillomas undergo rapid and predictable regression after maturity. Since the dog forms such an effective immune response to mucosal papillomavirus infection, knowledge of these events may lead to a better understanding of the key features required to clear persistent papillomavirus lesions, such as those seen in some HPV infections. COPV resembles some benign HPV infections, such as recurrent laryngeal papillomatosis, in its ability to cause persistent and recurrent infections in certain individuals (Nicholls *et al*., 1999). Furthermore, the dog has been used as a key model in the development of papillomavirus vaccines, including those based on heterologous wart extracts (Bell *et al*., 1994, Chambers *et al*., 1960), L1 virus-like particles (Ghim *et al*., 1995) and L1-encoding DNA vaccines (our unpublished observations). Clearly, to validate the canine model, it is important to establish whether the morphological events in regressing canine oral papillomas are similar to those seen in regressing human mucosal papillomas.

With the recent availability of an increasing number of immunological reagents for use in the dog (Moore & Rossitto, 1993, Moore *et al*., 1990, Moore *et al*., 1992,

Moore *et al*., 1994b), the immunology of canine oral papillomatosis is more amenable to evaluation. The aim of this study was to understand more clearly the immunological events in a mucosal papillomavirus model, especially with respect to the timing of leucocyte influx in relation to wart progression and regression. Weekly biopsies taken from experimental COPV infections allowed a prospective longitudinal immunohistochemical evaluation of the events occurring during wart progression and regression. The data indicate that an influx of both CD4+ and CD8+ lymphocytes begins just prior to wart regression, with maximum leucocyte influx correlating with rapid wart resolution.

Methods

Experimental infection

Two female beagles aged 10-12 weeks, obtained from a colony with no history of COPV infection, were housed in purpose-built, climate-controlled accommodation. To minimise the chances of cross-infection to and from other dogs in the unit, the dogs were housed in an airspace separate from other dogs in the building, with a virucidal (Virkon, Antec International, Suffolk, UK) footbath at the common entrance and exit. All staff changed their protective clothing (gown, gloves, boots) on entering the controlled area.

After a 10 day acclimatisation period, followed by overnight withdrawal of food, animals were examined and weighed before premedication by intramuscular injection of acepromazine maleate (0.03 mg/kg) as a sedative, and buprenorphine hydrochloride (10 µg/kg) as an analgesic. Anaesthesia was induced by intravenous sodium thiopentone (2.5%) at approximately 10 mg/kg to effect and maintained with

halothane and oxygen. The mucosa of the upper lip was lightly scarified over an area approximately 5 mm x 5 mm, using the tip of a scalpel blade, until a light ooze of blood occurred. An aliquot of 10 µl of homogenised canine oral papilloma extract (Bell *et al*., 1994), kindly donated by A. Bennet Jenson, was applied to each site by pipette and allowed to absorb for a few minutes. The site of challenge was marked by Indian ink tattoo points in a triangle centred on the site. Twelve sites were infected in each dog. Control blood samples and biopsies were taken prior to challenge and weekly thereafter. The maximum wart diameter was measured each week. At the end of the study, the animals were re-homed.

Blood sampling and mucosal biopsy preparation

Under general anaesthesia and after pre-infection control biopsies, further weekly biopsies of the oral mucosa were taken using a 6 mm biopsy punch or scalpel followed by suturing as appropriate. Where gross lesions had not yet developed, tissue was taken from the tattoo-marked sites. The biopsies were split and either fixed in 10% neutral buffered formalin or embedded in optimal cutting temperature compound (OCT, BDH) and frozen in isopentane cooled to its freezing point in liquid nitrogen. Formalin-fixed samples were processed after 4-8 hours to paraffin wax for routine (5-7 µm) sectioning. Frozen tissue was stored at -70ºC prior to cryostat sectioning. Blood samples were taken from the jugular vein during anaesthesia, and the serum frozen at -20ºC.

Immunohistochemistry of serial biopsies

Cyostat sections (7 µm) were fixed in acetone for 2 minutes, then immersed in 0.3% hydrogen peroxide in 0.1% w/v sodium azide for 10 minutes. After rinsing in PBS for 3 minutes, the sections were incubated in 10% normal goat serum in PBS for 30 minutes. All further incubations were for 30 minutes at room temperature in a humidified box. The blocking serum was tipped and blotted off before addition of the 1/10 diluted primary antibody (Table 1). Sections were washed in PBS (3 x 5 min.) before incubation with the 1/400 diluted biotinylated goat anti-mouse IgG1 or IgG2a (Amersham). After further washes in PBS (3 x 5 minutes), the sections were incubated with peroxidase-conjugated avidin-biotin complex (Vectastain Elite, Vector Laboratories) and developed with DAB (0.06% 3,3' diaminobenzidine tetrahydrochloride, 0.01% hydrogen peroxide in 0.1M Tris pH 7.5) according to the manufacturer's instructions. Sections were counterstained with Carazzi's haematoxylin and mounted in di-butyl-polystyrene-xylene (DPX). Frozen sections from lymph nodes were used as positive controls. Omission of the primary or secondary antibody was used as a negative control.

