Virology **283,** 31–39 (2001)
doi:10.1006/viro.2000.0789, available online at http://www.idealibrary.com on IDEAL®

Regression of Canine Oral Papillomas Is Associated with Infiltration of CD4+ and CD8+ Lymphocytes

Philip K. Nicholls,*[,1] Peter F. Moore,† Davina M. Anderson,* Richard A. Moore,* Nigel R. Parry,‡ Gerald W. Gough,‡ and Margaret A. Stanley*

**Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, United Kingdom; †Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California 95616; and ‡GlaxoWellcome Medicines Research Centre, Stevenage, Herts, SG1 2NY, United Kingdom*

Received September 20, 2000; returned to author for revision October 27, 2000; accepted December 6, 2000

Canine oral papillomavirus (COPV) infection is used in vaccine development against mucosal papillomaviruses. The predictable, spontaneous regression of the papillomas makes this an attractive system for analysis of cellular immunity. Immunohistochemical analysis of the timing and phenotype of immune cell infiltration revealed a marked influx of leukocytes during wart regression, including abundant $CD4^+$ and $CD8^+$ cells, with $CD4^+$ cells being most numerous. Comparison of these findings, and those of immunohistochemistry using TCR$\alpha\beta$-, TCR$\gamma\delta$-, CD1a-, CD1c-, CD11a-, CD11b-, CD11c-, CD18-, CD21-, and CD49d-specific monoclonal antibodies, with previously published work in the human, ox, and rabbit models revealed important differences between these systems. Unlike bovine papillomavirus lesions, those of COPV do not have a significant gamma/delta T-cell infiltrate. Furthermore, COPV lesions had numerous $CD4^+$ cells, unlike cottontail rabbit papillomavirus lesions. The lymphocyte infiltrate in the dog resembled that in human papillomavirus lesions, indicating that COPV is an appropriate model for human papillomavirus immunity.

Key Words: HPV; COPV; immunity; animal models; immunohistochemistry.

INTRODUCTION

Papillomaviruses have been associated with numerous benign lesions including warts of the skin, oral cavity, larynx, and anogenital region (Shah and Howley, 1996). Some of the lesions regress spontaneously but others prove refractory to treatment (Beutner and Ferenczy, 1997). The impact of benign papillomavirus infections is increased in people immunosuppressed 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 to complete the life cycle (Stanley, 1994a,b), 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 and 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. While 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 and Stanley, 1999), provide an opportunity to obtain a chronological series of biopsies spanning the entire wart life cycle from infection through to resolution. Canine oral papillomavirus is of particular interest because experimentally induced papillomas develop after only 4 to 8 weeks, and

[1] To whom reprint requests should be addressed at Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, South Street, Western Australia 6150. Fax: 61-(0)8-9310 4144. E-mail: nicholls@numbat.murdoch.edu.au.

0042-6822/01 $35.00

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 persisting papillomavirus infections, such as those seen in some HPV infections. Canine oral papillomavirus (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 viruslike 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 and Rossitto, 1993; Moore *et al.,* 1990, 1992, 1994), 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 leukocyte 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 leukocyte influx correlating with rapid wart resolution.

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 1 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 4 weeks after infection, so biopsies were taken randomly from the tattoo-marked infection sites. From week 5 postinfection, lesions were visible on the oral mucosa at the sites of infection not already biopsied. The size of the lesions was recorded

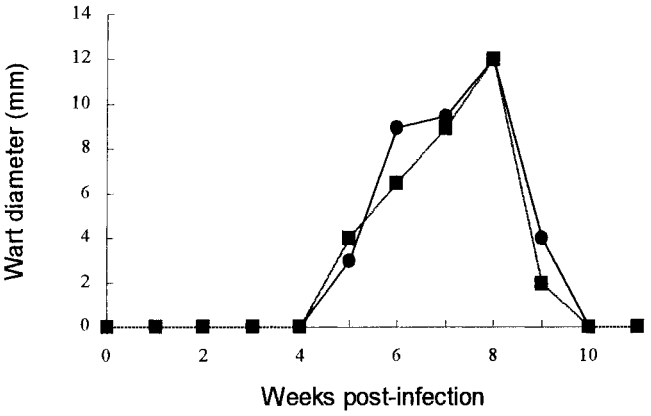

FIG. 1. Wart progression and regression after experimental infection. Infections in both animals showed a similar growth pattern. The maximum wart diameter was measured each week. Warts were visible 5 weeks after infection and grew rapidly to reach their greatest diameter at 8 weeks. Regression was rapid,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

(Fig. 1) to enable correlation with leukocyte 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 8 weeks mature papillomas with multiple projecting papillae were evident. In the mature 8-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 9 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 and 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 leukocyte 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. Preinfection control biopsies were used to establish the number of cells in normal tissues.

