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The Immunology of Animal Papillomaviruses

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Abstract

Papillomaviruses are species- and tissue-specific double-stranded DNA viruses. These viruses cause epithelial tumours in many animals, including man. Typically, the benign warts undergo spontaneous, immune-mediated regression, most likely effected by T cells (especially CD4, but also CD8 subsets), whereas humoral immunity can prevent new infections. Some papillomavirus infections fail to regress spontaneously, and others progress to malignant epithelial tumours. Additionally, the impact of these lesions is greater in immunosuppressed individuals. Many therapies are ineffective, and there is much interest in the potential for immunological intervention in papillomavirus infections of man and animals. Vaccination can be achieved with 'live' virus, formalin-inactivated virus, synthetic virus-like particles, and DNA vaccination. There has been much recent progress in the development of such vaccines for papillomavirus infections in the rabbit, ox and dog. Success in these animal models suggests that similar approaches may prove useful for prophylactic or therapeutic vaccination against the important human papillomaviruses involved in the development of cutaneous and anogenital warts, laryngeal papillomatosis, and cervical cancer.

1. INTRODUCTION

Papillomaviruses are highly species- and tissue- specific non-enveloped viruses, with a circular, double-stranded DNA genome of approximately 8 kilobases. They infect a wide variety of species, causing both benign and malignant

epithelial proliferations. Although the benign lesions (warts) typically undergo spontaneous regression, some infections have a prolonged or more extensive clinical course, occasionally progressing to cancer. The persistent benign warts can prove troublesome in both domestic animals and man. The human papillomaviruses are known to cause anogenital and cutaneous warts, and are known also to be a key factor in the development of cervical cancer. The impact of these diseases is huge: anogenital warts are the most common sexually-transmitted viral disease in the United Kingdom (Anonymous, 1989), and cervical cancer kills approximately 500,000 women every year (Howett *et al.*, 1997; Beutner and Tyring, 1997). Improved knowledge of the immunity of papillomavirus infections underpins the development of effective prophylactic and therapeutic vaccines. The study of animal papillomaviruses has proved central to the development of our understanding of the immunology of this important group of viral pathogens.

The study of animal papillomaviruses has a long history. One hundred years ago M'Fadyean and Hobday (1898) from the Royal Veterinary College in London undertook some simple transmission experiments using canine oral papillomavirus (COPV). Their failure to re-infect a bull terrier after its oral warts had regressed led them to conclude "the animal is left in a measure protected against a second infection of the same kind". This review analyses the historical and recent evidence for such immunity to papillomavirus infections in animals.

Wart regression

The simultaneous disappearance of many warts in individual rabbits (Kidd, 1938) is evidence for systemic immunity. After noting the spontaneous regression after 1 to 2 months of experimentally-induced canine oral papillomas (figure 1), M'Fadyean and Hobday (1898) proposed that "the credit claimed for some methods of treatment may be undeserved". Spontaneous regression of papillomas has been reported also in the pig (Parish, 1961), horse (Cook and Olson, 1951), ox (Knowles et al., 1996), sheep (Hayward et al., 1992), goat (Theilen et al., 1985), white-tailed deer (Sundberg et al., 1985), Indian elephant (Sundberg et al., 1981), rabbit (Kreider, 1963; Okabayashi et al., 1991) and opossum (Koller, 1972). This spontaneous and often unpredictable regression of papillomas has allowed many claims for therapeutic efficacy to flourish. Historically, some of the more colourful therapies for human warts include rubbing them with bacon and burying it, or tying knots on a piece of string, again followed by burying the string (Thomen, 1938). These 'sympathetic cures' relied on the belief that the warts could be transferred to some other object which then decays or is thrown away, taking the disease with it. Modern opinion on the therapy of papillomavirus infection is

reviewed elsewhere (Stanley et al., 1997; Phelps et al., 1998).

Immunity to reinfection

M'Fadyean and Hobday's original observation (1898) that dogs which recover from papillomas are immune to re-infection has been confirmed by others (DeMonbreun and Goodpasture, 1932; Chambers et al., 1960; Konishi et al., 1972). The same phenomenon is seen in the horse (Cook and Olson, 1951), rabbit (Shope, 1933) and cow (Olson et al., 1960). Experimentally, dogs cannot be re-infected from three weeks post-infection (Chambers et al., 1960; Konishi et al., 1972), despite the continued growth of existing warts. This indicates that a form of concomitant immunity exists, as is the case in cattle (Olson et al., 1960). Their inability to infect dogs in which warts were regressing prompted DeMonbreun and Goodpasture (1932) to suggest that it was host immunity which limited wart growth and initiated regression. The increased susceptibility of young dogs to COPV infections (Walder, 1992; DeMonbreun and Goodpasture, 1932) is further evidence that older animals have acquired immunity from a previous episode of papillomatosis (Chambers et al., 1960).

Early work with rabbits demonstrated that although the protective immunity seen in wart-bearing rabbits could be bypassed by infection with naked DNA or autografting of skin biopsies infected *in vitro*, even this was not possible on rabbits whose warts had regressed (Kreider, 1963). This quite clearly demonstrated a distinction between the ability to prevent a new infection and the ability to reject an established lesion. Immunity to re-infection is type-specific, as demonstrated by the ability of bovine papillomavirus (BPV)-2, BPV-5 and BPV-6 infected calves to succumb to infection with BPV-4 (Jarrett et al., 1990b). The multiplicity of viral types means that an individual may suffer successive infections by new viral types, despite the development of immunity to previous infections. Vaccine design will have to take into account the many viral types capable of causing disease.

Immunosuppression and papillomatosis

Early experimental studies failed to demonstrate increased persistence of papillomas in rabbits immunosuppressed using cortisone (Evans et al., 1962b). There are, however, occasional case reports of severe or generalised papillomatosis in animals immunosuppressed by prednisolone.