Cell counting from immunostained sections

Sections were examined by light microscopy with a x25 objective. Positive-staining cells were counted throughout the epidermis and the uppermost field of underlying dermis using an eyepiece graticule. All fields extending horizontally from one edge of the biopsy to the other edge were counted. Each field measured 400 µm square. Where cells within a graticule subdivision (40 µm square) were so abundant and closely packed that they were inumerable, the subdivision was allocated a count of ten

cells. The counts were repeated on two or more different sections and the mean count per field was calculated.

ELISA

Flexible PVC microplates were coated with 100 ng per well of purified COPV virions 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 minutes. Following further washing, 50 μl aliquots of tenfold serum dilutions, from samples taken at 0, 7, 8 and 11 weeks post-infection, 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 s-phenylene diamine/peroxide substrate in urea buffer (Sigmafast). Colour development was stopped with 20% sulphuric acid and absorbance levels were read at 490 nm using a Molecular Devices plate reader. The reciprocal of the $\log_{10}$ dilutions was plotted against the absorbance at 490 nm.

Results

To investigate the events during regression of mucosal papillomas, we obtained a chronological series of biopsies from beagles experimentally infected with canine oral papillomavirus. Histological sections from formalin-fixed biopsies enabled

morphological examination of papilloma regression, and cryostat sections from snap-frozen biopsies permitted immunophenotypical analysis of the infiltrating cells.

Wart development and regression

Scarified sites healed completely by one week and the dogs had no signs of discomfort, such as reluctance to eat, drink or play with their toys. No macroscopic lesions were found for the first four weeks after infection, so biopsies were taken randomly from the tattoo-marked infection sites. From week five post-infection, lesions were visible on the oral mucosa at the sites of infection which had not already been biopsied. The size of the lesions was recorded (Fig. 1) to enable correlation with leucocyte influx. The first lesions were raised, focal, smooth, domed, single or multiple masses similar in colour to surrounding mucosa. Lesions then increased in size, becoming more pale and firm compared with surrounding mucosa. The surface became irregularly textured and by eight weeks mature papillomas with multiple projecting papillae were evident. In the mature eight-week warts, apoptotic keratinocytes were common, and in places the tips of wart papillae had undergone focal coagulative necrosis. A prominent lymphocytic infiltrate was present in the regressing warts, and in many places the infiltrate obscured the dermo-epidermal interface, accompanied by apoptotic keratinocytes (Fig. 2). Both intracellular and intercellular oedema were present in keratinocytes within regressing lesions. The warts began to soften by week nine, and had sloughed or regressed leaving only a raised base by week 10. By week 11 there were no obvious signs of infection, and the histological appearance was similar to that of normal canine oral mucosa. No papillomas developed other than at inoculated sites.

Immunohistochemistry of regressing lesions

Preliminary studies on formalin-fixed, paraffin-embedded tissues (Nicholls & Stanley, 1996), using a CD3ε polyclonal antibody (DAKO), confirmed the presence of numerous T cells within regressing canine oral papillomas. To more accurately phenotype the inflammatory infiltrate and to establish the timing of leucocyte influx in relation to wart regression, a chronological series of biopsies was obtained from beagles infected experimentally with COPV. Frozen sections from weekly biopsies were processed for immunohistochemistry. Pre-infection control biopsies were used to establish the number of cells in normal tissues.

In pre-infection controls, small numbers of lymphocytes were present in the epithelium. Only a few CD4+ cells were present in the epithelium, with a few perivascular cells in the lamina propria. A few CD8α+ and CD8β+ cells were present along the basal layer of the epithelium, occasionally in the lamina propria too. TCRαβ+ cells had a similar distribution. TCRγδ+ cells were scarce, with only an occasional cell found within the epithelium. Antibodies to CD1a, CD1c, CD11a and CD11c detected dendritic cells both within the epithelium and lamina propria, whereas antibodies to CD11b detected dendritic cells in only the lamina propria. CD18+ cells were present in both epithelium and lamina propria. CD21+ cells were scarce and located in the lamina propria.