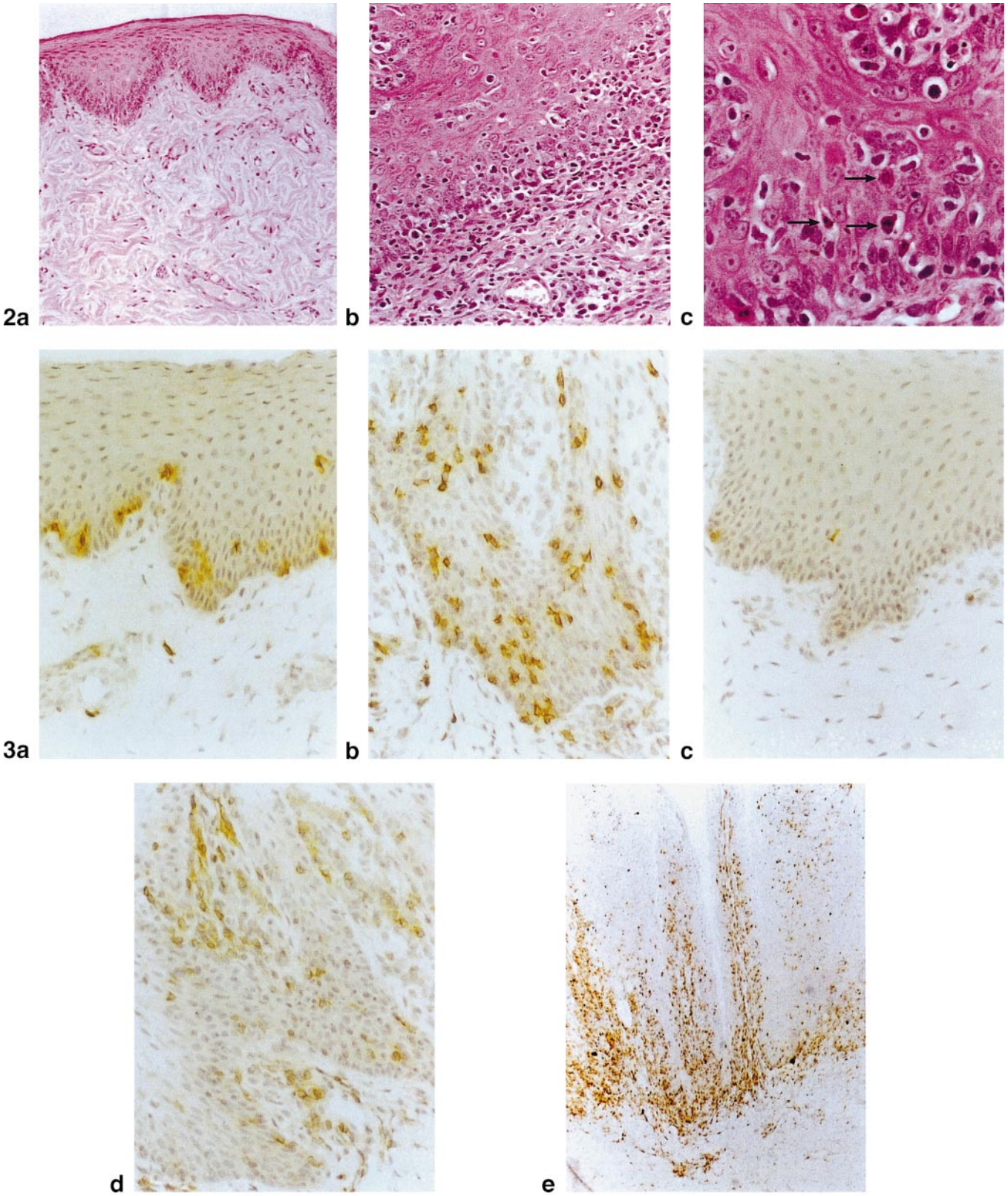

FIG. 2. Histological sections of regressing warts. Compared with normal canine oral mucosa (a), regressing papillomas were accompanied by a dense lymphocytic infiltrate obscuring the dermo-epidermal interface (b). Apoptotic keratinocytes were common (c). Haematoxylin and eosin. Original magnification (a) ×100, (b) ×200, (c) ×400.