Immunosuppression by corticosteroid therapy was implicated in the extensive oral and cutaneous papillomas of a young female dog (Sundberg et al., 1994). The cessation of corticosteroid therapy in conjunction with autogenous vaccination was followed by lesion regression. In a further case, similar regression of extensive canine cutaneous papillomas was seen three weeks after withdrawal of corticosteroid therapy (Le Net et al., 1997). Long-term anti-cancer chemotherapy has been associated with widespread canine cutaneous papillomatosis (Lucroy et al., 1998). Occasionally, severely affected animals show evidence of immunosuppression such as hypogammaglobulinaemia (Bredal, 1996) or IgM deficiency with impaired T-cell responses (Mill and Campbell, 1992). Recurrent papillomatosis, without the usual development of effective immunity, has been reported in the dog (Cierpisz et al., 1993). We have examined a similar case (figure 2) of severe, recurrent oral and cutaneous papillomatosis (Nicholls and others, in press). In this instance, the warts recurred despite the presence of abundant circulating antibodies to the virus, suggesting that either the animal was being infected by multiple viral types with no cross-reactive immunity, that a single latent infection was continually reactivating, or that the animal had defective cellular immunity. The virus was not an unusually pathogenic variant, as demonstrated by the uncomplicated spontaneous regression of warts after experimental infection of beagles with the isolated virus. In this and other cases where defective immunity has been suspected, the animals have not suffered from unusual fungal, protozoal or other infections, suggesting that any defect may be limited in its effects.

Papillomavirus infection in the domestic cat has been seen concurrently with feline immunodeficiency virus (FIV) infection (Egberink et al., 1992). This mirrors the situation in humans where infection with human immunodeficiency virus (HIV) is linked with enhanced papillomavirus-associated disease. Immunosuppression has been reported as a factor in papillomavirus infections in cattle (Duncan et al., 1975; Campo, 1987; Campo et al., 1994). Although Duncan's report is often cited, the original work is a case report describing abundant warts affecting a single one year old bull. Evidence of immunosuppression was based only on the lack of lymphocytic invasion within the warts, a failure to reject the warts after vaccination, and a negative tuberculin skin test following vaccination. More recent work has identified a link with bracken ingestion and the development of alimentary cancer, urinary bladder tumours, and enzootic haematuria in cattle (Campo, 1987; Campo et al., 1992; 1994). Chronic immunosuppression is thought to be a result of sesquiterpene pterosins and pterosides found in bracken (Evans et al., 1982, cited in Campo, 1997). Bracken-fed cattle developed neutropenia, severe enough to result in fatal septicaemia, as well as chronic lymphopenia.

Bracken-fed cattle developed cutaneous warts associated with BPV-1 or BPV-2 (Campo et al., 1994) and urinary bladder carcinomas or haemangiomas associated with BPV-2 (Campo et al., 1992). The immunosuppressing agent azathioprine has a similar effect in cattle (Campo et al., 1992). BPV-4 induced tumours in cattle fed on hay did not spread beyond the injection sites and regressed after a year. This contrasts with immunosuppressed cattle, in which the lesions became extensive, extending down the oesophagus to the rumen without regression. Feeding of bracken in conjunction with BPV-4 infection predisposes to transformation of the papillomas into carcinomas.

It is clear that the immune system plays an important role in modulating the severity of papillomavirus-associated disease. In order to develop appropriate immunotherapy, it is important to establish which components of the immune system are involved in prevention or removal of infection.

2. HUMORAL IMMUNITY

Cross reactive antibodies led to confusing results

Although it is now known that papillomaviruses share common cross-reactive epitopes (Dillner et al., 1991), the discovery of canine antibodies which precipitated human papillomavirus led earlier workers to conclude that dogs could transmit human warts (Pyrohnen, 1976). In addition to the discovery of cross-reactive epitopes, it is now known that there are significant species barriers to cross-infection (Parish, 1961; DeMonbreun and Goodpasture 1932).

Prevention of infection - neutralising antibodies in animal papillomaviruses

Passive transfer of immune serum prevents new infections but does not affect established lesions

In dogs recovering from oral papillomas, the ability of antibodies to neutralise infection was demonstrated 40 years ago by Chambers and others (1960). A key observation was that despite its ability to neutralise infection, passively-transferred immune serum failed to enhance papilloma regression. This indicated a role for cellular, rather than humoral, immunity in wart clearance. Shortly after Chambers' work in the dog, Parish (1962) established that neutralising antibodies were present in pigs injected with a wart extract. The neutralising ability of the serum was greatest in animals which had received multiple injections of the extract. The presence of neutralising antibodies coincided with immunity to reinfection, again suggesting that humoral immunity played a role in prevention of infection. Antibodies to pig warts, raised in rabbits, demonstrated viral antigen in pig warts only at the period of

maximum growth of the lesion. Although the significance of this finding may not have been clear at the time, it is likely that the antibodies were detecting the presence of the viral capsid protein, which is synthesised only in mature warts. Papillomavirus capsids are composed of a major (L1) and minor (L2) protein. It is antibodies to these proteins, especially L1, which prevent infection, as later work with the dog, ox and other animals has shown.

Other animal papillomavirus infections are associated with the development of antibodies, which can be protective. The development of serum antibodies was demonstrated in deer experimentally infected with papillomas (Sundberg et al., 1985). In a rodent (*Mastomys natalensis*), viral infectivity was neutralised by preincubation in serum from an immune animal (Muller and Gissmann, 1978). Early work by Shope (1937) demonstrated the existence of neutralising antibodies in rabbits immune to reinfection. More recently, antibodies to cottontail rabbit papillomavirus (CRPV) L1, and to a lesser extent L2, have been shown also to have neutralising ability (Lin et al., 1992). Additionally, passive transfer of serum from immune rabbits can protect naïve rabbits from infection (Breitburd et al., 1995).