No changes in staining patterns were seen over the first six weeks. By week seven, foci of inflammation were seen at the edges of the immature papilloma. At this stage, the central region of the papilloma had very few leucocytes or dendritic cells, compared with the inflamed edges and the adjacent normal epithelium. The

inflammatory foci were positive after immunostaining for CD4, concentrated mostly in the lamina propria. Increased staining for CD8α, CD8β, TCRαβ and CD49d was seen within both lamina propria and epithelium. Both CD1a and CD1b showed focal increases in staining in this region, although the effect was not marked. Focal increases in number of CD11a, CD11c and CD18 positive cells were seen, distributed in epithelium and lamina propria, whereas CD11b staining was concentrated in the lamina propria. Although focal increases in staining of CD1 and CD11 antibodies were seen, the total counts in the sections were not obviously different from those in earlier weeks. No increase in TCRγδ or CD21 staining was seen.

The amount of staining for CD4, CD8, TCRαβ, TCRγδ, CD18 and CD49d further increased during week eight (Fig. 3). CD4+ cells were the most abundant and were at their maximal levels at this week. TCRαβ, TCRγδ, CD18 and CD49d were also at their peak number at week eight, but were less numerous than CD4+ cells.

At week nine, CD4, CD8, TCRαβ, and CD18 -positive cells remained numerous, with CD8+ and TCRγδ+ cells reaching their peaks. At this point, the amount of staining for CD1a, CD1c, CD11a, CD11b, CD11c and CD21 peaked also. The number of TCRγδ+ CD79a and CD21+ cells was comparatively small, comprising below 10% of the activity of any other antibody. The wart tissue had almost completely regressed by week nine and there was ulceration over a large fraction of the epithelium. The ulceration and loss of epithelium made it difficult to determine the location of cells in densely inflamed areas.

By week 10, the intensity of staining with most of the antibodies had subsided markedly, although CD18 and CD49d were still prominent. By week 11, all antibodies

showed levels of staining indistinguishable from the pre-infection control sections. The cell counts are summarised in Fig. 4.

ELISA

The serum sample taken as a pre-infection negative control showed low levels of reactivity to COPV viral particles. Samples taken seven and eight weeks after infection showed similarly low levels of anti-COPV IgG antibody. By week 11, however, a marked increase in titre to COPV virions was seen (Fig. 5).

Discussion

The spontaneous regression of COPV infections described here is typical of lesions induced by this virus, with only very rare exceptions (Nicholls *et al*., 1999). The marked increase in COPV-specific IgG by week 11 after infection is typical of the response to COPV. Although COPV L1 capsid protein was detected immunohistochemically in small amounts at week 7 and large amounts at week 8 (data not shown), there is clearly a lag before significant levels of COPV-specific IgG are detectable in the circulation. The late expression of viral L1 protein, and its expression in only superficial keratinocytes of the stratum granulosum, may help the virus evade the immune response by maintaining a state of immune ignorance until the viral life cycle is nearing completion. Immunity to re-infection seems solid in the dog, although, as with other papillomavirus infections, it seems that COPV may enter a state of latency, since we have detected COPV DNA by PCR in oral mucosa after spontaneous lesion regression (unpublished data). The immunohistochemical analyses showed a prominent T-cell infiltrate in regressing canine oral papillomas. Previous

work in the rabbit (Okabayashi *et al*., 1991, Selvakumar *et al*., 1997), ox (Knowles *et al*., 1996) and human (Coleman *et al*., 1994) has demonstrated increased lymphocytes in regressing warts compared with progressing warts, but the chronological analysis described here in the dog has allowed a more detailed examination of these events. In the dog, the influx of lymphocytes began just prior to wart regression, and became maximal during lesion resolution, before returning to pre-infection levels when the lesions had resolved. Although similar increased leucocyte numbers have been reported in other species, some important differences are present. In regressing cottontail rabbit papillomavirus (CRPV)-induced papillomas, dense T-lymphocyte infiltrates were seen within the basal region of the epidermis and in adjacent dermis (Okabayashi *et al*., 1991). This distribution was similar to that seen in COPV lesions, in which the infiltrate often obscured the interface zone. This pattern of interface dermatitis is typical of immune-mediated skin disease in the dog and human. In contrast to COPV lesions, the infiltrate in CRPV warts comprised predominantly CD8+ lymphocytes within the basal and suprabasal layers of epithelium (Selvakumar *et al*., 1997) with no CD4+ cells demonstrable. The scarcity of CD4+ cells in the CRPV lesions is noteworthy, considering their abundance in the COPV lesions. This may represent a true biological difference between rabbit and other animals including dog and human. However, whilst the anti-rabbit CD4 antibody was reported to work well on spleen sections, it was described as being non-specific on the papilloma sections, suggesting that further work in the rabbit may be required to address this issue. Numerous apoptotic keratinocytes were demonstrable in regressing COPV lesions, with occasional necrosis of the tips of filiform epithelial papillae. It seems that keratinocyte apoptosis may play an important role in lesion regression, since in this study it correlated both spatially and temporally with lymphocytic infiltration and

