STUDIES ON IMMUNITY TO BOVINE PAPILLOMAVIRUSES

A thesis submitted for the Degree of Master of Science (Veterinary Science) in the faculty of Veterinary Medicine of the University of Glasgow.

by

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Dedication

This thesis is dedicated to the memory of Nancy O'Neil, who was a true friend and loving mother, until her untimely death in March 1986. I gratefully acknowledge the generous support and encouragement that she gave throughout my life, which has resulted in numerous treasured memories.

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Declaration

The work recorded in this was carried out in the Department of Veterinary Pathology at the University of Glasgow. I declare that all the results were obtained by the author unless otherwise stated in the text.

I also hereby certify that no part of this thesis has been submitted previously in any form to any university.

Brian William O'Neil

Glossary and Abbreviations

Acanthosis:	Diffuse hyperplasia and thickening of the prickle cell layer of the epidermis (stratum spinosum).
AGID:	Agar gel immunodiffusion
AP:	Alimentary papilloma
BAPV:	Bovine alimentary papillomavirus
BF:	Buffered formalin
Bowen's Disease:	Squamous carcinoma **in-situ** affecting both genital and non-genital skin. On the penis or vulva it is synonymous with intraepithelial neoplasia grade 111.
BPV:	Bovine papillomavirus
Carcinoma <u>in-situ</u>:	A malignant lesion which occupies the full thickness of the epithelium but has not invaded adjoining tissue.
Cervical Intra-Epithelial Neoplasia (CIN):	A spectrum of intraepithelial change which begins as a generally well differentiated neoplasm which has traditionally been classified as mild dysplasia and ends with invasive carcinoma.
CMI:	Cell mediated immunity
CsCl:	Caesium chloride
DAB:	Diaminobenzidine tetrahydrochloride

DNA:	Deoxyribonucleic acid
Dysplasia:	Disturbances of differentiation of the squamous epithelium of lesser a degree than carcinoma in-situ.
Dyskeratosis:	Abnormal or premature keratinisation in squamous cells.
ELISA:	Enzyme-labelled immunosorbent assay
EM:	Electron microscope
EV:	Epidermodysplasia verruciformis
H&E	Haematoxylin and eosin
HPV:	Human papillomavirus
HSV:	Herpes simplex virus
IB:	Inclusion bodies
IM:	Intramuscular
kb:	Kilobases
Koilocyte:	A squamous cell showing cytopathic changes; hyperchromatic, pyknotic nuclei with prominent perinuclear halos.
ME:	Mercaptoethanol
OD:	Optical density
Parakeratosis:	Keratinising squamous cell which retains its nucleus.

PAP:	Peroxidase anti-peroxidase
PBS:	Phosphate buffered saline
PTA:	Phosphotungstic acid
PV's:	Papillomaviruses
RCPV:	Rabbit cutaneous papillomavirus
RDE:	Receptor destroying enzyme
TEM:	Transmission electron microscope
SDS:	Sodium dodecyl sulphate

Summary

In the last few years there has been an intensive study of papillomavirus infections in both humans and animals. To date this has resulted in the recognition of 51 distinct types and several sub-types of human papillomaviruses (HPV's) and 7 types of bovine papillomaviruses (BPV's). This interest has stemmed from the fact that all these viruses are oncogenic and several are potentially carcinogenic in their natural hosts, which could possibly provide a valuable insight into the molecular mechanism of neoplasia.

Cattle provide us with an excellent animal model system for papillomavirus infections in humans, as several manifestations are common in the two systems; multiple virus types, specificity of lesions, malignant progression of some papillomas and the involvement of cofactors.

The studies described in this thesis record a series of experiments which help to detail the pathogenesis of BPV's and the subsequent immunity by the injection of purified virions. **Chapter 1** reviews the features of naturally occurring HPV and animal papillomavirus infections. It details the various isolates, their physical properties, their replication cycle, their host range and their potential oncogenicity. A detailed description of the main papillomavirus infections in both the human and the bovine system are given.

Chapter 2 describes the development of the techniques and reagents used in the subsequent experiments. The methodology provided the purified virus to; 1) experimentally induce BPV infections, 2) to localise BPV antigens and 3) to immunise against BPV infections.

In **Chapter** 3 study of the detailed pathogenesis of papillomavirus infections caused by BPV's was possible by serially biopsying the experimentally transmitted tumours. This permitted the study of the tumour cycle; infection -> tumour induction -> tumour regression by BPV-2, 4, 5 & 6.

In the BPV-2 induced infection the first stage of tumour development was first reported at 7 weeks after infection. This was a fibromatous lesion with no thickening of the overlying epithelium and no viral antigen could be detected by the peroxidase anti-peroxidase (PAP) technique. However, within 7 days there was thickening of the squamous epithelium and the establishment of plexiform acanthosis, coupled with the presence of a small focus of cells expressing viral antigen. The biopsy at 11 weeks after infection revealed a fully developed fibropapilloma with an abundance of viral antigen in the upper granular layer of the epithelium. By the 19th week calf 50 had rejected its tumours but calf 49 had still a virally active lesion. The process of regression of this tumour (calf 49) was slow and it was not until a year after the challenge had elapsed before the skin returned to its normal state.

Tumour induction by BPV-4 was first recorded 2 weeks after infection. These were small, opalescent lesions covering the palate. By the 6th week there was a virally active epithelial papilloma. The absence of viral antigen was first recorded in the biopsy taken 30 weeks after infection. These epithelial papillomas remained on the palate, pharynx and oropharynx for more than 1 year but no viral antigen could be detected in any cells of the biopsies taken after week 30.

The BPV-5 induced lesion was first recognised 26 weeks after infection. This biopsy revealed a typical plaque lesion with a marked epithelial thickening and cytopathic cells in the upper granular layer but with no involvement of the sub-

epithelial tissue. The latency of this particular infection and the development of the tumour was much longer than that of BPV-2, 4 and 6. The `rice grain' tumour, a plaque lesion with a protruding papillomatous structure, was first identified 37 weeks after infection. By 46 weeks the typical BPV-5 induced teat fibropapilloma was well established. However, at this stage there was also the emergence of a marked mononuclear reaction in the perivascular spaces, which by week 52 had started to destroy the basal cells. This reaction might well indicate the onset of the rejection process.

In the experimentally-induced BPV-6 infection, tumour development was first seen 4 weeks after infection but no viral antigen could be detected. A virally active lesion was confirmed at the week 18 biopsy , which is much later than the infections induced by BPV-2 and 4 but the positive antigen reaction remained for approximately for 1 year before the regression process started.

Chapter 4 investigates the status of infection and reinfection among different papillomavirus types. The animals selected had all completed the cycles of oncogenesis from infection to rejection for BPV-1, 2, 5 & 6 and were challenged with BPV-4.

All animals developed lesions within 4 weeks of virus application. These were small, projectile lesions with no demonstrable viral antigen. However, by 8 weeks the lesions had developed into typical BPV-4 induced epithelial papillomas with numerous cells containing viral antigen. The lesions were examined at regular intervals over 1 year and virus was consistently demonstrated until the 40th week.