Antibody development during progression from papilloma to carcinoma – the rabbit model

The rabbit has provided the opportunity to study host antibody responses during progression from papilloma to carcinoma, assayed using bacterial fusion proteins in an immunoblot. Antibodies to viral early proteins E1 and E2 (involved in viral DNA replication), as well as E6 and E7 (responsible for altering the host cell cycle to maximise viral replication), were seen in the papilloma stage, with E1 and E2 antibody levels remaining constant whilst those to E6 and E7 declined later. There was only a low response to the structural proteins L1 and L2 during the benign phases. The L1 neutralising epitopes were conformational, since only native fusion proteins blocked immunoprecipitation. With progression to carcinoma came a marked increase in response to the capsid antigens, without significant changes in early protein responses (Lin et al., 1993b). The decline in antibody responses to E6 and E7, and their low antibody levels compared with those to E2, cannot be explained by differences in levels of expression, because mRNA levels for E6 and E7 are higher than those for E2, and are the same in papilloma and carcinoma (Wettstein, 1987). Assuming the mRNA levels reflect protein expression, it is possible that the difference in antibody levels could reflect tolerance or impaired MHC presentation of the relevant peptides. Conversely, an abundance of viral antigen could have obscured antibody levels. No humoral response to E4 (a protein possibly involved in viral DNA replication or viral release from cells) or E5 (a protein able to increase cell growth) was seen in either domestic rabbits or cottontail rabbits, although

E4 mRNA is less abundant than that for L1 and L2 in the rabbit (Nasseri and Wettstein, 1984). In some regressor rabbits, E2 was the only antigen which generated a response (Lin et al., 1993b). Antibody responses to E2 were greater in rabbits with regressing rather than progressing lesions (Selvakumar et al., 1995a). The role of E2 as an immunogen is clearly important in the rabbit infections, although the lack of correlation between E2 antibody levels and regression suggests a cell-mediated response is more important than a humoral response during regression (Selvakumar et al., 1995b). This work further supported the earlier reports that passive transfer of serum from immune rabbits (Evans et al., 1962a) and dogs (Chambers et al., 1960) does not enhance regression.

The capsid antigens (L1 and L2) can elicit protective IgG antibodies

The early work on passive transfer has recently been extended. Passive transfer of serum immunoglobulin from immune dogs was able effectively to prevent infection in naïve dogs (Suzich et al., 1995). Assay of serum IgG from pre-immune and immune dogs, using intact COPV virus as an ELISA reagent, demonstrated the development of IgG antibodies and neutralising serum in animals with regressing oral papillomas (Ghim et al., 1997a). The use of native COPV virions as an ELISA reagent indicated that antibodies to conformational capsid (L1) epitopes were likely to be the main effective antibody. Similar results were seen in the ox, in which antibodies to L1, or L1 and L2, were protective against BPV-4 challenge (Kirnbauer et al., 1996). These antibodies were not associated with regression of established lesions. In addition to these experimental studies, naturally-occurring papillomavirus infections offer important insight into the role of humoral immunity. For example, the presence of multiple non-regressing crops of warts in a natural COPV infection, despite the demonstration of high levels of virion-specific antibody (using native COPV virions as an ELISA reagent), demonstrated that humoral immunity plays little role in wart regression in natural infections (Nicholls and others, in press). The findings that, although effective prophylactically, anti-L1 antibodies are ineffective in wart clearance have important implications for vaccine design. This is especially true for the syndrome of recurrent respiratory papillomatosis (RRP) of humans in which the recurrent crops of mucosal papillomas, similar to those described occasionally in the dog, are unlikely to be treatable by vaccination against the viral L1 protein.

Mechanisms of antibody-mediated viral neutralisation

Although neutralisation by blocking of viral binding sites appears to be an important mechanism, there seem to be other modes of antibody-mediated prophylaxis. The mouse xenograft system, in which target tissue of the appropriate

species is incubated with its host-specific virus (with or without pre-incubation in immune serum) prior to grafting onto immunodeficient mice, confirmed the neutralising ability of antibodies, raised in rabbits, to intact CRPV or BPV-1 (Christensen and Kreider, 1990). Interestingly, it seemed that neutralisation could be achieved despite the virus attaching to the cell (Christensen et al., 1995). A similar conclusion was reached by Roden and others (1994a). Although four monoclonal antibodies to BPV-1 L1 neutralised viral infectivity, only three of them prevented adhesion to the cell surface. Antibodies to the amino-terminal of BPV-4 L2 also were shown to have a neutralising effect despite presence of detectable viral DNA, but no lesion, at the challenge site (Gaukroger et al., 1996). This agreed with the suggestion that antibodies to BPV capsid proteins could neutralise virus by a mechanism other than prevention of adhesion to the cell surface. That some antibodies can work by virus neutralisation is well illustrated by the work of Lin and others (1993a). They showed that antibodies to L1 prevented infection by virus, but not by naked DNA-induced papillomas, the induction of which effectively bypasses virus neutralisation and interaction with cell-surface receptors. This observation was largely pre-empted by much earlier work demonstrating the ability of papilloma- or carcinoma-bearing rabbits to be re-infected by viral DNA, but not virus, in the face of neutralising antibodies (Evans and Ito, 1966). The mechanisms by which neutralising antibodies prevent infection have been studied. Antibodies to conformational epitopes are required for neutralisation, both *in vitro* (Roden et al., 1994b) and *in vivo* (Suzich et al., 1995). Although both L1 and L2 antibodies neutralise infection, antibodies against conformational epitopes on L1 VLPs, but not anti-L2 antibodies, prevent virus attachment to the cell (Roden et al., 1994b). Two antibodies to L1 which neutralise by different mechanisms, with only one preventing attachment to the cell, have been shown to bind to different sites on the viral capsid (Booy et al., 1998). Virus binding and internalization is a complex multistep process (Haywood, 1994), and presumably antibodies to L2 inhibit one of the post-attachment steps such as secondary binding, virion entry, or uncoating (Unckell et al., 1997).

In vitro techniques for study of virus neutralisation

Some *in vitro* systems have been developed for the assay of neutralising antibodies. The focus-forming ability of bovine papillomaviruses in NIH/3T3 cell cultures has been used to confirm and map the neutralising abilities of anti-BPV antibodies (Cason et al., 1993). A cottontail rabbit epidermal cell line was used to demonstrate type-specific neutralising activity by monoclonal antibodies to CRPV, but not HPV-11 (Angell et al., 1992). The neutralisation was attributed to failure of virus to penetrate the cells, since a reduced amount of CRPV DNA was demonstrated within the

neutralised cultures. A further system used BPV-1 virions made *in vitro* using vaccinia virus-derived L1 and L2, which self-assemble into virus-like particles (VLPs). These L1/L2 VLPs were able to package BPV-1 DNA from a cell-line containing the episomal viral genome, before being used to infect mouse fibroblasts. Infection was prevented by neutralising antibody, providing a system in which to investigate virus neutralisation (Zhou et al., 1993). The discovery that L1 protein alone, expressed *in vitro*, spontaneously self-assembles into virus-like particles, similar to those seen in natural infections (figure 3) allowed the creation of reagents for assay of antibody responses in a variety of systems. Antibodies to yeast-expressed CRPV L1 VLPs have been demonstrated in rabbits, with immune serum capable of neutralising the virus *in vitro* (Jansen et al., 1995).