wart regression. No obvious necrosis was seen associated with the cellular infiltrate in CRPV lesions (Selvakumar *et al*., 1997) in which it was thought that the CD8+ cells within the epithelium were releasing cytokines such as IL-2 or interferon-γ, reducing viral spread. Secreted cytokines may have attracted leucocytes into the dermis, where they released TNFα (Hagari *et al*., 1995), perhaps reducing epidermal growth and viral gene expression.

CRPV affects haired skin, so the results obtained in the dog need comparing with another mucosal papillomavirus infection. Bovine papillomavirus type 4 (BPV-4) provides such an opportunity. In fact there were some important differences from the data obtained in the rabbit and dog. The infiltrate in regressing BPV-4 papillomas had numerous CD4+ cells in the dermis (Knowles *et al*., 1996), a finding confirmed here in the COPV lesions. In the more superficial layers of the bovine epithelium there were more CD8+ than CD4+ cells, whilst the basal layers of epithelium had similar numbers of CD4+ and CD8+ cells. There were increased TCRγδ+ cells in the superficial epithelium in BPV-4 lesions. The COPV lesions did have an increase in TCRγδ+ cells, although these formed only a tiny fraction of the total infiltrate.

In the bovine lesions, lymphocyte numbers correlated with regression, with CD4+ cells being the most numerous type (Knowles *et al*., 1996). The predominance of CD4+ cells seen also with COPV lesions suggests they are playing a key role, perhaps with T_H1 CD4+ cells activating macrophages, or by cytokine-mediated inhibition or killing of infected keratinocytes. The timing of the CD4+ cell appearance seen in the dog is consistent with their playing a primary role, since they appear just before the onset of regression. This contrasts with the situation seen for CD1+, CD11a-c+ and CD21+ cells, which peaked only once regression was underway. This suggests a

secondary function for these cells, perhaps responding to secondary infection from the loss of epithelial integrity, or responding to tissue necrosis in the regressing wart.

The CD4+ cells in regressing BPV-4 papillomas were present mostly as sub-epithelial clusters within the dermis, sometimes surrounded by CD8+ and TCRγδ+ cells, but migrating more into the epithelium once the basal lamina had been breached (Knowles *et al*., 1996). Although sub-epithelial foci of CD4+ cells were seen in the canine lesions, the pattern described for BPV-4 lesions was not prominent. A major departure from the findings noted in BPV-4 lesions was the paucity of TCRγδ+ cells in COPV lesions. In the dog, gamma/delta T cells appear to play an important role in various infectious, immune-mediated, inflammatory and metabolic skin diseases (Cannon *et al*., 1998), and the majority of canine epitheliotropic cutaneous T cell lymphomas are of gamma/delta phenotype (Moore *et al*., 1994a). Normal canine oral mucosa has increased numbers of gamma/delta T cells compared with normal canine haired skin (Cannon *et al*., 1998) although, from the findings reported here, their role in clearance of papillomavirus infections seems minor, at least with respect to the number of cells involved. Ruminants have abundant TCRγδ+ cells, and these were more numerous than CD8+ cells in the BPV-4 lesions. Immunostaining for the interleukin-2 receptor, an indicator of T cell activation, showed that half of the CD4+ and CD8+ cells, and three quarters of the TCRγδ+ cells, were positive (Knowles *et al*., 1996).

Numerous lymphocytes are seen in regressing human anogenital warts (Coleman *et al*., 1994). The scarcity of B-cells (CD21+) seen in COPV lesions was noted also for the HPV lesions. An increase in number of both CD4+ and CD8+ cells was common to regressing lesions of both COPV and HPV, as was an increase in the number of

intraepithelial CD4+ cells during regression. Although lymphocytes were the most common infiltrating cell in the regressing HPV lesions, macrophages were seen also. No changes in Langerhans cell number were seen in the regressing HPV lesions, although dendrites appeared blunted. A different study reported a reduction in CD1a+ Langerhans cell number in low and high grade cervical disease, compared with normal cervical epithelium (Mota *et al*., 1998). The Langerhans cells remaining in cervical disease appeared to upregulate HLA-DQ expression. The COPV lesions had a late increase in Langerhans cells and dermal dendritic cells, noted after regression had started. Interestingly, week seven canine papillomas appeared to have fewer Langerhans cells within the wart epithelium compared with adjacent normal epithelium, although this effect was not reflected in the mean for the section. The reduced numbers of Langerhans cells might have been due to their activation and migration to the draining lymph nodes.