These experiments confirmed that all the bovine viruses are immunologically different and that immunity to tumour induction by BPV-1, 2, 5 and 6 is **type-specific.**

Chapter 5 describes the various prophylactic vaccination agents used in order to assess their ability to induce rejection of a papillomavirus infection. The attempt to show protection by immunising with bovine cells transformed in vitro failed. However it was demonstrated that cattle could be protected against homologous infection and oncogenesis by vaccines prepared from virus and tumour extracts. There was no obvious advantage in administering virus along with the cellular suspension. Prophylactic vaccination using purified virions from BPV-2, 4 and 6 was very successful and it also showed that this was a type-specific immunity.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

General Introduction and Literature Review

Papillomaviruses have until recently formed one genus of the Papovaviridae family. (Melnick, 1962). The papillomaviruses (PV's), polyomavirus and the simian vacuolating agent (SV 40) each provide the first two letters to form the group name PAPOVA. The viruses are named by host specificity and in those hosts where more than one papillomavirus exists, by type number.

Papillomaviruses are widespread amongst humans and animals inducing epithelial or fibroepithelial proliferations of the skin or of the mucosa. As all are oncogenic and several are potentially carcinogenic in their natural hosts, there is considerable interest in the possibility that they will provide valuable insights into some of the molecular mechanisms of neoplasia.

Physical Properties of Papillomaviruses

The papillomaviruses range between 50-55nm in diameter and have an external icosahedral protein capsid consisting of 72 capsomeres, with the width of each being 8nm (Pfister, 1984). Within this protective coating there is a double stranded circular and supercoiled molecule of DNA. They have a genomic length of approximately 8000 base pairs ($5x10^6$ daltons molecular weight); all have the same genetic organisation (Giri & Danos, 1986); they are not rapidly inactivated at high temperatures (eg. 56-65^{o}C for 30 mins); they will survive well either frozen or in 50% phosphate buffered saline/glycerol.

Papillomaviruses are considered to be of distinct types if they show less than 50% homology by liquid hybridization under stringent conditions.

Papillomavirus Isolates

Papillomaviruses which infect different species are serologically distinct but have many properties in common. Virus isolates from human, bovine, canine, equine and rabbit were studied by density gradient centrifugation followed by phosphotungstic acid negative staining, and visualisation in the transmission electron microscope (TEM) ; all were found to be almost identical in appearance (Crawford & Crawford, 1963). They were tested serologically by agar gel immunodiffusion (AGID) (Le Bouvier et al, 1966) and were found to be distinguishable on a host species basis. The assumption then became common that each host species had one or occasionally two papillomaviruses (Rowson & Mahy, 1967). The major factor which allowed further discrimination between viruses within a species was the development of restriction enzymes, molecular cloning and genome mapping techniques. The first maps of the human virus were produced by Gissman et al, 1976 and Orth et al, 1977 and of the bovine by Lancaster, 1979; Jarrett et al, 1980 and Campo et al, 1981.

There has been an exponential increase in the number of new human viruses isolated since then and this has resulted in the recognition to date of 51 distinct types and several sub-types. Seven bovine virus types have been identified.

Replication

It is now accepted that any one species may have a number of different and often partly related papillomaviruses causing various lesions in a variety of animals (cattle, horses, rabbits, dogs, sheep, goats, deer, hamsters and monkeys). Usually the tumours show a limited growth span and regress spontaneously.

Papillomavirus virion replication is largely confined to

sites producing keratin as the viral cycle is linked to the differentiation of epithelial cells (Orth et al, 1971). The virus is assembled in the granular and corneal layers of the skin but not in the keratinocyte or basal layers although viral genomes can be detected there by the use of **in situ** hybridization techniques. However some tumours are virus induced without virion replication and structural antigen production, notably the mesenchymal tumours induced by bovine viruses eg. these tumours contain non-integrated papillomavirus genomes (Howley et al, 1980 and Moar et al, 1981).

In Vivo ; Host Range and Oncogenicity

Papillomaviruses display a wide range of oncogenicity **in vivo.** Some viruses produce benign, epithelial, hyperplastic lesions restricted to their host species (Rowson and Mahy, 1967), while others are oncogenic in a number of animal species (Campo, et al, 1980; Kidd & Rous, 1940; zur Hausen, 1976). In general these latter members produce tumours restricted to epithelial and mesenchymal tissues and to their equivalents in the central nervous system.

The progression of papillomas to carcinomas is stimulated by genetic and/or environmental factors. The best examples of these are: the cottontail rabbit/domestic rabbit, where the genetic make up of the host is an important factor determining the extent and rate of malignant conversion (Kreider, 1980): the progression to carcinomas of the Epidermodysplasia verruciformis (EV) lesions in areas exposed to sunlight in individuals with impaired cell-mediated immunity (Orth et al, 1980); and the transition of papillomas to carcinomas in the upper alimentary tract of cattle feeding on the bracken fern (Jarrett et al, 1978). Oriel in 1971 recognised the malignant transformation of anogenital warts whereas recently serious study has been made of the

association between cervical cancer and cervical intra-epithelial neoplasia (CIN) and papillomavirus infection (zur Hausen, 1977; Morin et al, 1981; Crum et al, 1984).

Recently there has been renewed interest in some of the human papillomaviruses (HPV's) as carcinogenic agents in humans. Examples of tumours in which HPV's have been isolated include common warts (Green et al, 1981), genital warts (de Villiers et al, 1981; Gissman et al, 1982), cervical dysplasias and carcinomas (zur Hausen, 1976; Meisels et al, 1982), various non-verrucoid skin tumours (Kawashima et al, 1986; Spradbrow et al, 1983; Scheurlen et al, 1986; Gassenmaier et al, 1986), conjuctival neoplasia (McDonnell et al, 1986) and oesophageal carcinoma (Kulski et al, 1986).

Human Infections

To date 51 distinct HPV's and several sub-types have been identified. Most of the papillomavirus DNA's under investigation were molecularly cloned from biopsy material and not from purified virions. They are regarded as viral DNAs if they satisfy the following criteria (1) they are in the size range of seven to eight kilobases (kb); (2) they persist in the tumour as extrachromosomal, circularly closed DNA; (3) they have partial sequence homology with other papillomaviruses; and (4) have a genomic organisation similar to other papillomaviruses. A virus isolate is considered novel if it shows less than 50% DNA sequence homology with other viral types under stringent conditions, whereas a sub-type is defined as having greater than 50% homology with a known virus but a different restriction endonuclease map (Coggins and zur Hausen, 1979). The human papillomaviruses have been divided into seven major groups (A-G) based on their molecular relationship ie. they share at least 1% DNA sequence homology and on their clinical and pathological characteristics (Pfister, 1984).

Group A has HPV-1 type virus which is associated with deep plantar warts, verruca plantaris.

Group B includes HPV-2, 3 & 10 which are found in the common and flat warts; verruca vulgaris, verruca plana and epidermodysplasia verruciformis (EV).

Group C has HPV-4 which is found in verruca vulgaris lesions.

Group D includes HPV-5, 8, 9, 12, 14, 15 & 17 which are associated with pityriasis-like lesions of EV and are also found in squamous carcinomas.

Group E contains HPV-6,11 & 13 which are the venereally transmitted agents of genital warts of the condyloma accuminata type. HPV 11 is involved in laryngeal papillomatosis.

Group F contains HPV-7 associated with butchers' warts.

Group G contains HPV-16 and 18 implicated in cervical intraepithelial neoplasia (CIN), genital cancer and Bowen's disease.