Synthetic virus-like particles are an important tool

The ability to synthesise virus-like particles *in vitro* has allowed studies on the role of humoral immunity in human papillomavirus infections. Prior to VLP development, antibody responses to only HPV-1 and HPV-11 could be examined, since they were the only lesions from which sufficient virus could be isolated as the ELISA reagent (Steele and Gallimore, 1990; Bonnez et al., 1991). The generation of VLPs allowed studies of the correlation between lesion status and antibody prevalence (for reviews see Stanley, 1997; Carter and Galloway, 1997). Despite the progress made in studies of immunity to human papillomaviruses since the development of VLP-based ELISAs, experimental studies of animal papillomaviruses continue to provide important data which are difficult to obtain by clinical studies of HPV infections. Although not all patients with HPV-associated lesions have detectable antibodies, the proven immunogenicity of HPVs injected into rabbits (Christensen et al., 1994) suggests that the lack of consistent antibody response in natural infections in humans reflects poor presentation of viral antigens to the immune system. Similar work has been undertaken in primates, demonstrating that HPVs are certainly immunogenic under the right conditions (Lowe et al., 1997).

Experimental studies in cattle have provided useful insights into possible reasons for the ineffective immunity seen in many papillomavirus infections. For example, there seems to be good correlation between HPV-16-associated cervical cancer and the presence of antibodies to E6, E7 and to a lesser extent E2 and E4 (Mann et al., 1990; Dillner et al., 1994). The significance of these antibodies is difficult to establish, although antibodies to HPV-16 E7 seem to indicate a poorer prognosis (Gaarenstroom et al., 1994). This is of interest in the light of a chronological study of the response

to E7 in cattle infected with BPV-4 (Chandrachud et al., 1994). In the BPV-4 study, a response to E7 was seen only late in the infectious cycle, despite a good response when used as a vaccine, suggesting that the protein is poorly presented to the immune system in natural infections. Animal models have demonstrated the presence of neutralising antibodies to human papillomavirus infections. HPV-11 virions were neutralised by incubation with specific polyclonal antiserum (Christensen and Kreider, 1990; Bryan et al., 1997) or monoclonal antibodies (Christensen et al., 1990), prior to xenografting human skin under the renal capsule in an athymic mouse system. In this procedure, the immunosuppressed environment permits propagation of HPV-infected xenografts, circumventing the significant difficulties involved in tissue culture based systems. Using this technique, neutralising antibodies were found to be directed to external non-linear epitopes. Virion pseudotypes, using HPV-16 L1 and L2 expressed by recombinant Semliki forest virus to package BPV-1 DNA, have been used to demonstrate neutralising antibodies against HPV-16 (Roden et al., 1996). The pseudotype virus is incubated in the test serum prior to assay by focus-formation on fibroblast cultures. More recent work used HPV-16 virions generated from murine xenografts for a neutralisation assay. The neutralising ability of polyclonal sera, raised in rabbits against HPV VLPs, was assayed by the detection of early viral transcripts in keratinocytes infected *in vitro* after the virus had been preincubated in serum (White et al., 1998). Neutralisation was type-specific.

3. CELLULAR IMMUNITY

Cellular immunity and lesion regression

Early work highlighted the different roles of humoral and cellular immunity in papillomavirus infections

As discussed above, the inability to enhance wart regression by passive transfer of immune serum in both the dog (Chambers et al., 1960) and rabbit (Kidd, 1938; Evans et al., 1962a) suggested that lesion regression was probably effected by cellular, rather than humoral, immunity. Further evidence for the role of cellular immunity came from the resistance of regressor rabbits to infection by naked DNA, which would be able to bypass the immunity due to neutralising antibodies (Evans and Ito, 1966). Infection with naked DNA, or autografting of skin biopsies infected *in vitro*, was successful on wart-bearing rabbits, whereas viral challenge by scarification was prevented due to neutralising antibodies. Once the warts had regressed, DNA and grafting were unable to cause lesions, due presumably to the development of cellular immunity (Kreider, 1963).

Early work by Parish (1962) indicated that cellular immunity played a role in papillomavirus lesion regression. Parish noted that injection of wart filtrate into recovered immune pigs resulted in a type of lesion typical of a delayed-type hypersensitivity reaction. Parish's conclusion that "It is probable that immunity depends on cellular resistance rather than on humoral antibodies" now seems to be true as far as wart regression is concerned.

Wart regression is associated with lymphocyte infiltration

Morphological evidence for the role of lymphocytes in papilloma regression comes from histological demonstration of cellular infiltrates associated with wart resolution. This has been noted in many species including the pig (Parish, 1961), horse (Hamada et al., 1990), deer (Sundberg et al., 1985), sperm whale (Lambertsen et al., 1987), ox (Jarrett et al., 1991; Knowles et al., 1996), and lesions of both cottontail (CRPV) (Kreider, 1963; Okabayashi et al., 1991; 1993a) and rabbit oral papillomavirus (ROPV) (Harvey et al., 1998). Analysis of regressing CRPV-induced papillomas revealed dense T-lymphocyte infiltrates within the epidermis itself, near the basement membrane and in adjacent dermis (Okabayashi et al., 1991). In the regressing CRPV lesions, the prominent dermal infiltrates (mostly T lymphocytes) appeared not to be dividing significantly (as demonstrated by BrdU and Ki67 immunostaining), whereas the epidermal T lymphocytes were actively cycling. Additionally, the epidermis of regressing papillomas had a lower division rate in the upper layers, as measured by the same technique, suggesting that regression was associated with reduced cell proliferation in the upper layers of the epidermis (Okabayashi et al., 1993a). The infiltrate in CRPV lesions was found to consist mostly of CD8+ lymphocytes within the basal and suprabasal layers of epithelium (Selvakumar et al., 1997) with no CD4+ cells demonstrable. The absence of CD4+ cells in the CRPV lesions is remarkable, considering their abundance in regressing COPV (Nicholls and others, unpublished data), BPV (Knowles et al., 1996) and HPV (Coleman et al., 1994) lesions. The antibody used to detect CD4+ cells in the rabbit worked well on spleen sections, but was described as being non-specific on the papilloma sections. Further work in the rabbit is needed to confirm these data.