FIG. 3. Immunohistochemistry of COPV lesions. Only small numbers of CD8α^+ (a) and CD4$^+$ (c) lymphocytes are found in normal canine oral mucosa. Nine weeks after infection, regressing COPV lesions contain increased numbers of both CD8α^+ (b) and CD4$^+$ (d) lymphocytes. At low magnification, the dense infiltrate of CD4$^+$ lymphocytes is seen to follow the dermo-epidermal interface (e). Original magnification (a–d) ×200, (e) ×40.

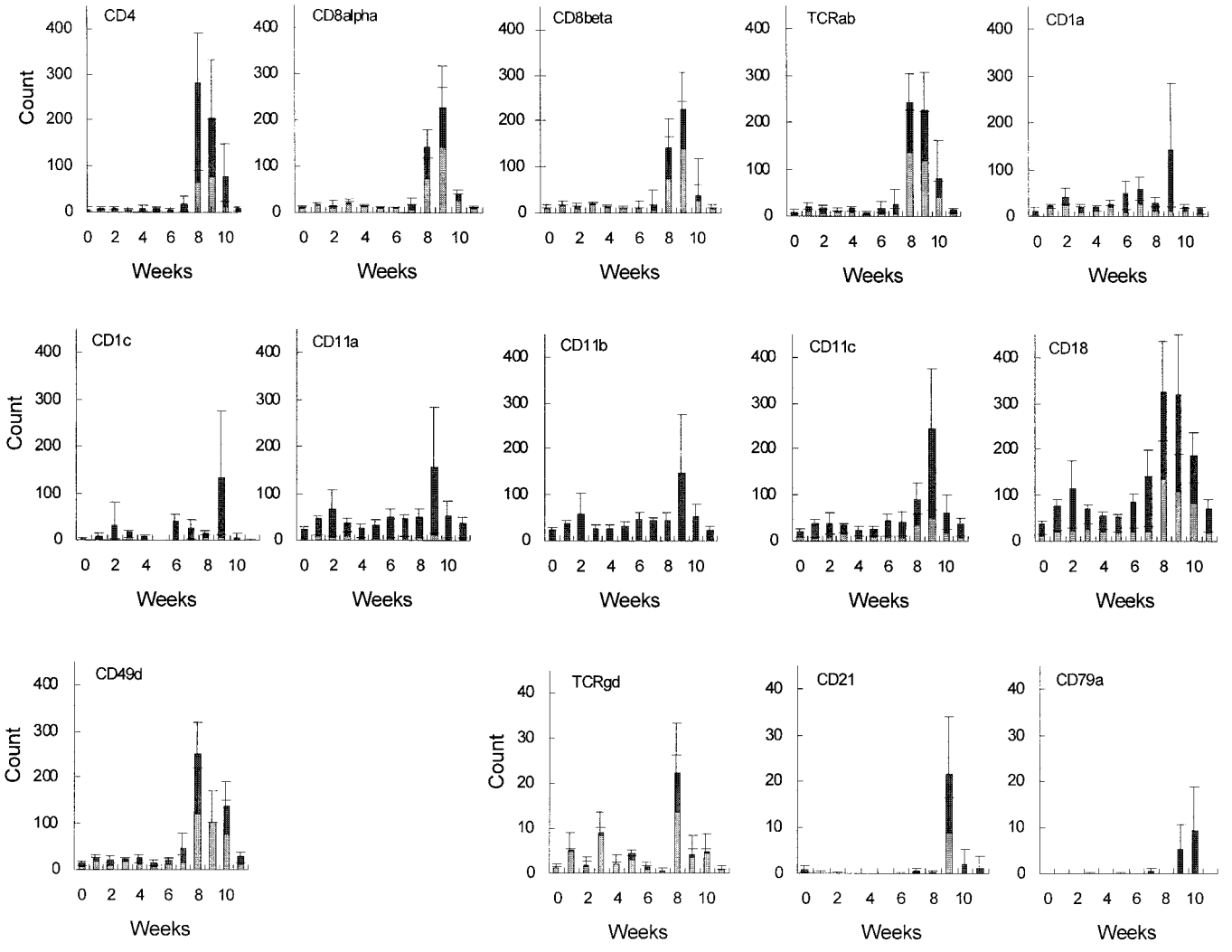

FIG. 4. Cell counts during wart progression and regression. Cell counts were undertaken on weekly biopsies stained with each of the antibodies shown. The mean count per field is plotted against time. In H&E stained sections, an increase in infiltrating lymphocytes can be seen from week 8. Quantitative immunohistochemistry reveals that $CD4^+$ lymphocytes reached maximum number at this time.