The dog is a useful model of human immunity, playing an important role in toxicity trials and development of drugs acting on the immune system. Transplant biology also has been investigated extensively using a canine model. Dogs develop a spectrum of immunological diseases similar to that seen in humans, and therefore act as a key large animal model for immunology and immunopathogenesis. Previously, there has been only a limited number of canine immunological reagents, although the situation has now changed due to the increasing availability of reagents for use in the dog (reviewed in Williams, 1997). It is important to exercise caution in extrapolating data from animal to human models. For example, the immune system of the ruminant has important differences from that of humans. In humans, gamma/delta T-cells form only a minority of the peripheral T-lymphocyte pool, whereas they are present in strikingly

increased numbers in the peripheral blood of ruminants, especially young cattle (Hass *et al*., 1993, Hein & Mackay, 1991, Wyatt *et al*., 1994). Additionally, the normal white cell count of cattle is different from that of humans, and generally comprises fewer neutrophils, more lymphocytes, and more eosinophils (Hope, 1998, Jain, 1993). Canine normal white cell counts fall within the normal ranges quoted for humans (Hope, 1998, Jain, 1993) and in the dog, the low numbers of gamma/delta T-cells in normal haired skin (Cannon *et al*., 1998) are similar to the findings reported in humans (Alaibac *et al*., 1992). In the rabbit also, the gamma/delta T cell proportion in peripheral blood (23%) is relatively high compared with the human (Sawasdikosol *et al*., 1993). The bovine neutrophil has a unique third type of cytoplasmic granule and, unlike human neutrophils, lacks lysozyme (reviewed in Roth, 1994). The biology of bovine mast cells differs also from that in the human (reviewed in Pastoret, 1998). Furthermore, the placental structure of cattle is different from that of humans and other species. The inability to transfer immunoglobulins across the bovine placenta means that the ox differs from the human in the composition of colostrum and the leukocyte distribution in neonatal peripheral blood (reviewed in Pastoret, 1998). Although the rabbit continues to play an important role in studies of papillomavirus immunity and vaccine development, it should be remembered that B-cell diversification in the rabbit, unlike that in other mammals, occurs in the lymphoid tissue of the appendix in a manner analagous to that occurring in the Bursa of Fabricius in birds (Pastoret *et al*., 1998).

In summary, these data showed that a predominantly T-cell infiltrate appeared predominantly at the dermo-epidermal interface just prior to morphological regression, becoming maximal during the period of rapid wart regression. CD4+ cells

were more abundant than CD8+ cells, and keratinocyte apoptosis was common during regression. These observations support the findings reported for regressing human anogenital warts (Coleman *et al*., 1994) and confirm that the canine system is likely to be a good model for papillomavirus immunology studies and vaccine development. Since the response was clearly very effective in clearing mucosal papillomas, effective therapeutic strategies should aim at stimulating this type of immunity.

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Figure legends

Fig. 1. Wart progression and regression after experimental infection. The maximum wart diameter was measured each week. Infections in Dog 1 (●) and Dog 2 (■) showed a similar growth pattern. Warts were visible five weeks after infection, and grew rapidly to reach their greatest diameter at eight weeks. Regression was rapid, with the lesions disappearing by ten weeks post-infection.

Fig. 2. Histopathology of wart regression. Regressing papillomas were accompanied by numerous apoptotic keratinocytes (a), identifiable by their intensely eosinophilic cytoplasm and dense, fragmented nucleus (arrowhead). A dense lymphocytic infiltrate obscured the dermo-epidermal interface (b). Haematoxylin and eosin. Original magnification (a) x 200, (b) x 100.

Fig. 3. Immunohistochemistry of COPV lesions. Only small numbers of CD8α+ (a) and CD4+ (c) lymphocytes are found in normal canine oral mucosa. Eight weeks after infection, regressing COPV lesions contain increased numbers of both CD8α+ (b) and CD4+ (d) lymphocytes. Original magnification (a, c) x 200, (b, d) x 100.

Fig. 4. Cell counts during wart progression and regression. The mean cell count per field for each cell type shown is plotted against time after infection with COPV. In H&E stained sections, an increase in infiltrating lymphocytes can be seen from week 8, at the onset of wart regression. Quantitative immunohistochemistry reveals that CD4+ lymphocytes reached maximum number at this time. For clarity, counts for the scarce TCRgd, CD21 and CD79a cells are plotted on a different scale

Fig. 5. COPV ELISA of sequential serum samples during experimental infection. Pre-infection control serum (●)had low levels of antibody to native COPV virions. Although viral L1 capsid protein can be detected immunohistochemically in lesions at week 7 (■) and week 8 (▲), no increase in capsid antibody is detectable at these points. By week 11(□), after wart regression, the serum contains high levels of antibody to native COPV virions.