The infiltrate in regressing BPV-4 papillomas had numerous CD4+ cells in the dermis (Knowles et al., 1996). In the more superficial layers of the 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 TCR$\gamma\delta$+ cells in the superficial epithelium. The CD4+ cells were present mostly as clusters subepithelially within the dermis, sometimes surrounded by CD8+ and

TCRγδ+ cells, but migrating more into the epithelium once the basal lamina had been breached. In BPV-4 lesions, lymphocyte numbers correlated with regression, with CD4+ cells being the predominant type. 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). Preliminary studies on formalin-fixed, paraffin-embedded tissues using a CD3 polyclonal antibody (Nicholls et al., 1997) confirmed the presence of numerous T cells within spontaneously-regressing naturally-occurring canine oral papillomas. More recent work with experimental COPV infections has demonstrated a marked influx of CD4+ and CD8+ lymphocytes (figure 4) in spontaneously-regressing canine oral papillomas (Nicholls and others, unpublished data). Lymphocyte infiltrates correlated both spatially and temporally with wart regression. The predominance of CD4+ cells seen in both BPV-4 and COPV lesions suggests they are playing a key role in clearance of mucosal papillomas. T_H1 CD4+ cells could help clear viral infections by activating macrophages, or by cytokine-mediated inhibition or killing of infected keratinocytes. Heavy infiltration of lymphocytes was seen also in regressing BPV-2 and BPV-4 induced papillomas following vaccination with L2 and E7 respectively (Jarrett et al., 1991; Campo et al., 1993). There appeared to be some downregulation of MHC-I on BPV-4 induced cancer cells (Gaukroger et al., 1991), implying that by this mechanism the cells may escape CTL-mediated killing. That these observations are applicable to human papillomavirus infection is supported by the presence of lymphocytic infiltrates in both benign (Coleman et al., 1994) and malignant (Hilders et al., 1994) HPV-associated lesions, with loss of MHC-I expression in cervical carcinoma (Connor and Stern, 1990).

Studies of T-cell function in papillomavirus immunity

Skin tests and lymphoproliferative assays demonstrate active cellular immunity

The demonstration of lymphocytic infiltrates in regressing warts clearly indicates their role in lesion clearance. Several animal papillomaviruses have provided functional data to support these findings. Evidence from the CRPV model, in which seroconversion was not required for regression, indicates that regression is a T-cell mediated event (Selvakumar et al., 1995b). The positive skin test in pigs injected with wart filtrate, noted by Parish (1962), was one of the earliest functional assays of cell-mediated immunity but is still used in more recent studies, sometimes in conjunction with other functional assays. For example, the positive skin tests using viral proteins in regressor rabbits (Hopfl et al., 1993), together with *in vitro* responses of peripheral blood lymphocytes to viral proteins, clearly indicate active cellular immunity in the rabbit. T-cell proliferative responses to the viral E1, E2, E6 and E7 proteins have been seen in the

rabbit, with those to E2 being the strongest (Selvakumar et al., 1995a; Han et al., 1997). With progression from papilloma to carcinoma, an increased lymphoproliferative response to L1 and L2 proteins was seen, despite the low levels of mRNA for these proteins in the domestic rabbit infections (Lin et al., 1993b; Selvakumar et al., 1994). The increased immune response as tumours progress presumably reflects better presentation of epitopes as the malignant cells disseminate throughout the body.

Lymphoproliferative assays demonstrated E7-specific T-cells in cattle with naturally-regressing papillomas (Chandrachud et al., 1994; McGarvie et al., 1995), although the response was much lower than that of cattle vaccinated with E7 fusion protein, perhaps reflecting poor natural presentation of the antigen. T-cell responses to L2 proteins have been demonstrated in the same model (Chandrachud et al., 1995).

T-cell lymphoproliferative responses to COPV L1 protein have been documented both in infected and VLP-vaccinated dogs (Cohn et al., 1997), although their role in infections has not been established. Although destruction of the mature keratinocytes in which L1 protein is expressed would not by itself clear infection from lower layers of the epithelium, it is possible that a bystander effect could allow greater impact.

Rodent models can demonstrate effects of T-cell immunity

Various rodent models have been used to investigate T-cell responses to papillomavirus proteins. Immunization with E7 can induce cytotoxic lymphocyte-mediated regression of HPV-16 tumour cells in mice (Chen et al., 1991; Meneguzzi et al., 1991; Feltkamp et al., 1993). The ability to use allografts of human lymphocytes in xenografted SCID mice means that T-cell responses could be examined in this system (Brandsma et al., 1995). The rodent models which use tumours to present papillomavirus antigens may not accurately reflect the situation in natural wart infections, in which immune ignorance of viral antigens may be an important factor. However, it is possible to mimic the natural presentation of viral proteins in infected keratinocytes by using mouse models in which a cutaneous graft of transfected keratinocytes presents papillomavirus proteins in a more biologically-relevant manner (Chambers et al., 1994;McClean et al., 1993). Experimentally, most papillomavirus antigens can be made to elicit an immune response, depending on the mode and route of delivery. This may not reflect the situation in natural infections, during which viral antigens may be either shielded from the host immune system, for example by being expressed only superficially, or may be

expressed on keratinocytes in the absence of costimulatory molecules, leading to anergy (Malejczyk et al., 1997). In a murine model, HPV-16 E7 expressing cells cotransfected with B7 caused regression of HPV-16 E7 expressing tumours, indicating the key role of costimulation in effective antigen presentation (Chen et al., 1992). Because of some of the uncertainties involved in using these experimental models of cellular immunity, whole animal models based on infection by the appropriate host-specific virus still play an important role in the study of natural and induced immunity. The role of cellular immunity in human papillomavirus infections (reviewed in Malejczyk et al., 1997) seems similar to that found with animal papillomaviruses. T-cell proliferative responses to both early (de Gruijl et al., 1996) and late (Shepherd et al., 1996) proteins of HPV-16 have been demonstrated in humans and CTLs specific for E7 peptides have been isolated directly from HPV-associated cervical cancer tissue and regional lymph nodes (Evans et al., 1997). Similar studies in HPV-6 associated genital warts have demonstrated CTL activity against both L1 and E7 in infiltrating lymphocytes (Hong et al., 1997). The association of proliferative responses to E6 and E7 with the ability to clear infection (Kadish et al., 1997) and the presence of CTL activity against these antigens in a human trial of a vaccinia virus encoding E6 and E7 (Borysiewicz et al., 1996) suggest some promise for therapeutic vaccination.