Examples of infiltrating lymphocytes in preinfection controls and regressing warts are given in Fig. 3. The cell counts are summarised in Fig. 4. In preinfection controls, only a few $CD4^+$ cells were present in the epithelium, with a few perivascular cells in the lamina propria. A few $CD8\alpha^+$ and $CD8\beta^+$ cells were present along the basal layer of the epithelium, occasionally in the lamina propria too. $TCR\alpha\beta^+$ cells had a similar distribution. $TCR\gamma\delta^+$ cells were scarce, with only an occasional cell found within the epithelium. Antibodies to CD1a, CD1c, CD11a, and CD11c detected dendritic cells within both the epithelium and the 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 6 weeks. By week 7, foci of inflammation were seen at the edges of the immature papilloma. At this stage, the central region of the papilloma had very few leukocytes 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 $CD8\alpha$, $CD8\beta$, $TCR\alpha\beta$, 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 and 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 $TCR\gamma\delta$ or CD21 staining was seen.

The amount of staining for CD4, CD8, $TCR\alpha\beta$, $TCR\gamma\delta$, CD18, and CD49d further increased during week 8 (Fig.

3). $CD4^+$ cells were the most abundant and were at their maximal levels at this week. TCR$\alpha\beta$, TCR$\gamma\delta$, CD18, and CD49d were also at their peak number at week 8, but were less numerous than $CD4^+$ cells.

At week 9, CD4-, CD8-, TCR$\alpha\beta$-, and CD18-positive cells remained numerous, with $CD8^+$ and TCR$\gamma\delta^+$ cells reaching their peaks. At this point, the amount of staining for CD1a, CD1c, CD11a, CD11b, CD11c, and CD21 peaked also. The number of TCR$\gamma\delta^+$ 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 9 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 preinfection control sections.

ELISA

The serum sample taken as a preinfection negative control showed low levels of reactivity to COPV viral particles. Samples taken 7 and 8 weeks after infection showed similarly low levels of anti-COPV IgG antibody. By week 11, however, a marked increase in titer to COPV virions was seen (Fig. 5).

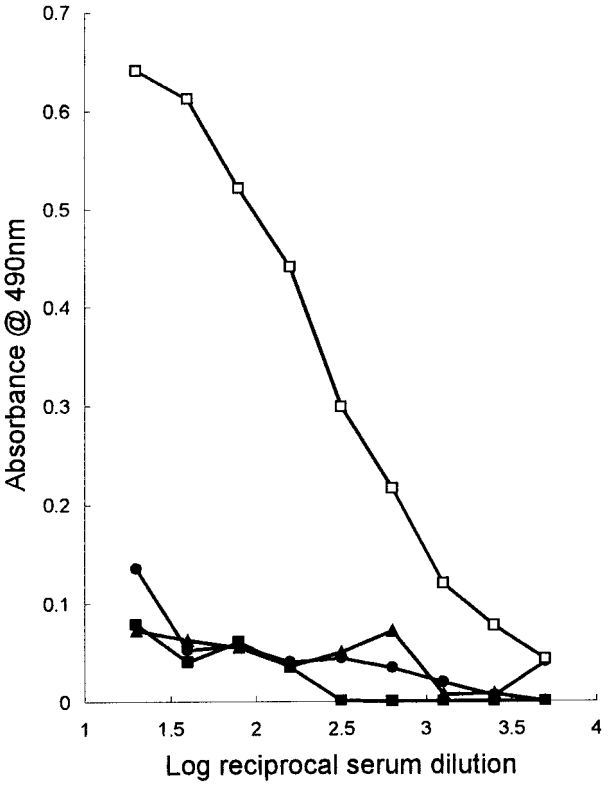

FIG. 5. COPV ELISA of sequential serum samples during experimental infection. Preinfection serum (●) had low levels of antibody to native COPV virions. Although viral L1 capsid protein can be detected immunohistochemically in lesions at weeks 7 (■) and 8 (▲) (data not shown), no increase in capsid antibody is detectable at these points. By week 11 (□), after wart regression, the serum contains high levels of antibody to native COPV virions.

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). 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. Immunity to reinfection 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 (our unpublished data).

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 regression, peaked during lesion resolution, and returned to preinfection levels when the lesions had resolved. Although similar increased leukocyte numbers have been reported in other species, some important differences are present, although care should be taken when comparing studies with different methodologies, due to possible differences in timing of the biopsies and sensitivity of the immunohistochemistry. 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, while the anti-rabbit CD4 antibody was reported to work well on spleen sections, it was described as being nonspecific on the papilloma sections, suggesting that further work in the rabbit may be required to address this

issue. It seems that keratinocyte apoptosis may play an important role in COPV 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).