Common Warts

Verruca plantaris and verruca vulgaris are the commonest virally induced tumours found in the general population, where approximately 30% of individuals have antibodies to the viruses involved. It has been found that HPV's 1 and 2 are involved in these lesions and can be detected as episomes within the tumour cells. These tumours mainly occur in school children and during early adolescence ; they are totally benign in nature with the exception of two cases

where there was a malignant transition into squamous carcinoma (Grussendorf and Gahlen, 1975; Shelley and Wood, 1981). With so very few reports of such cases it would lend credence to the theory that papilloma virus types found in the common warts do not induce malignant growth in epidermal cells. Carcinomas arising after such infections occur only after long latency periods from sites without earlier wart development. The incidence of verrucae vulgaris is increased in patients with immunodeficiency eg. those who have had renal transplants and more than one year of immunosuppression (Spencer and Andersen, 1970); these may progress to squamous carcinoma.

Genital Lesions

For many years it had been suggested that there was a link between promiscuous sexual activity and the development of cervical cancer. Kessler's epidemiological study in 1983 showed that cervical neoplasia is a sexually transmitted disease. Until relatively recently, herpes simplex type II virus was thought to be the aetiological agent involved in cervical cancer. This was based on data which observed a high correlation between the incidence of cervical cancer and the elevated serum antibody titre to the herpes virus (Rawls et al, 1969). However in 1976 zur Hausen, using molecular hybridization techniques, suggested that specific types of papillomaviruses might be the aetiological agent involved.

i) **Condyloma Acuminata**

Condyloma acuminata, the anogenital wart of man, is venereally transmitted (Teokharov, 1962; Marino, 1964; Oriel 1971 & 1971b; Waugh, 1972; zur Hausen et al, 1975) and is prevalent in populations of high sexual promiscuity (Underwood et al, 1971; Waugh, 1972). Condylomas are generally benign but invasive conversions have been regularly reported (Siegal,

1962; zur Hausen, 1977; Morin et al, 1981; Crum et al, 1984). HPV-6 and 11 are the predominant viral types involved and episomal DNA can be detected in 90% of the lesions (Gissman et al, 1983; zur Hausen, 1985). The viruses have also been identified in low grade cervical dysplasias (CIN) which tend to regress rather than progress to malignancy (Crum et al, 1984). HPV 16 and 18 have also been identified in condylomas but to a lesser extent than in high grade cervical dysplasias (CIN 11 and 111) and carcinomas.

ii) Vulvar, Penile and Vaginal Neoplasia

HPV 16 and 18 have been associated with a range of epithelial atypias, with penile warts, with squamous carcinoma of the penis (Durst et al, 1983), with Bowenoid papulosis (BP) of the vulva and penis (Wade et al, 1979; Steffen, 1982; Ikenberg, 1983; Guillet et al, 1984; Gross et al, 1985), with vulvar and vaginal intraepithelial neoplasia, and with squamous carcinomas (Gissman and Schwarz, 1986). These infections have become a serious clinical problem in sexually promiscuous young women (Oriel, 1981; Syrjanen, 1984; Okagaki, 1984). The correlation of several different manifestations of genital carcinomas with papillomaviruses is now very convincing (Syrjanen, 1986).

iii) Cervical Neoplasia

Epidemiological evidence indicates that cervical carcinoma and other high-grade dysplastic lesions of the female genital tract are sexually transmitted diseases. Cervical cancer has not been reported in nuns

and other virgins (Rigoni-Stern, 1842) whereas the sexual partners of men whose first wives had cervical cancer are at a much greater risk themselves (Gross et al, 1985b; Kessler, 1986). Many of the risk factors for these cancers are indirectly related to certain lifestyles but are indicative of a venereal aetiology. These are the onset of sexual relations at an early age, sexual promiscuity, use of oral contraceptives, lower socioeconomic status and multiple kinds and episodes of infections (Brinton, 1986; Doll, 1986). Cervical dysplasias and carcinomas are more frequent among smokers than non-smokers (Vesey, 1986). The microorganisms which have been implicated as the causative or contributory agents include Treponema pallidum (syphillis), Neisseria gonorrhoeal, Chlamydia trachomatis, Trichomonas vaginalis, herpes simplex and cytomegalovirus, but proof of carcinogenesis has been lacking in every case.

Cervical intraepithelial neoplasia (CIN) is histologically diagnosed in the following stages: 1) CIN grade I: mild dysplasia; 2) CIN grade II: moderate dysplasia; 3) CIN grade III: severe dysplasia and carcinoma **in situ.** These may be followed by invasive carcinoma. The current concept of squamous carcinoma of the cervix is that it is a multi-stage disease developing over a 10 to 15 year period. It is recognised as a three stage disease by the pathologists: a preinvasive, a microinvasive and an invasive stage.

HPV infection of the uterine cervix produces flat acetowhite lesions commonly described as atypical condylomas, which come into the category of CIN (Meisels et al, 1977). These condylomatous lesions may be classed as being (a) of a mild cytological atypia characterised by a high degree of koilocytosis with diploid or polyploid chromosomes. These are likely to regress and have been associated with HPV-6, 10

or 11; (b) a marked cytological atypia which has a low degree of koilocytosis containing aneuploid chromosomes. These are more likely to progress through CIN to squamous carcinomas and are associated with HPV 16 and 18 (Crum et al, 1984; McCance et al, 1985). Cervical cancer has an extremely high association with the presence of HPV 16 or 18, the figure ranging from between 60-90% of cases in various studies (Durst et al, 1983; McCance et al, 1985b; Boschart et al, 1984). This strongly suggests the involvement of these viruses in the aetiology of cervical cancer.

It has also recently been demonstrated, by Kreider and his colleagues (1985), that HPV-II infections induce changes characteristic of koilocytotic dysplasia upon infection of human cervical tissue heterografted beneath the renal capsule of nude mice. Viral production was observed, therefore Koch's postulates were fulfilled for HPV-II being the causative agent of this kind of lesion. Moreover, Kreider et al, (1986) reported that HPV-II particles are produced in considerable amounts in such heterotransplants which offers the possibility of producing viral particles of different HPV's even when they are replicating very poorly under natural conditions. It is anticipated that this technique will establish the causative role of HPV's in CIN and therefore point to a aetiological role of these agents in a clearly pre-malignant disease. Since HPV 16 and 18 have been directly isolated from cervical carcinoma biopsies, it is an important question to clarify their role in this type of cancer.

Interestingly, in condylomatous and CIN lesions of all grades, the HPV-DNA is free and unintegrated, whereas in the majority of malignant tissues HPV types 16 and 18, it is integrated into the host cell genome (Durst et al, 1983; McCance et al, 1985). Although Tooze (1980) wrongly stated

that integration of animal DNA tumour viruses is a pre-requisite for cell transformation **in vitro** and tumour production **in vivo**, it still has to be demonstrated that integration of HPV-DNA accounts for the malignant transformation of cervical cells.