4. HOST FACTORS IN LESION REGRESSION

The outcome of any viral challenge depends on the balance of both viral and host factors. Variation in lesion duration and rate of regression is seen in both natural and experimental infections of several species. In the dog, considerable variability in host immune response to COPV vaccination or infection has been noted (Cohn et al., 1997). Variation in antibody and T-cell responses was seen both in dogs vaccinated with COPV L1 VLPs and in dogs infected with COPV. A similar phenomenon is seen in CRPV infections. It seems that progression or regression of CRPV-induced warts in rabbits may be linked to MHC-II allotype (Han et al., 1992). Studies on rabbits homozygous for three DQA haplotypes revealed that the outcome of CRPV infection (regression or malignant progression) was linked with the host haplotype (Breitburd et al., 1997). In one group of rabbits a fraction of the original warts persisted. The persisting warts were all associated with CRPV of the prototype strain (CRPVa), despite it being present as only a minor component in the pooled inoculum. In the same individuals, warts arising from a new strain, CRPVb, underwent regression (Salmon et al., 1997). The ability of some individuals to reject warts of one strain but not another suggests that the basic mechanisms of immune recognition are essentially intact and functional, but that antigens from certain viral strains are ineffectively presented by some hosts.

The ability of the same COPV isolate to cause persisting severe infections in some individuals but not others (Nicholls and others, in press) clearly highlights the importance of host factors in viral infections, as is the case with human papillomavirus infections.

5. VACCINATION AGAINST ANIMAL PAPILLOMAVIRUSES

Autogenous vaccination

Autogenous vaccines, prepared by injection of homogenised wart into the original animal, have been used in the ox (Narayana et al., 1973), dog (Chambers et al., 1960; Cierpisz et al., 1993; Sundberg et al., 1994), goat (Lloyd, 1982; Rajguru et al., 1988), parrot (Cooper et al., 1986) and rabbit (Evans et al., 1962a). In some cases the lesions could have regressed spontaneously but other controlled experiments indicate a positive effect (Evans et al., 1962a). The technique is still used today (Agut et al., 1996).

Heterogenous wart extracts

Early work with rabbits demonstrated that vaccination using a crude wart suspension could generate antiviral immunity, with serum neutralising antibodies (Shope, 1937). As well as being protective, both heterogenous and autogenous crude wart extracts were able to induce regression of warts (Evans et al., 1962a).

Forty years ago, a crude canine oral papilloma extract, injected with adjuvant intramuscularly or subcutaneously, was shown to be effective prophylactically (Chambers et al., 1960). Recent work has confirmed the efficacy of systemically-administered formalin-inactivated papilloma extract (Bell et al., 1994). Successful vaccination with "live" COPV extract, however, was occasionally associated with development of squamous cell carcinoma or other neoplasms at the injection site (Bregman et al., 1987; Meunier, 1990).

Crude wart vaccines have a long history of usage in cattle (Olson et al., 1960) and more recent work demonstrated that homogenised BPV-2 fibropapilloma protected cattle from the homologous viral infection (Jarrett et al., 1990a).

Purified virus as a vaccine

Intramuscular vaccination of calves with purified virions of BPV-2 (Jarrett et al., 1990a), BPV-4 and BPV-6 (Jarrett et al., 1990b) protected animals from subsequent challenge by homologous virus infection. The ability of BPV-1 to infect the BPV-6-vaccinated animals demonstrated type-specific protection, an important consideration in papillomavirus vaccine design considering the multiplicity of viral types within a species. The demonstration that purified virus was protective indicated that viral capsid proteins alone could make an effective vaccine.

Recombinant proteins as vaccines

Bacterial-expressed proteins and CRPV vaccination

Vaccination studies in rabbits showed both L1 and L2 fusion proteins to be protective, accompanied by a neutralising antibody response which was greater for L1 than L2 (Lin et al., 1992). Presumably critical conformational epitopes can be retained in the L1 and L2 fusion proteins. The role of conformational epitopes was highlighted by the failure of L1 subfragments, expressed as fusion proteins, to protect rabbits from papillomas and latency (Lin et al., 1993a). The protection afforded by the full-length L1 fusion protein could be bypassed by DNA infection, indicating that viral particle uptake was being neutralised. This protection was abolished by heat denaturation, indicating that the neutralisation epitopes were conformational. Other means of generating viral proteins have proved successful in vaccination trials. Vaccinia-expressed L1 generates an antibody response which inhibits papilloma formation in rabbits (Lin et al., 1992).

In addition to the prophylactic immunity demonstrated for L1, the non-structural proteins E1, E2, and E6, but not E7, were found to enhance regression of viral papillomas (Lathe et al., 1989; Selvakumar et al., 1995b). Vaccinated rabbits still developed warts as frequently as the controls, but these regressed more rapidly. There was no correlation between antibody levels and regression, indicating that the response was cell-mediated. These results contrast with those described below for BPV-4, in which therapeutic vaccination with E7, but not E2, is effective.