CRPV affects haired skin and the results obtained in the dog need comparing with another mucosal papillomavirus infection. BPV-4 provides such an opportunity, and 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), as in the COPV lesions.

The predominance of $CD4^+$ cells in BPV and 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. $CD1^+$, CD11a–c^+, and $CD21^+$ cells, however, peaked only once regression was underway, perhaps responding to secondary infection from the loss of epithelial integrity, or responding to tissue necrosis in the regressing wart. This important difference in the timing of leukocyte influx is apparent only with frequent sequential biopsies and is an issue not addressed in previous studies.

The $CD4^+$ cells in regressing BPV-4 papillomas were present mostly as subepithelial clusters within the dermis, sometimes surrounded by $CD8^+$ and TCR$\gamma\delta^+$ cells, but migrating more into the epithelium once the basal lamina had been breached (Knowles *et al.,* 1996). Although subepithelial 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 TCR$\gamma\delta^+$ cells in COPV lesions. Ruminants have abundant TCR$\gamma\delta^+$ cells, and these were more numerous than $CD8^+$ cells in the BPV-4 lesions (Knowles *et al.,* 1996). 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.

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. 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 COPV lesions had a late increase in Langerhans cells and dermal dendritic cells, noted after regression had started. Although not reflected in the mean for the section, week 7 canine papillomas appeared to have fewer Langerhans cells within the wart epithelium compared with adjacent normal epithelium, suggesting activation and migration to the draining lymph nodes.

It is important to exercise caution in extrapolating data from animal to human models. Although there are important differences in the immune systems of dog and human, such as the expression of CD4 on canine neutrophils (Moore *et al.,* 1992), there is much overlap in cell surface antigen markers and cell function both in lymphocytes and in antigen presenting cells (Marchal *et al.,* 1995). Functional overlap is suggested also by the clinical and immunological similarities between canine and human immune-mediated diseases, including X-linked severe combined immunodeficiency (Felsburg *et al.,* 1998; Hartnett *et al.,* 2000), bullous pemphigoid (Xu *et al.,* 2000), systemic lupus erythematosus (Chabanne *et al.,* 1995), and gluten-sensitive enteropathy (Hall and Batt, 1990). Furthermore, the dog has a long history as a model for the development of organ transplantation in the human (Ladiges *et al.,* 1990; Starzl, 2000; Wagner *et al.,* 1999). 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 and 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 is relatively high compared with the human (Sawasdikosol *et al.,* 1993). 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 analogous to that occurring in the Bursa of Fabricius in birds (Pastoret *et al.,* 1998).

In summary, these data show that a predominantly T-cell infiltrate appeared mainly at the dermo-epidermal junction just prior to morphological regression, becoming maximal during the period of rapid wart regression. $CD4^+$ cells were more abundant than $CD8^+$ cells. Addi-

tionally, frequent biopsy appears to be required to avoid confusion of primary immune infiltrates with likely secondary inflammatory infiltrates. 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 human papillomavirus immunity 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.

MATERIALS AND 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 crossinfection to and from other dogs in the unit, all dogs in this study 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 with 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 × 5 mm, using the tip of a scalpel blade, until a light ooze of blood occurred. An aliquot of 10 μl of homogenized 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 rehomed.

Blood sampling and mucosal biopsy preparation

Under general anaesthesia and after preinfection control biopsies, further weekly biopsies of the oral mucosa were taken using a 6-mm punch biopsy 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 (BDH) cooled to its freezing point in liquid nitrogen. Formalin-fixed samples were processed after 4–8 h to paraffin wax for routine (5–7 μm) sectioning. Frozen tissue was stored at −70°C prior to cryostat sectioning. Blood samples were taken from the jugular vein during anaesthesia, and the serum frozen at −20°C.

Immunohistochemistry of serial biopsies

Cryostat sections (7 μm) were fixed in acetone for 2 min and then immersed in 0.3% hydrogen peroxide in 0.1% w/v sodium azide for 10 min. After rinsing in PBS for 3 min, the sections were incubated in 10% normal goat serum in PBS for 30 min. All further incubations were for 30 min 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 × 5 min) before incubation with the 1/400 diluted biotinylated goat anti-mouse IgG1 or IgG2a (Amersham). After further washes in PBS (3 × 5 min), the sections were incubated with peroxidase-conjugated avidin–biotin complex (Vectastain Elite, Vector Labs.) and developed with DAB (0.06% 3,3′ diaminobenzidine tetrahydrochloride, 0.01% hydrogen peroxide in 0.1 M Tris, pH 7.5) according to the manufacturer's instructions. Sections were counterstained with Carazzi's haematoxylin and mounted in di-butyl-polystrene-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 ×25 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^2. Where cells within a graticule subdivision (40 μm^2) were so abundant and closely packed that they were innumerable, the subdivision was allocated a count of 10 cells. The counts were repeated on two or more different sections and the mean count per field was calculated.