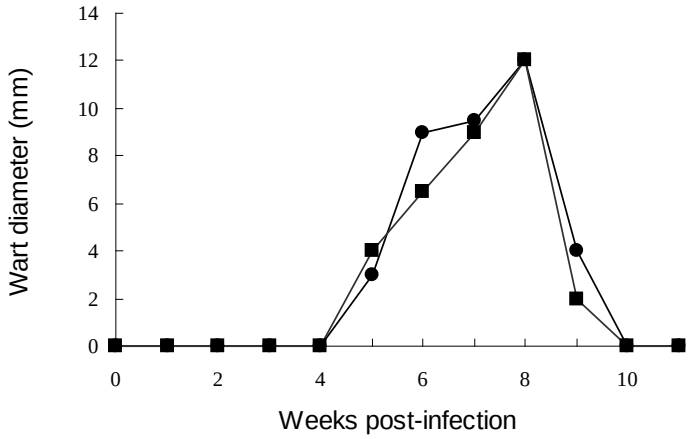
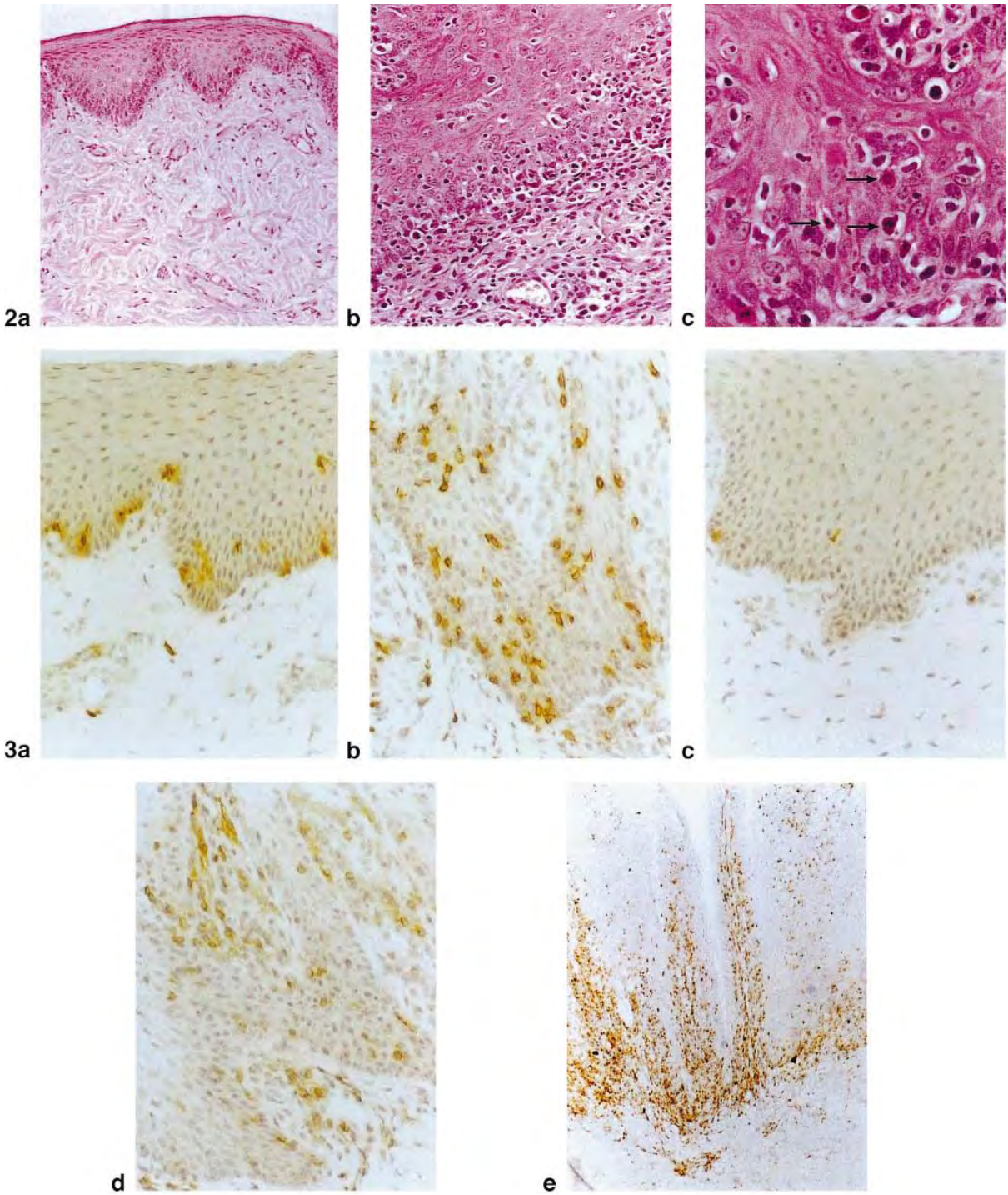