Bacterial-expressed proteins and BPV vaccination

BPV-2 L1 and L2 proteins expressed as *E. coli* β-galactosidase fusion proteins were trialled in calves (Jarrett et al., 1991). Vaccination with L1, but not L2, generated serum-neutralising antibodies, and prevented tumour formation when given prophylactically. L2 vaccination seemed to promote tumour regression, accompanied by tumour-

infiltrating lymphocytes, when given either prophylactically or after challenge. L2 vaccination did stimulate antibody production, although these were ineffective at neutralisation, as assessed by a cell transformation inhibition assay. The ability of L2 to cause regression is surprising since L2 appears not to be expressed in dividing cells. It is possible that the response initiated by the L2 vaccine also stimulated a host response to other viral proteins as a bystander effect, causing regression. Although BPV-2 L2 was not effective prophylactically, a later study was able to show protection from BPV-4 using an L2 fusion protein (Campo et al., 1993). This later study used full-length L2, rather than the N-terminal truncated protein used with the BPV-2 trial. Protection was mediated via neutralising antibodies to the N-terminal (Chandrachud et al., 1995; Gaukroger et al., 1996), a finding confirmed by the lack of neutralising ability of serum depleted of L2 antibodies. Although antibodies were raised to the C-terminal region, they were not protective, perhaps because the C-terminal region is internal and interacts with DNA (Zhou et al., 1994). Interestingly, unvaccinated infected calves did not develop antibodies to L2, indicating that it may not be well-recognised by the immune system during natural infection. Antibodies to the amino-terminal of BPV-1 L2 react with BPV-1 virions and prevent *in-vitro* transformation by the virus (Roden et al., 1994a). This study showed that some L1 monoclonal antibodies appeared to neutralise infection by a post-attachment mechanism, since binding of virions to the cell surface was not markedly inhibited. Gaukroger and others (1996) reached a similar conclusion for BPV-4 L2 antibodies.

As with the CRPV system, vaccination using fusion proteins from early viral genes has been evaluated in the bovine model. Preliminary experiments with BPV-4 β-galactosidase fusion proteins failed to show an effect for E2. In contrast with the failure of E7 to cause regression in rabbits, the BPV-4 E7 protein promoted early rejection when given either two weeks before or after challenge (Campo et al., 1993). Further work with the BPV-4 E7 fusion protein mapped B- and T-cell epitopes and confirmed that the vaccine retarded papilloma development and promoted early regression in calves when given prior to challenge (Chandrachud et al., 1994; McGarvie et al., 1995). Peripheral blood mononuclear cell proliferation assays demonstrated a positive response to E7 in the vaccinated group, as well as IgG antibody production by two weeks after boosting. The E7 antibodies were not neutralising, and their role in regression is unknown. Non-vaccinated infected cattle had only a weak cellular and humoral response to E7 which developed only during the later stages of infection. Some unvaccinated infected animals appeared not to develop antibodies to E7 (Chandrachud et al., 1994). It seems likely that viral E7 is poorly presented to the immune system during natural infection.

Virus-like particles as vaccines

Virus-like particles and COPV

COPV L1 VLP vaccination protected dogs from infection (Ghim et al., 1995). Serum from immune dogs protected naïve dogs in passive transfer experiments (Suzich et al., 1995; Ghim et al., 1997a). A denatured L1 vaccine made antibodies but did not prevent infection, demonstrating the need for conformational epitopes. HPV-11 L1-VLPs were not protective, demonstrating the type-specificity of the neutralising antibodies. VLPs made from the L1 protein of COPV display type-specific conformational epitopes (Chen et al., 1998). The ability to form VLPs remains even when the protein is truncated sufficiently to abolish expression of the neutralising conformational epitopes, demonstrating that not all VLPs may be useful as vaccines.

Virus-like particles and BPV

The ability of BPV VLPs to generate serum neutralising antibodies was demonstrated by vaccination of rabbits followed by use of the serum for *in vitro* neutralisation assays. The ability to neutralise virus depended upon conformational epitopes (Kirnbauer et al., 1992). VLPs composed of either L1 alone or L1 with L2 were effective at generating antibody responses and preventing BPV-4 infection in calves. The vaccines did not effectively initiate regression of established lesions, and although the lesions of vaccinated animals did show a tendency to regress more rapidly than those of controls this did not reach statistical significance (Kirnbauer et al., 1996). As with COPV, this work with BPV-4 demonstrated the ability of VLPs to prevent mucosal papillomavirus infections.

Virus-like particles and CRPV

Vaccination with CRPV L1 VLPs made in yeast cells (Jansen et al., 1995), and CRPV L1 or L1-L2 VLPs made by baculovirus in insect cells (Breitburd et al., 1995; Christensen et al., 1996b) protects rabbits. This protection is long-term, lasting for at least one year (Christensen et al., 1996b). ELISA using native CRPV L1-L2 VLPs demonstrated a marked response within a week of the second boost, whereas control rabbits had only a smaller rise in antibody titre after CRPV challenge. Protection is mediated via virus-neutralising IgG and requires a conformational epitope (Breitburd et al., 1995). Protection is type-specific, since BPV L1-L2 VLPs (Breitburd et al., 1995) and HPV-11 VLPs (Christensen et al., 1996b) failed to protect rabbits from experimental challenge.

Virus-like particles and EcPV

Virus-like particles prepared from the L1 protein of equine cutaneous papillomavirus (EcPV-1) have been used as reagents for ELISA studies and generation of monoclonal antibodies (Ghim et al., 1997b). Sarcoid or BPV-1 sera were not reactive with EcPV-1 VLPs. The recombinant VLPs carried conformational type-specific epitopes as well as sequential type-specific epitopes on the surface and acted as an effective prophylactic vaccine.

Other VLP-based vaccines

The ability to delete portions of BPV-L1 without affecting its ability to form VLPs (Paintsil et al., 1996) enables various epitopes, up to 60 amino acids (Muller et al., 1997), to be incorporated into the particle as 360 copies. This was put into practice using BPV-1 L1 VLPs carrying two different CTL epitopes, including one for HPV-16 E7, fused to the L1 C-terminus. Immunised mice generated a CTL response to the E7 epitope as well as a neutralising antibody response to the BPV-1 VLPs. The functional significance of the E7 CTL response was proven by the ability of immunised mice to resist challenge from an E7-transfected tumorigenic cell line (Peng et al., 1998). Recent work has shown that chimaeric BPV-1 L1/E7 VLPs, administered intranasally to mice, resulted in both systemic and mucosal antibody production (Liu et al., 1999). The recent demonstration that oral delivery of VLPs in mice generated type-specific, conformationally-dependant antibodies, which had neutralising ability based on an *in vitro* assay (Rose et al., 1999), opens a further avenue for exploration in the field of VLP research. The ability of VLPs to be effective via several routes, and to act as chimaeric particles and deliver both systemic and mucosal immunity, demonstrates their flexibility and there is much current interest in the potential of VLP vaccines against human papillomavirus infections (reviewed in Schiller, 1999).