Laryngeal Papillomas and Carcinomas

Laryngeal papillomas are rapidly growing, benign epithelial neoplasms caused primarily by HPV's 6 and 11 (Gissman et al, 1982, Mounts et al, 1982). They are usually located on the true vocal cords and epiglotis, but sometimes involve all of the larynx, the trachea, bronchial tubes and even the lungs. These papillomas are generally subdivided into "juvenile" and "adult" types due to the bimodal age distribution, with one peak between 2-5 years old and the second between 40-60 years old (Quick, et al, 1978). Juvenile papillomas are rare tumours in Europe and the United States, whereas the prevalence is much higher in South American Indians where there is a 33-85% incidence rate (Soneira & Fonesca, 1964). Although the incidence is relatively low it has however a high degree of morbidity and mortality. The juvenile papillomas are often multiple and recur but they do tend to regress spontaneously at puberty. This remission can be temporary or can last the lifetime of the patient. There is little doubt that these papillomas are induced by a papillomavirus ; typical viral particles have been demonstrated by electronmicroscopy (Boyle et al, 1971 and 1973). These juvenile papillomas are almost certainly caused by infection during birth, since the mothers of these children frequently have genital warts (Duff, 1971; Boyle et al, 1973). Further evidence for the association of laryngeal and anogenital papillomas comes from a comparison of their viral DNA's (zur Hausen et al, 1974; Gissman et al, 1977 and 1983). The studies of Steinberg's group (1983) have suggested that the virus is present not only in the

papillomas but in apparently normal tissues taken from both patients with active disease and from patients in spontaneous remission.

The adult papilloma syndrome appears to present itself as a pre-malignant disease. The tumours occur twice as frequently in males as in females. In longitudinal studies the conversion to malignancy is more than 20% (Kleinasser, 1973). How the infection is transmitted in these cases is unknown but it is possible that infection is acquired during the birth process and remains latent or subclinical until the onset of symptoms decades later (Mounts & Shah, 1984).

In the past the treatment of choice was radiotherapy. However it was observed that after an incubation period ranging from 5-40 years, many papillomas became malignant, suggesting a synergistic relationship between x-rays and viral infection (zur Hausen, 1977). Florid oral papillomatosis may also occasionally convert into squamous cell carcinomas (Samitz et al, 1967) without the intervention of x-rays or other treatments.

Whilst episomal forms of HPV-11 DNA can be identified and isolated from benign papillomas, viral DNA had not until recently been identified in laryngeal carcinomas. The development of increasingly sensitive probes has enabled the discovery of integrated and rearranged viral genomes within malignant cells (Scheurlen et al, 1986 & 1986b).

Epidermodysplasia Verruciformis (E.V.)

E.V. is a dermatosis where verruca plana papules appear usually by puberty and disseminate to a great extent in early adult life (Jablonska & McLewsky, 1957). Although perhaps

one of the rarest HPV-associated conditions, it is amongst the most well documented and widely studied. It is seen as two distinct types, flat warts and pityriasis versicolour-like lesions. As the affected individuals get older (20-40 years) around 30% develop malignant skin carcinomas of the Bowenoid or squamous type (Orth et al, 1979). Squamous cell carcinomas develop first at light-exposed sites of caucasians (Schellender & Fritsch, 1970; Ruiter & Van Mullem, 1970 and 1970b; Jablonska et al, 1972) and it has therefore been suggested that sunlight and in particular U.V. radiation might play a role in the development of malignancy. This theory is further supported by the observation that EV is much rarer and has a much better prognosis with respect to the development of malignancy in black races (Jacyk & Subbuswany, 1979). It has also been noted that 27% of caucasian patients have malignant conversion of papillomas after more than 5 years of existing verrucosis generalisata (Touraine, 1961).

Genetic factors too are believed to play a role as there is frequently a family history of EV and an autosomal recessive gene may be involved (Lutzner, 1978). Defects in cell-mediated immunity, which occur in many of these patients, have also been implicated in the aetiology of this condition (Glinski et al, 1976; Prawer et al, 1977), although the presence of an abnormal immune response may not correlate with the development of malignancy as patients with carcinomas have been found to have apparently normal cell mediated immunity (Orth et al, 1980).

Although papillomaviruses have been suspected of being implicated in squamous cell cancer of the skin and mucosa of man for some time (Aaronson & Lutzner, 1967; zur Hausen, 1977) the first demonstration of papillomavirus in such cancers was in 1980 when Orth's group detected HPV-5 DNA in patients with E.V. To date, over 25 different types of

papillomaviruses have been isolated from E.V. patients; however only specific types are associated with the development of malignancy. The flat warts are generally associated with HPV 3 and 10 which tend not to undergo malignant transformation, whereas HPV 5 and 8 are mainly isolated from the pityriasis versicolour-like lesions and they frequently become carcinomatous (Orth et al, 1979). Other HPV's were also found to be potentially oncogenic: HPV 14, 17 and 20 (Kremsdorf et al, 1984; Tsumari et al, 1983; Gassenmaier et al, 1984). Papillomavirus-like particles can be identified in benign lesions by microscopy but not in malignant lesions (Aaronson & Lutzner, 1967); only HPV DNA in genomic form is found in the latter (Orth et al, 1980). An E. V. patient may have benign lesions infected by multiple HPV's of the specific types: HPV - 3, 5, 8, 9, 10, 12, 14, 15, 17, 19, 20, 21, 22, 23 and 24 (Kremsdorf et al, 1982 and 1983; Lutzner et al, 1984; Orth et al, 1980; Pfister et al, 1981 and 1983).

Miscellaneous

A) **Butcher's Warts :**
Butcher's warts, which involve HPV-7, are found almost exclusively on the hands and forearms of veterinary surgeons, meat-handlers and butchers although there is no evidence to suggest that HPV-7 is related to any of the bovine papillomaviruses. Jablonska et al in 1982 found a proportion of butchers (25 of 83) to be infected with two and sometimes three HPV types which generally localised to the palmar and dorsal sites of the hands. They are not malignant and regress spontaneously (Orth et al, 1981).

B) **Focal Epithelial Hyperplasia of Heck** : This is an extremely rare condition in Caucasian races, which was

first identified in North American Indian tribes. It affects the buccal mucosa causing focal hyperplasia and occurs mainly in children and young adults with frequent manifestations in the same family, which may indicate that adults have an acquired immunity against it. They may persist for several years but do not become malignant and tend to regress spontaneously. This lesion has HPV-13 involvement.

Animal Infections

The rabbit papilloma virus is one of the best documented of the papilloma virus group (Shope, 1933). This virus induces skin warts in cottontail rabbits under natural conditions and in domestic rabbits under experimental conditions. These may develop into carcinomas in up to 25% of the cottontail rabbits and in up to 75% of the domestic rabbits (Rous & Beard, 1935; Syverton, 1952). Only two leporine viruses have as yet been identified.

In the model system, papillomas may be induced by intradermal infection of the virus. Malignant progression is enhanced by the application of tar to the papillomas (Rous & Kidd, 1938). Virus on its own can produce papillomatous lesions, whilst tar cannot, but neither the virus or the tar alone can initiate malignancy. However, when applied together to the skin, they induce aggressive malignant neoplasms.

Besides the human and rabbit systems, papillomaviruses have been found in cattle (Olson & Cook, 1951), which will be dealt with in greater detail in this thesis; goats (Davis & Kemper, 1936), horses (Montes & Vaughan, 1975), dogs (Cheville & Olson, 1964), monkeys (Koller & Olson, 1972), hamsters (Graffi et al, 1969), sheep (Gibbs et al, 1975), chaffinches (Osterhaus et al, 1977), and also in mice (Muller & Gissman, 1978).