ELISA

ELISA was undertaken as previously described (Nicholls *et al.,* 1999) using 100 ng per well of purified COPV particles in carbonate buffer and 50 μl aliquots of 10-fold serum dilutions, from samples taken at 0, 7, 8, and 11 weeks postinfection, added to duplicate wells of the coated plates. After incubation and washing, peroxidase-conjugated, rabbit anti-dog IgG (1/500, ICN Immunobiologicals) was added to all wells. Following incubation and a final wash, the plates were developed using an *s*-phenylene diamine/peroxide substrate in urea buffer (Sigmafast). The reciprocal of the $\log_{10}$ dilutions was plotted against the absorbance at 490 nm.

TABLE 1

Origin and Specificity of Primary Antibodies

Clone	Isotype	Specificity and references
CA15.8G7	IgG1	TCR$\alpha\beta$, T-cell receptor, major subset (Moore and Rossitto, 1993)
CA20.8H1	IgG2a	TCR$\gamma\delta$, T-cell receptor, minor subset (Moore *et al.,* 1994)
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 and inflammatory T_H1 T cells, neutrophils (Moore *et al.,* 1992)
CA9.JD3	IgG2a	CD8α, MHC-I coreceptor, cytotoxic T-cells (Cobbold and Metcalfe, 1994; Moore *et al.,* 1992)
CA15.4G2	IgG1	CD8β, MHC-I coreceptor, cytotoxic T-cells (Cobbold and 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 and Metcalfe, 1994, Moore *et al.,* 1992)
CA4.5B3	IgG1	CD49d, α^4 integrin, VLA-4, activated Langerhans cells (Cobbold and Metcalfe, 1994; Moore *et al.,* 1992)

ACKNOWLEDGMENTS

This work was supported by the Biotechnology and Biological Sciences Research Council (Grant S08647). P.K.N. was supported by a BBSRC Veterinary Research Fellowship. The COPV wart homogenate was a generous gift from A. B. Jenson, Georgetown University, Washington, D.C. The monoclonal antibodies specified in Table 1 were from the laboratory of Peter F. Moore. Madeline Fordham and Andrea Kemp, Histology, Dept. of Clinical Veterinary Medicine, undertook the paraffin section preparation.

REFERENCES

Alaibac, M., Morris, J., Yu, R., and Chu, A. C. (1992). T lymphocytes bearing the gamma/delta T-cell receptor: A study in normal human skin and pathologic skin conditions. *Br. J. Dermatol.* **127,** 458–462.

Anonymous. (1996). "Human Papillomaviruses." IARC Monographs on the Evaluation of Carcinogen Risks to Humans, Vol. 64. World Health Organization International Agency for Research on Cancer, Lyon, France.

Bell, J. A., Sundberg, J. P., Ghim, S. J., Newsome, J., Jenson, A. B., and Schlegel, R. (1994). A formalin-inactivated vaccine protects against mucosal papillomavirus infection—A canine model. *Pathobiology* **62,** 194–198.

Beutner, K. R., and Ferenczy, A. (1997). Therapeutic approaches to genital warts. *Am. J. Med.* **102,** 28–37.

Campo, M. S. (1997). Vaccination against papillomavirus in cattle. *Clin. Dermatol.* **15,** 275–283.

Cannon, A. G., Olivry, T., Ihrke, P. J., Naydan, D. K., and Moore, P. F. (1998). Gamma delta cells in normal and diseased canine skin. *In* "Advances in Veterinary Dermatology" (K. W. Kwochka, T. Willemse, and C. von Tscharner, Eds.), Vol. 3, pp. 137–143. Butterworth and Heinemann, Oxford, U.K.

Chabanne, L., Fournel, C., Caux, C., Bernaud, J., Bonnefond, C., Monier, J. C., and Rigal, D. (1995). Abnormalities of lymphocyte subsets in canine systemic lupus erythematosus. *Autoimmunity* **22,** 1–8.

Chambers, V. C., Evans, C. A., and Weiser, R. S. (1960). Canine oral papillomatosis. II. Immunologic aspects of the disease. *Cancer Res.* **20,** 1083–1093.

Chow, L. T., and Broker, T. R. (1997). In vitro experimental systems for HPV: Epithelial raft cultures for investigations of viral reproduction and pathogenesis and for genetic analysis of viral proteins and regulatory sequences. *Clin. Dermatol.* **15,** 217–227.