DNA vaccination against animal papillomaviruses

The induction of specific immunity after injection of antigen-encoding DNA into mouse skin heralded a novel approach to vaccination (Tang et al., 1992). Both intramuscular injection and particle bombardment of skin are effective, and the immunity is long lasting (reviewed in Tuting et al., 1998).

In a study investigating the immune response to nucleic-acid induced papillomas, the warts of two rabbits in an

experimental group regressed shortly after DNA inoculation (Evans and Ito, 1966). This could have been a coincidental spontaneous regression, since there were no controls, but it stimulated thought as to the possibility of inducing immunity by DNA vaccination.

In rabbits, cutaneous gene-gun delivery of DNA plasmids encoding the CRPV L1 capsid protein elicited a strong antibody response (Sundaram et al., 1996). The recent findings that intramuscular (Donnelly et al., 1996) and cutaneous gene gun (Sundaram et al., 1997) vaccination with a DNA plasmid encoding CRPV L1 were able to prevent infection in rabbits has broadened the options for vaccine development. These studies have been extended recently to demonstrate protection after vaccination with a DNA plasmid encoding the E6 gene (Sundaram et al., 1998). The DNA was attached to 1-3 µm gold particles and delivered into the dorsal skin by a helium-driven "gene-gun", with boosting three weeks later. Antibodies to E6 were not detectable by ELISA after vaccination, but there was a greater E6-specific *in vitro* proliferative response in three of six E6 vaccinated rabbits compared with controls. This response correlated with protection from subsequent viral challenge, with two rabbits showing complete protection, and one rabbit developing only two tiny papillomas out of nine challenged sites. The remaining three vaccinated rabbits showed partial protection as judged by delayed onset and reduced number and size of papillomas. DNA vaccines encoding a combination of viral early proteins may prove more effective than vaccines based on single genes. This is suggested by recent work in the domestic rabbit (with CRPV challenge), in which DNA vaccination with a combination of genes encoding E1, E2, E6 and E7 proved appeared more effective than DNA vaccines encoding only a single protein (Han et al., 1999b). In Han's study, gene-gun vaccination did not elicit detectable humoral responses to the encoded antigens, although T-cell lymphoproliferative responses were seen to each of the encoded antigens. The lack of humoral response to the encoded antigens was seen when DNA was delivered by either intracutaneous gene gun (Han, et al., 1999b), or intramuscular injection (Han et al., 1999a).

In addition to their ability to alter the course of cutaneous papillomavirus infections, DNA vaccines are also efficient prophylactically in mucosal papillomavirus models. We have shown that vaccination of dogs, using a DNA construct encoding the L1 protein, elicits both humoral and cell-mediated immunity and is effective in preventing the development of oral papillomas after mucosal challenge with virus (unpublished observations). Clearly, DNA vaccines have the potential to play an important role in the future armamentarium against papillomavirus infections.

6. CONCLUDING REMARKS

In summary, *in vitro* and *in vivo* studies on both human and animal papillomaviruses show that antibody responses occur in the natural infection, and that antibodies to conformational epitopes on the viral capsid can neutralise viral infectivity in a type-specific manner. Humoral immunity appears to play little part in wart regression. Cellular immunity, however, is crucial in mediating wart regression, with E2 and E7 being implicated as important antigens. These findings are clearly of fundamental importance for vaccination development. It should be borne in mind that immunological strategies may be less useful in those suffering from severe papillomavirus infections due to immunosuppression. In this respect, it is noteworthy that not all wart regression need be mediated by the immune system. There is evidence from studies in the rabbit that treatment of warts with podofilox causes regression by a direct toxic effect on keratinocytes, rather than by stimulation of host immunity (Okabayashi et al., 1993b). Studies of natural and experimental disease in animals have demonstrated the basic roles of humoral and cellular immunity in prevention and regression of papillomavirus infections. Additionally, the demonstration of effective prophylactic vaccination against bovine, canine and rabbit papillomaviruses holds some promise for reducing the impact of human papillomavirus-associated disease. Despite these successes, there remain many important issues to be addressed. These include the role of cytokines in lesion regression, and their potential as immunomodulatory agents for therapeutic vaccination (Gaspari et al., 1997; Tan et al., 1999). The availability of recombinant cytokines (Zucker et al., 1993; Okano et al., 1997) or reagents for the study of cytokines in animals (Buttner et al., 1998; Gröne et al, 1998) provides the tools for addressing some of these issues. Novel methods of immunotherapy, including DNA vaccination, already show some promise in altering the course of papillomavirus infection in animals. Increased knowledge of the mechanisms underlying tolerance and immunity in animal disease models provides hope for the many people suffering the serious effects of human papillomavirus infection.

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948
949 Figure legends
950 Figure 1. Spontaneous regression of canine oral papillomas.
951 Experimentally, canine oral papillomas appear from 4 weeks after infection. Early lesions are raised, multiple or
952 confluent smooth nodules (a). The mature papillomas appear at approximately 8 weeks (b) and are more pale and firm,

with multiple projecting filiform papillae. Regression occurs spontaneously, in this case starting at week 9, with a softening and shrinking of the papilloma (c). The bulk of the papilloma then sloughs to leave a raised base (d) at 10 weeks, which resorbs to leave normal intact mucosa. Scale bars = 1 cm.

Figure 2. Naturally-occurring, non-regressing canine oral papillomatosis.

Occasionally spontaneous regression fails, with multiple crops of warts throughout the oral cavity (a), including the tongue and oesophagus (b).

Figure 3. Papillomavirus virions.

In the natural infection, virions are assembled in the nucleus of superficial keratinocytes within the stratum granulosum. The virions are abundant, often forming crystalline arrays, as seen here. Particles with similar morphology can be generated by *in vitro* expression of the L1 capsid protein, which then assembles spontaneously into virus-like particles (VLPs). Bar = 1 μm.

Figure 4.

Cellular immunity in wart regression.

Many species, including man, have lymphocytic infiltration in regressing warts. In this example, from a dog, pre-infection control oral mucosa has only scant alpha/beta T cells in the epidermis and dermis (a). During early wart regression, T cells begin to increase in number both intraepithelially and in the superficial dermis (b). x 20 objective.