Bovine Papillomavirus Infections - Range and Tissue Specificity

Bovine papillomaviruses (BPV's) have been extracted from a variety of lesions occurring in different anatomical sites in cattle. BPV was found to be the causative agent of cutaneous fibropapillomas (Creech, 1929); genital fibropapilloma (McEntee, 1952); and it may be associated with bladder tumours of cattle (Olson et al, 1965). "Atypical" skin papillomas have been shown to contain BPV (Barthold et al, 1974). BPV's have been extracted and characterised from alimentary papillomas, alimentary fibropapillomas, penile papillomas, 3 types of teat papillomas and fibropapillomas (Jarrett, 1978; Campo et al, 1981; Jarrett et al, 1984). Experimentally BPV induces meningiomas in calves (Gordon & Olson, 1968); "sarcoid" tumours in the horse (Olson & Cook, 1951; Lancaster et al, 1977); fibroblastic and liposarcomatous tumours in the hamster (Boiron, et al, 1964; Cheville, 1966; Robl et al, 1972; Robl & Olson, 1968; Moar et al, 1986) and fibromatous tumours in $C_3H/1B$ mice (Boiron et al, 1964).

Papillomavirus In Vitro

Bovine papillomaviruses have been shown to transform murine cells in tissue culture (Black et al, 1963; Thomas et al, 1964; Campo et al, 1983); bovine kidney and foetal bovine conjunctiva (DBC) cell lines (Moar et al, 1981b); secondary cultures of foetal bovine heart (Black et al, 1963); foetal bovine skin cells and secondary fibroblastic cultures of various strains of foetal mouse (Thomas et al, 1963 and 1964). BPV-1 which causes fibropapillomas of the teat and fibropapillomas of the penis can transform fibroblasts **in vitro** and have been found to contain viral DNA (Campo et al, 1980). Foetal equine fibroblasts are also transformed with

BPV-1 (Wood & Spradbrow, 1985). BPV-2 which represents the virus isolated from the classical cutaneous fibropapilloma found on the neck and shoulders of cattle has been shown to transform bovine conjunctiva and palatine cultures (Moar et al, 1981).

The Immunology of Bovine Papillomaviruses

Virus antigen was first detected in fibropapillomas using immunofluorescence (Smithies & Olson, 1961). Immunodiffusion was also employed in an attempt to estimate the quantities of bovine papillomaviruses and anti-viral antibodies during the regression of fibropapillomas and the relationship of the latter to anti-viral antibody in the serum. It was however an insensitive technique (Koller et al, 1974). No correlation was found and the fibropapillomas continued to grow in the face of anti-virion antibody (Barthold & Olson, 1974b). The fibroma cells of BPV-induced fibropapillomas lacked viral antigens detectable by their current immunocytochemistry and electron microscopy (Smithies & Olson, 1961; Robl & Olson, 1968), but it was claimed that they possessed a surface antigen detectable by indirect immunofluorescence on live, unfixed, BPV-induced fibroma cells grown **in vivo** and **in vitro** (Barthold & Olson, 1974b). Serially sampled fibropapillomas show considerable histopathological evidence of a major role played by cell-mediated immunity (CMI) in the regression of at least the fibroma component of bovine fibropapillomas (Barthold & Olson, 1974b). Humoral responses (Segre et al, 1955; Lee & Olson 1969 and 1969b; Barthold et al, 1976) have also been documented but again the antibodies present in the sera of these animals do not appear to be related to growth or regression of papillomas. A role for CMI has been supported by the observations that persistent papillomatosis in cattle is associated with a CMI deficiency syndrome (Duncan et al, 1975). Most ofthe data now forth-coming on humoral responses

to BPV's have been obtained by measuring antibody responses to cutaneous and alimentary papillomas using a variety of techniques e.g. immunocytochemistry (PAP technique), complement fixation, ELISA, serum neutralisation and immunoblot assay (Western), the results of which will be discussed later.

Physiochemical Characteristics of Bovine Papillomaviruses

Three physiochemical aspects are relatively important; 1) the ability to agglutinate red blood cells; 2) virus particle diameter and 3) virus theremostability. The above aspects were important formerly due to the fact that these characteristics were used to differentiate between individual papovavirus members. However today the molecular weight of the viral DNA is used to type the different papillomaviruses. Again until recently haemagglutination represented one of the principal methods of quantitating virus and anti-viral antibodies. BPV has been shown to agglutinate mouse RBC's at $4^{o}C$ between pH6.8 and pH 8.4. BPV is eluted at $37^{o}C$. RBC receptors are resistant to receptor destroying enzyme (RDE) and influenza neuraminidase. Haemagglutination is associated with both full and empty viral particles (Favre et al, 1974). We have shown that while BPV-1 has haemagglutinating receptors, BPV-4 has not. However haemagglutination is not a sensitive assay technique.

In searching through the relevant literature it is apparent that there is a wide range of estimates of virus particle diameter using both negative staining technique and thin sections by electron microscopy. There are inherent difficulties in interpreting these preparations as negatively stained specimens may contain flattened virions; in the thin section technique, diameter estimates may be low due to the shrinkage caused by dehydration and embedding (Gross L, 1970). Estimates therefore may vary considerably due to

preparatory methods.

On the thermostability aspect of the virus it has been found that most members are able to survive at least 30 minutes at 60^{o}C. It is of interest that the DNA can withstand high temperatures for long periods largely because of its unique, closed circular form. Crawford et al, 1964, noted that at high temperatures the DNA disassociates into single strands but reassociates on cooling. The increased thermostability of viral DNA over whole virus may be due to the earlier denaturing of the viral capsid protein.

No **in vitro** system of virus replication has yet been developed and virus purification depends entirely on the isolation from tumours (Gissman & zur Hausen 1976; Gissman et al, 1977; Orth, et al, 1977; Jarrett et al, 1980).

The question of whether BPV can infect man has still to be resolved. Papillomaviruses found in the hand warts of butchers were studied by molecular hybridization, restriction enzyme analysis and immunofluorescence (Jablonska et al, 1980). HPV-1, 2, 3 and 4 were detected ; one papillomavirus now called HPV-7 whose DNA did not anneal with any of the RNA's complementary to either HPV-1 to 5 or to BPV-1 was found. The question as to whether this virus is of human or animal origin is still open.

Bovine Papillomavirus Infections

In the last few years an intensive study of BPV's has been undertaken by Professor W F H Jarrett and his group. To date 7 BPV's have been recognised and 6 characterised. Each is associated with specific lesions (Campo et al, 1981; Jarrett et al, 1984 and 1985). The viruses fall into two subgroups : subgroup A causing fibropapillomas, and subgroup B causing true epithelial papillomas (Table 1).

Table 1

Lesion Specificity of Bovine Papillomaviruses

	Virus	Lesion
Group A	BPV-1	1) Penile fibropapilloma 2) Teat frond fibropapilloma 3) Adjacent skin fibropapilloma
Group A	BPV-2	1) Classical skin fibropapilloma 2) Alimentary fibropapilloma
Group A	BPV-5	Teat "Rice Grain" fibropapilloma
Group B	BPV-3	Skin papilloma
Group B	BPV-4	Alimentary (true) papilloma
Group B	BPV-6	Teat (true) frond papilloma

The viruses in Group A transform both fibroblasts and epithelial cells whereas the viruses in Group B transform only keratinocytes. The subgroups can also be clearly distinguished immunologically and by differences in genome size and organisation. In both the fibropapilloma and the squamous papilloma, the viral DNA is present in cells in a non-integrated episomal form. Viral replication takes place only in keratinocytes which are undergoing terminal differentiation. A brief description of the differentiation of the epithelium is necessary to understand the natural progression of viral infection. The epidermis generally matures over a 10-14 day interval by progressive vertical differentiation of basal cells. As they mature they form a prickle cell layer which in turn begins a succession of changes in keratin gene expression (Franke et al, 1986). Superficial to the prickle cell layer comes the granular layer of two to four rows of polygonal cells which lie parallel to the skin surface. At this level the death of the epidermal cells begins. The dead or superficial cells are anucleate and contain a precursor of keratin derived from keratohyalin. Finally, on the surface, the keratin-containing cells become devoid of structure and constantly desquamate ; they are replaced from beneath. Internal mucosal squamous epithelium expresses a different set of keratins as it differentiates ; in the human it does not develop but in the bovine there is cornification.