Cobbold, S., and Metcalfe, S. (1994). Monoclonal antibodies that define canine homologues of human CD antigens. Summary of the First International Canine Leukocyte Antigen Workshop (CLAW). *Tissue Antigens* **43,** 137–154.

Coleman, N., Birley, H. D., Renton, A. M., Hanna, N. F., Ryait, B. K., Byrne, M., Taylor Robinson, D., and Stanley, M. A. (1994). Immunological events in regressing genital warts. *Am. J. Clin. Pathol.* **102,** 768–774.

Danilenko, D. M., Moore, P. F., and Rossitto, P. V. (1992). Canine leukocyte cell adhesion molecules (LeuCAMs): Characterization of the CD11/CD18 family. *Tissue Antigens* **40,** 13–21.

Felsburg, P. J., Somberg, R. L., Hartnett, B. J., Henthorn, P. S., and Carding, S. R. (1998). Canine X-linked severe combined immunodeficiency. A model for investigating the requirement for the common gamma chain (gamma c) in human lymphocyte development and function. *Immunol. Res.* **17,** 63–73.

Frattini, M. G., Lim, H. B., and Laimins, L. A. (1996). In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependant late gene expression. *Proc. Natl. Acad. Sci. USA* **93,** 3062–3067.

Ghim, S.-J., Newsome, J., Jenson, A. B., Schlegel, R., Suzich, J., Tamura, J., White, W., Hill, F., Warrener, P., Bell, J. A., and Sundberg, J. (1995). Formalin-inactivated oral papilloma extracts and recombinant L1 vaccines protect completely against mucosal papillomavirus infection: A canine model. *Vaccines 95* **25,** 375–379.

Hall, E. J., and Batt, R. M. (1990). Development of wheat-sensitive enteropathy in Irish setters: Morphologic changes. *Am. J. Vet. Res.* **51,** 978–982.

Hartnett, B. J., Somberg, R. L., Krakowka, S., Ochs, H. D., HogenEsch, H., Moore, P. F., Weinberg, K. I., and Felsburg, P. J. (2000). B-cell function in canine X-linked severe combined immunodeficiency. *Vet. Immunol. Immunopathol.* **75,** 121–134.

Hass, W., Pareira, P., and Tonegawa, S. (1993). Gamma/delta cells. *Ann. Rev. Immunol.* **11,** 637–685.

Hein, W. R., and Mackay, C. R. (1991). Prominence of gamma/delta T cells in the ruminant immune system. *Immunol. Today* **12,** 30–34.

Hope, R. A., Ed. (1998). "Oxford Handbook of Clinical Medicine." 4th ed. Oxford Univ. Press, Oxford, U.K.

Jain, N. C. (1993). Comparative hematology of common domestic ani-

mals. *In* "Essentials of Veterinary Hematology," pp. 20–21. Lea and Febiger, Philadelphia.

Knowles, G., O'Neil, B. W., and Campo, M. S. (1996). Phenotypical characterisation of lymphocytes infiltrating regressing papillomas. *J. Virol.* **70,** 8451–8458.

Ladiges, W. C., Storb, R., and Thomas, E. D. (1990). Canine models of bone marrow transplantation. *Lab. Anim. Sci.* **40,** 11–15.

Marchal, T., Saint-Andre, I., Magnol, J. P., Dezutter-Dambuyant, C., and Schmitt, D. (1995). Dendritic cells in dogs and cats: Models of study in human pathology. *Pathol. Biol. (Paris)* **43,** 910–920.

Meyers, C., Frattini, M. G., Hudson, J. B., and Laimins, L. A. (1992). Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science* **257,** 971–973.

Moore, P. F., and Rossitto, P. V. (1993). Development of monoclonal antibodies to the canine T cell receptor complex (TCR/CD3) and their utilisation in the diagnosis of T cell neoplasia. *Vet. Pathol.* **30,** 457.

Moore, P. F., Rossitto, P. V., and Danilenko, D. M. (1990). Canine leukocyte integrins: Characterization of a CD18 homologue. *Tissue Antigens* **36,** 211–220.

Moore, P. F., Rossitto, P. V., Danilenko, D. M., Wielenga, J. J., Raff, R. F., and Severns, E. (1992). Monoclonal antibodies specific for canine CD4 and CD8 define functional T-lymphocyte subsets and high-density expression of CD4 by canine neutrophils. *Tissue Antigens* **40,** 75–85.