FIG. 1. Wart progression and regression after experimental infection. Infections in both animals showed a similar growth pattern. The maximum wart diameter was measured each week. Warts were visible 5 weeks after infection and grew rapidly to reach their greatest diameter at 8 weeks. Regression was rapid, with the lesions disappearing by 10 weeks postinfection. Infections in both animals showed a similar growth pattern.



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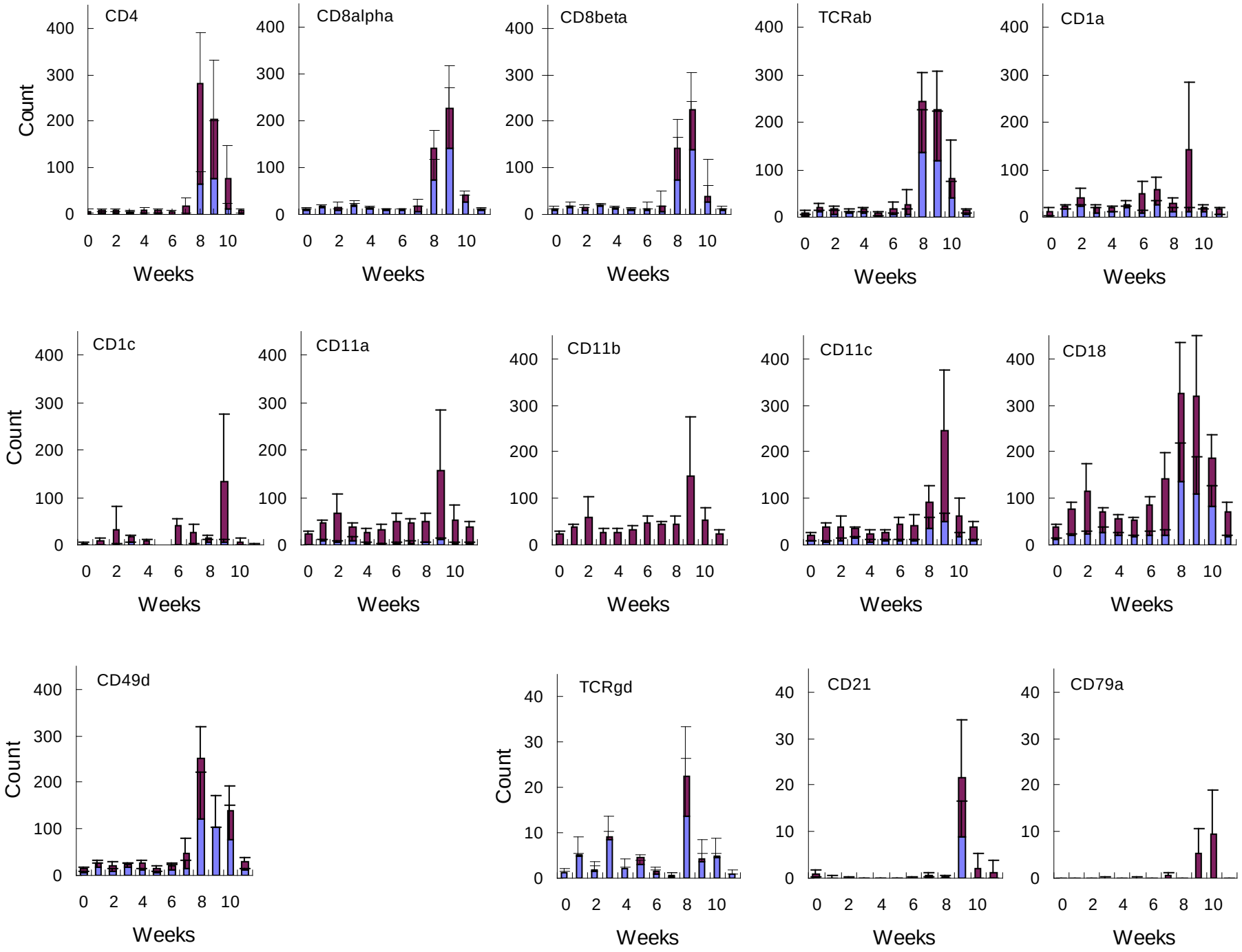
CD4
CD8alpha
CD8beta
TCRab
CD1a
CD1c
CD11a
CD11b
CD11c
CD18
CD49d
TCRgd
CD21
CD79a
Count
Weeks
0
2
4
6
8
10
100
200
300
400
10
20
30
40