Infection with papillomavirus apparently requires direct access to one or more basal epithelial cells (Jarrett, 1985). The virus can be demonstrated replicating in the nuclei of cells in the stratum spinosum and in the stratum granulosum (Almeida et al, 1962). After partial disintegration of the cell structures, aggregates of virus are readily demonstrated in the stratum granulosum and corneum by immunocytochemical techniques (Orth, et al, 1971). In other words, epidermal cells are non-permissive for

papillomavirus replication in the beginning of their differentiation process but become more permissive with increasing differentiation. The specific factors allowing the development of papillomavirus replication are still unknown.

BPV-1 Tumours

Jarrett et al, (1980) found that (1) BPV-1 was primarily associated with paragenital lesions because 40% of animals had such tumours on their teats and (2) BPV-2 was associated with cutaneous fibropapillomas in cattle reared in the United Kingdom; Lancaster and Olson, (1978) did a small survey in the USA and found both BPV-1 and BPV-2 in naturally occurring fibropapillomas of the head, neck and flank. Pfister, 1980 obtained similar results but he also found both BPV-1 and 2 in fibropapillomas of the udder.

A comprehensive review of bovine papillomavirus infections has been given by Jarrett (1985).

The basic morphology of cutaneous fibropapillomas induced by BPV-1 and 2 are identical. Experimental infections with purified virus were carried out and the development of the tumours observed by serial biopsies (Jarrett 1985). In these situations it was found that the time between infection and the appearance of the tumour depended on the concentration and infectivity of the virus and the depth of skin scarification made before applying the virus. In the first 2 to 3 weeks after infection, the scarification wound healed but thereafter there was obvious proliferation of fibroblasts which continued for a further 4 weeks. The cells, many of which were undergoing mitosis, had large hyperchromatic nuclei and abundant cytoplasm. At this stage there was no thickening of the granular layer and there was no evidence of either proliferation or cytopathic changes.

This stage could persist for several months in which the fibroma may range from 3 to 20 cm. The study of serial biopsies showed that transformation was taking place in the subepithelial cells and extended by infiltration of the tumour among the hair follicles and skin adnexae. The latter disappeared and the tumour developed as a solid bulbous form. Histologically this was seen as a benign fibroma. As the tumour developed the fibroblasts under the basal layer of the epithelium became very large and contained multiple copies of the viral genome when examined by **in situ** hybridization or DNA extraction. However in our studies, even after numerous attempts by electron microscopy, virus isolation and immunocytochemistry, we failed to detect either virus antigen or virions. In the growth phase of the fibromatous component of the tumour the proliferating epithelium appeared as a thickened crust on the surface of the tumour which in places penetrated deeply into the fibromatous tissue. This downward growing network of epithelium is termed plexiform acanthosis by Professor W F H Jarrett. The final stages of development consisted of a thickened granular cell layer, enlarged keratohyalin granules, hydropic degenerated cell cytoplasm and marginated nuclear chromatins. The latter is associated with the replication of virus particles and these virus containing nuclei move upwards into the hyperkeratotic keratin layer. At this point papillomatosis developed with projecting processes which consisted of proliferating keratinocytes, granular and keratin layers with a central core of blood vessels and connective tissue. Fibropapillomas could last up to 12 months before regressing and all have been associated with BPV-1.

BPV-1 has also been associated with fibropapillomas of the teats and udders of cattle. In 1984 Lindholm et al, carried out a survey of 1000 cattle and found that 37% had teat tumours and of these 28% were attributed to BPV-1. Histologically the development of the lesion was identical to

that of the penile lesion. Three different types of papillomas have been found on teats and udders of cattle and the associated viruses, BPV-1, 5 and 6, have been isolated and characterised (Jarrett et al, 1980; Campo et al, 1981; Jarrett et al, 1984). BPV-5 and 6 will be detailed later.

BPV-2 Tumours

Cutaneous Fibropapilloma

These tumours are commonly to be found on the forehead, neck, upper thorax and back but they can also appear all over the body. Again infection appears to depend on the concentration and infectivity of the virus as well as the depth of the skin scarification made before applying the virus. They manifest themselves as hard, rough and spherical and may number from a few to hundreds. They also may be sessile or pedunculated, smooth or or papillomatous depending on the stage. The development of the cutaneous fibropapilloma is as described for the BPV-1 lesions. By immunocytochemistry, viral antigen can be demonstrated in the cytopathic cells of the stratum granulosum in the final stages of development.

Alimentary Fibropapillomas

In an extensive study of papillomas of the upper tract of cattle which were processed through the Glasgow Abattoir; 7,746 animals were examined and it was found that 19% had tumours (Jarrett et al, 1978). A further study of 100 oesophaguses which had tumours revealed that 78% had squamous papillomas (BPV-4) and 22% were fibropapillomas which were later found to be associated with BPV-2 (Jarrett et al, 1978 and 1984). A detailed study of 60 cases of alimentary fibropapillomas was embarked upon. These tumours ranged in size from 1mm to 30cm and usually occurred in a multiple form

as against the finding of a single tumour. The lesion was white and generally smooth but occasionally ulceration of the surface was seen. This benign tumour was found in the oesophagus, oesophageal groove and the rumen. Some cases were pure fibromas of the subepithelial fibrous tissue. Others showed the development of acanthosis (thickening of the prickle cell layer), parakeratosis (retention of nuclei in the cells of the keratin) and hyperkeratosis (thickening of the horny layer) of the epithelium overlying the fibroma; pegs of this epithelium branched into the fibromatous tissue. Despite the extensive use of immunocytochemical techniques with a range of antisera to different BPV's no evidence of replicating virus was found. The same result was achieved when examined by the electron microscope. However BPV-2 viral genome was detected by blot hybridization in all of the specimens examined (Campo et al, 1982; Jarrett et al, 1984). The BPV-2 DNA was present in the fibropapilloma cells as multiple episomal copies. When sections were examined by **in situ** hybridization techniques using cRNAs from BPV-1,2,4 & 5 genomes there was no evidence of a degree of viral DNA replication which would be found in a productive viral infection; however the tumour cells did contain low numbers of episomal copies of the viral genome.

BPV-3 Tumours

This virus was isolated in Germany from a cutaneous papilloma found in Australian cattle (Pfister et al, 1979). The tumour appeared as an epithelial lesion with no fibromatous component; the virus is distinct from the subgroup members, BPV-4 and 6.