Moore, P. F., Rossitto, P. V., and Olivry, T. (1994). Development of monoclonal antibodies to canine T cell receptor-1 (TCRgd) and their utilization in the diagnosis of epidermotropic cutaneous T-cell lymphoma. *Vet. Pathol.* **31,** 597.

Mota, F. F., Rayment, N. B., Kanan, J. H., Singer, A., and Chain, B. M. (1998). Differential regulation of HLA-DQ expression by keratinocytes and Langerhans cells in normal and premalignant cervical epithelium. *Tissue Antigens* **52,** 286–293.

Nicholls, P. K., Klaunberg, B. A., Moore, R. A., Santos, E. B., Parry, N. R., Gough, G. W., and Stanley, M. A. (1999). Naturally occurring, non-regressing canine oral papillomavirus infection: host immunity, virus characterization, and experimental infection. *Virology* **265,** 365–374.

Nicholls, P. K., and Stanley, M. A. (1996). Canine oral papillomavirus—A model for human papillomavirus regression. BBSRC Comparative Immunology Workshop Proceedings, p. 19. Biotechnology and Biological Sciences Research Council, Swindon, U.K.

Nicholls, P. K., and Stanley, M. A. (1999). Canine papillomavirus—A centenary review. *J. Comp. Pathol.* **120,** 219–233.

Okabayashi, M., Angell, M. G., Christensen, N. D., and Kreider, J. W. (1991). Morphometric analysis and identification of infiltrating leucocytes in regressing and progressing Shope rabbit papillomas. *Int. J. Cancer* **49,** 919–923.

Palefsky, J. M., Holly, E. A., Ralston, M. L., and Jay, N. (1998). Prevalence and risk factors for human papillomavirus infection of the anal canal in human immunodeficiency virus (HIV)-positive and HIV-negative homosexual men. *J. Infect. Dis.* **177,** 361–367.

Pastoret, P. P., Griebel, P., Bazin, H., and Govaerts, A. (1998). Immunology of Lagomorphs, Chapter VI. *In* "Handbook of Vertebrate Immunology," pp. 223–260. Academic Press, London.

Sawasdikosol, S., Hague, B. F., Zhao, T. M., Bowers, F. S., Simpson, R. M., Robinson, T., and Kindt, J. (1993). Selection of rabbit CD4- CD8- TCR$\gamma\delta$ cells by in vitro transformation with HTLV-1. *J. Exp. Med.* **178,** 1337–1345.

Selvakumar, R., Schmitt, A., Iftner, T., Ahmed, R., and Wettstein, F. O. (1997). Regression of papillomas induced by cottontail rabbit papillomavirus is associated with infiltration of CD8+ cells and persistence of viral DNA after regression. *J. Virol.* **71,** 5540–5548.

Shah, K. V., and Howley, P. M. (1996). Papillomaviruses. *In* "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2077–2109. Lippincott–Raven, Philadelphia.

Stanley, M. A. (1994a). Replication of human papillomaviruses in cell culture. *Antiviral Res.* **24,** 1–15.

Stanley, M. A. (1994b). Virus-keratinocyte interactions in the infectious cycle. *In* "Human Papillomaviruses and Cervical Cancer—Biology and Immunology" (P. L. Stern, and M. A. Stanley, Eds.), pp. 116–131. Oxford University Press, Oxford, U.K.

Starzl, T. E. (2000). History of clinical transplantation. *World J. Surg.* **24,** 759–782.

Wagner, J. L., Burnett, R. C., and Storb, R. (1999). Organization of the canine major histocompatibility complex: Current perspectives. *J. Hered.* **90,** 35–38.

Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., Snijders, P. J., Peto, J., Meijer, C. J., and Munoz, N. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189,** 12–19.

White, W. J., Wilson, S. D., Bonnez, W., Rose, R. C., Koenig, S., and Suzich, J. A. (1998). In vitro infection and type-restricted antibody-mediated neutralization of authentic human papillomavirus type 16. *J. Virol.* **72,** 959–964.

Wyatt, C. R., Madruga, C., Cluff, S., Parish, S., Hamilton, M. J., Goff, W., and Davis, W. C. (1994). Differential distribution of $\gamma\delta$ T cell receptor lymphocyte subpopulations in blood and spleen of young and adult cattle. *Vet. Immunol. Immunopathol.* **40,** 187–199.

Xu, L., O'Toole, E. A., Olivry, T., Hernandez, C., Peng, J., Chen, M., and Chan, L. S. (2000). Molecular cloning of canine bullous pemphigoid antigen 2 cDNA and immunomapping of NC16A domain by canine bullous pemphigoid autoantibodies. *Biochim. Biophys. Acta* **1500,** 97–107.