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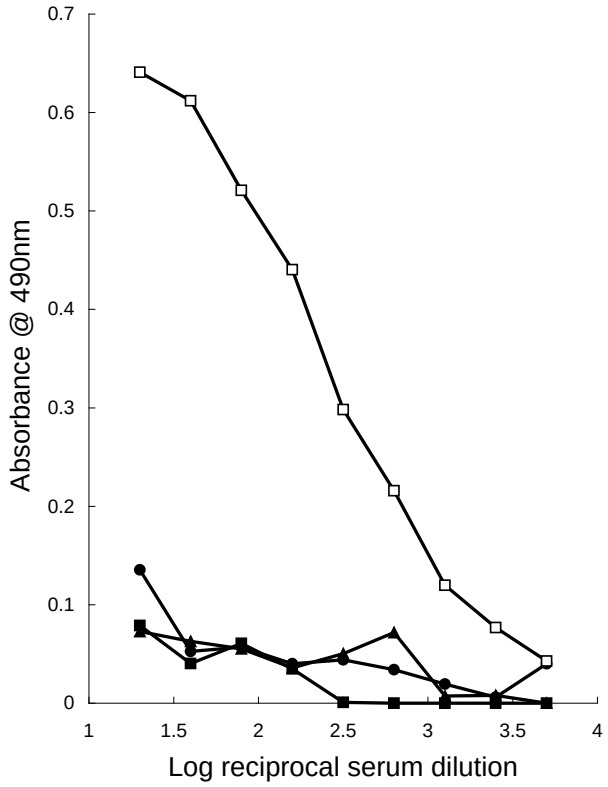

TABLE 1. Origin and specificity of primary antibodies

Clone	Isotype	Specificity and references
CA15.8G7	IgG1	TCRαβ, T-cell receptor, major subset (Moore & Rossitto, 1993)
CA20.8H1	IgG2a	TCRγδ, T-cell receptor, minor subset (Moore *et al*., 1994b)
CA9.AG5	IgG1	CD1a, Langerhans cells, dermal dendritic cells (Danilenko *et al*., 1992, Marchal *et al*., 1995, Moore *et al*., 1996)
CA13.9H11	IgG1	CD1c, Langerhans cells, dermal dendritic cells (Danilenko *et al*., 1992, Marchal *et al*., 1995, Moore *et al*., 1996)
CA13.1E4	IgG1	CD4, Helper T_H2 & inflammatory T_H1 T cells, neutrophils (Moore *et al*., 1992)
CA9.JD3	IgG2a	CD8α, MHC-I co-receptor, cytotoxic T-cells (Cobbold & Metcalfe, 1994, Moore *et al*., 1992)
CA15.4G2	IgG1	CD8β, MHC-I co-receptor, cytotoxic T-cells (Cobbold & Metcalfe, 1994, Moore *et al*., 1992)
CA11.4D3	IgG1	CD11a, LFA-1, granulocytes, monocytes, T-cells, NK-cells, Langerhans cells, follicular dendritic cells, Kupffer cells (Danilenko *et al*., 1992, Marchal *et al*., 1995, Moore *et al*., 1996)
CA16.3E10	IgG1	CD11b, Mac-1, granulocytes, monocytes, some lymphocytes (NK cells?), not Langerhans cells (Danilenko *et al*., 1992, Moore *et al*., 1996)
CA11.6A1	IgG1	CD11c, monocytes, Kupffer cells, granulocytes, Langerhans and dermal dendritic cells (Danilenko *et al*., 1992, Marchal *et al*., 1995, Moore *et al*., 1996)
CA1.4E9	IgG1	CD18, β2-integrins, thymocytes, most peripheral leucocytes, dermal and epithelial dendritic cells (Danilenko *et al*., 1992, Marchal *et al*., 1995, Moore *et al*., 1990)
CA2.1D6	IgG1	CD21, B- cells (Cobbold & Metcalfe, 1994, Moore *et al*., 1992)
CA4.5B3	IgG1	CD49d, α^4 integrin, VLA-4, activated Langerhans cells (Cobbold & Metcalfe, 1994, Moore *et al*., 1992)