BPV-4 Tumours

Alimentary squamous papillomas are warts of the frond type with no fibromatous component or plexiform acanthosis

(Jarrett et al, 1978). These alimentary papillomas were studied in great detail by Professor W F H Jarrett and his group after it was found that large numbers of these wart-like lesions were present in alimentary carcinoma cases (Jarrett et al, 1978 and 1978b). A very high incidence of squamous cell carcinomas of the upper alimentary tract in cattle grazing on the bracken infested upland areas of Britain was found. As a result an abattoir survey was instigated to determine the incidence and aetiology of squamous papillomas of the alimentary tract of these areas. The upper alimentary cancers were confined to five specific sites:

1) dorsum of the tongue - 7%;
2) soft palate - 5%;
3) oropharynx - 8%;
4) oesophagus - 50%;
5) anterior part of the stomach - 30%.

A noticeable feature of these cases were the presence, at the same sites as the cancers, of squamous papillomas (Jarrett et al, 1978). These benign tumours had not been reported in Britain and were not thought to be common in cattle ; they have been noted in Germany, America and Kenya (Thorsen et al, 1974). This was understandable as the majority of cases seen in cattle have contained a single lesion and could have easily been overlooked. However we have had a few cases where several hundred papillomas have been found in the mouth, tongue, pharynx and oesophagus.

Alimentary papillomas in cattle are associated with bovine papillomavirus 4 (BPV-4);(Jarrett et al, 1978b) which could be isolated from young tumours and were distinguished from other BPV's by both molecular biological techniques and by immunocytochemical methods (O'Neil, 1982). Alimentary papillomas have the typical structure of a frond papilloma.

Each tumour is composed of a number of sub-units, or fronds of transformed cells each terminating in a keratinised tip. Virus replicates at an early stage, when cytopathic changes are taking place in the immediately sub-keratin layer. This appeared in cells which were undergoing cytopathic changes as a large intranuclear inclusion body when examined by light microscopy. On E.M. examination these inclusion bodies were composed of crystalloid arrays of virus particles. As the papilloma grew these individual sub-units or fronds extended upwards and at this stage there was a lower virus content. The virus was restricted to sites of active keratinisation, which only occurred between and near the base of the fronds. By **in situ** hybridization techniques there were large copy numbers of viral genome found in the keratinocytes, however after approximately 8 months duration both replicating virus and viral genome was difficult to locate in the epithelium of the lesion.

BPV-5 Tumours

"Rice Grain" fibropapilloma is a very common lesion in cattle and from this our group has isolated and characterised the associated virus which is designated BPV-5 (Jarrett et al, 1980; Campo et al, 1981). The name "rice grain" was derived from the tumour size and the fact that it had a smooth, white, elongated appearance. Unlike BPV-1 and 6 induced tumours, the rice grain lesion does not adopt the colour of the skin in which it is located, but is always white.

This lesion has also three phases of development when studied by light microscopy. The first phase that can be visualised is a small fibroma of 2-3 mm diameter. Secondly, over this fibroma develops a plaque of epithelium, largely keratinocytes, about 50 cells thick. This plaque stage has no increased granular layer, no cytopathic features and no virus replication was visualised either by electron microscopy or

immunocytochemistry. Thirdly, at a later development stage, the granular layer thickens to 25 - 30 cells. Cytopathic changes now appear in the mid-area of this thickened granular layer and the keratohyalin granules become large and distorted. As the cells migrate towards the surface, virus replication could be seen to develop in them, whereas in the lower layers there was a marked increase in the genome number per cell which could be detected by **in situ** hybridization. This is a very common tumour which often appears as multiple papillomas but the majority of animals examined were found to have multiple infections i.e. BPV-1, 6 and 5.

BPV-6 Tumours

There is another very common infection which frequently occurs in association with BPV-1 and 5 (Lindholm et al, 1984) on the skin of the teats and udder of cattle and is known as frond epithelial papilloma. An abattoir survey was conducted of 1000 unselected slaughtered cattle to determine the incidence of BPV infection; and it was found that 37.3% had teat tumours and 86.2% of these had multiple infections ; BPV-6 virus was isolated and characterised from 92.3% of these lesions.

In pure frond epithelial papillomatosis there is no fibroma, no plaque formation and no acanthosis. The tumour has a pedunculated base and the line of basal cells forms a curved boundary with the underlying normal dermis. The next layer consists of keratinocytes which are arranged in columns at right angles to the base of the tumour producing the individual fronds; similar to those induced by BPV-4. These fronds are enclosed by a layer of keratin. Where the fronds merge together at the base of the lesion there is an area of union of the keratin of adjacent fronds and the underlying granular layer where there is also active incorporation of keratinising cells into the horny layer: this is called the

incorporation zone (Jarrett et al, 1984). In experimental studies we have shown that virus can be detected quite easily in the first 30 days of development of the tumour, when there is cytopathic changes occurring in the thickened granular layer. After about 90 days a similar pattern to that of the BPV-4 lesion emerges. There is a disappearance of virus containing cells from the granular-keratin layer interface and the areas of viral cytopathic effect are confined to incorporation zones.

The Association of Bovine Papillomaviruses with Cancer in Animals

Jarrett et al, (1978) showed that in the upland areas of Scotland and the North of England there is an extremely high incidence of alimentary cancer in beef cows whereas in the lowland areas the disease is virtually unknown. The environmental factor which is thought to be involved in this phenomenon is bracken fern as the farms with higher infestations of bracken inevitably have higher incidences of carcinoma and other tumours. Another surprising feature evident to the group was that on studying intestinal lesions 40% of the squamous carcinoma cases had adenomas and 16% adenocarcinomas of the intestines which were identical in type to those found in man (Jarrett, 1978).

Bracken fern contains a carcinogen that experimentally can induce tumours in laboratory animals (Evans, 1972) and are mutagenic (Bjeldanes & Chang, 1977; Sugimura et al, 1977). Bracken feeding can induce urinary bladder neoplasms in cattle (Rosenberger, 1971; Pamukcu et al, 1976) and these have also been found in the same herds as the squamous carcinoma cases investigated by Jarrett and his workers (1978). These animals showed clinical signs of haematuria, haemorrhagic anaemia and a wasting syndrome. Experimentally, we have consistently been able to induce, by

the feeding of bracken for periods of 1 to 2 years, carcinomas of the transitional epithelium and haemangioendotheliomas of the subadjacent connective tissue. Olson et al, (1959 & 1965) and Brobst, (1965) showed that suspensions of skin papillomas could induce urinary bladder tumours and conversely that suspensions of bladder tumours could induce papillomas of the skin. In naturally occurring and experimentally induced bladder tumours in cattle, we have demonstrated the presence of BPV-2 genome but we have never been able to locate any replicating virus or viral antigens (Jarrett et al, unpublished data). We have also been unable to demonstrate papillomavirus genomes in samples of normal bladder epithelium.

Bracken fern has induced tumours in mice, rats, guinea pigs and quail experimentally (Evans, 1972; Pamukcu et al, 1976; Miyakawu et al, 1975). When fed dried bracken it induced intestinal and urinary tumours in rats and lung adenomas and lymphoid neoplasia in mice. The milk from bracken-fed cows has been implicated in the induction of urinary bladder carcinoma in mice and rats. Bracken fern is eaten by humans especially in Japan and such preparations retain carcinogenicity for rats. Certain areas in Japan have a high incidence of gastric carcinomas in humans and there is epidemiological evidence to suggest that eating bracken increases the risk of oesophageal cancer in the human (Pamukcu et al, 1976).

The other BPV which offers an excellent **in vivo** model system for the virally induced carcinogenesis story, is BPV-4. This has been studied in depth in recent years and it was found that there was a high incidence of alimentary squamous carcinoma in cattle at the same sites where they found the squamous papillomas (Jarrett et al, 1978). BPV-4 virions have been isolated from these benign papillomas originating in the upper gastrointestinal tract of cattle (Campo et al,

1980). However we have never been able to detect BPV-4 genome in any of the carcinoma cases that we have investigated. It is therefore currently accepted that the BPV-4 genome is not required for progression to, or maintenance of the malignant phenotype (Campo, et al, 1985). A few hypotheses have been offered to explain the interaction of the virus found in pre-malignant lesions, and the association of the malignant development of the lesions in the presence of bracken fern :

1) BPV-4 acts as an initiator causing changes in cellular DNA which is then acted upon by the bracken fern to promote a full progression to malignancy.

2) BPV-4 stimulates an increased number of keratinocytes and expression of proto-oncogenes, which would increase the chances of a malignant conversion if confronted with a carcinogen.

Recently in Kenya our group isolated a papillomavirus from a lesion of the eyelid in cattle. This papillomatous lesion of the periocular skin is very common in Kenya and other sunny areas of the world. This lesion was found at the same site, ie periocular areas in which squamous carcinoma also develops. These lesions are initially found as plaque like or papillomatous lesion of the eyelid before the onset of a malignant lesions is diagnosed. Australian scientists have also isolated a papillomavirus from similar lesions (Spradbrow et al, 1983 and 1983b). The virus we isolated has not been characterised therefore the association of the virus and the malignant development cannot be explained at this moment.

Equine sarcoid is a common tumour found mainly on the legs of the horse and donkey. It develops into a fibrosarcoma with a form of plexiform acanthosis of the overlying epithelium.

Olson and Cook (1951) experimentally induced similar lesions by intradermal inoculations of bovine papillomavirus, which gave credence to the idea that sarcoid tumours might be associated with papillomavirus infections. Lancaster and his group (1977 & 1979) found the presence of BPV-1 and 2 type DNA in both natural and experimental equine tumours.

CHAPTER 2

DEVELOPMENT OF TECHNIQUES FOR BOVINE PAPILLOMAVIRUS INFECTIONS

Development of Techniques for Bovine Papillomavirus Infections

This chapter deals in detail with the development of the methodology to purify, transmit, immunise and localise bovine papillomavirus antigens.

As in all research into infectious diseases, it is important to develop or adapt and improve existing techniques to suit the current investigations. The purity and sensitivity of all the materials used is extremely important when attempting to mimic a natural biological disease and for the understanding of the responses.

These techniques are consistent for all the experimental work detailed in this thesis unless otherwise stated.

Purification of Bovine Papillomaviruses

Since papillomaviruses cannot be grown in cell culture, virus has to be obtained by extraction of tumours. Maximum extraction of virus has to be achieved on account of the expense that the technique involves; this demanded improvement of the maceration methods.

The literature contains many methods for extracting papillomavirus but most of these employed as starting material, large quantities of pooled tumours from several cases of known virus content (Crawford, 1965,; Favre et al, 1975). The use of pooled tumours from different cases was precluded in this work because of the possibility of mixing different viruses from the same anatomical site (but see chapters 3 & 5)

In any investigation into an infectious disease, the quantity and infectivity of the challenge virus, the degree of application together with the route of administration of that challenge is important. The route of challenge in all the experiments was at the same sites as the tumours occurred naturally.

Some variability in the infection following application of the virus cannot be totally unexpected considering the wide range of severity found in natural outbreaks of papillomatosis in cattle. Many factors are likely to influence the development of these lesions including a) the antibody status of the recipient, b) the quality of the virus used for transmission and or challenge and c) the amount of virus administered. The amount of virus given as a transmissible agent or as a challenge dose was difficult to quantify and was variable due to many factors; the lack of biological assays using cell cultures, the difficulty of obtaining virus spreads which give statistically accurate virion counting techniques such as latex particle mixing and finally the presence of low virus yields from certain tumour types.

In view of the relative durability of papillomaviruses, a purification method had to be devised which would produce a high yield coupled with a high degree of purity without an appreciable loss of infectivity. To try and fulfill these objectives many methods were attempted and discarded except for the following 2 techniques which involved the use of density centrifugation.

In the first method of centrifugation, rate zonal separation, the sample is layered on to a preformed sucrose gradient. Under centrifugal force the particles sediment through the gradient at a rate which is determined by their mass and size. To achieve separation using this technique, the

densities of the sample particles must be greater than the density of the gradient at any given point and centrifugation must be terminated before any of the separated zones reaches the bottom of the tube.

In the second method of centrifugation, the isopycnic technique, the sample is layered onto a preformed density gradient, which encompasses the densities of all particles in the sample. The gradient is formed using Caesium Chloride solutions of increasing densities. Under centrifugal force the particles sediment to the position in the gradient where the density of the solution is exactly equal to their own. Separation using this technique is on the basis of particle density and size only affects the rate at which particles reach their position of isodensity. This is independent of time, after sufficient time has been allowed for each particle to reach its isopycnic position.

Materials and Methods

All tumours were stored in 50% phosphate buffered saline in glycerol for fourteen days or more, as this softens the tumours and aids the extraction procedure. The tumours were collected, unless otherwise stated, from individual animals.

Purification by Rate Zonal Centrifugation

The tumours were cut up finely with scissors before being weighed so that the final weight per volume was adjusted to 10%. Tris buffer (0.1M NaCl, 1mM Tris pH 8.0, 1mM EDTA) containing 0.5% sodium deoxycholate and 0.5% Nonidet 40 (NP40) was used as the diluent. The tumours were homogenised on ice using a Silverson homogeniser for 20 minutes, the resultant supernatant being clarified at 2,000 rpm in a bench centrifuge for 10 minutes. This was followed by a further

clarification in a Sorvall OTD 50 ultracentrifuge at 10,000 rpm for 10 minutes at 4^{o}C, using a SW-41 rotor.

Sucrose velocity gradients were made in SW-41 Ultra-Clear tubes (Beckman) by layering 5ml of 10-20% sucrose on top of a 1ml bed of 1.5g/cc caesium chloride (CsCl). The clarified material was carefully layered on top of the gradient and centrifuged at 40,000 rpm for 20 minutes. The gradients were examined against direct light and if in sufficient concentration, the virions could be visualised as a light-scattering band, which was found at the interface of the caesium chloride and the sucrose. The virus was collected by piercing the bottom of the tube with a needle. The virus was then passed through an equilibrated Sephadex G-25 column. The virus was subjected to a further sucrose velocity gradient purification procedure as described above, before being checked quantitatively by transmission electron microscopy using phosphotungstic acid negative staining.

Tris Buffer Saline (TBS) x 10

Sodium chloride	116.9g
Tris	24.2g
Ethylenediaminetetra acetic acid (EDTA)	6.7g

The above chemicals were dissolved in 1900ml of distilled water and adjusted to pH 8.0 with concentrated hydrochloric acid (HCl) before making up to 2 litres with distilled water.

TBS x 1 for use

To 200ml of TBS x 10, 1800ml of distilled water was added and the pH adjusted to 8.0.

20% Sucrose

216.2g of sucrose were dissolved in 1 litre of distilled water and the refractive index was ascertained to be 1.3638 and the density (D) 1.0829.

10% Sucrose

The 20% sucrose was diluted with equal parts of distilled water and the refractive index was ascertained to be 1.3478 and the density 1.04.

Caesium Chloride - 1.5g/cc

6.7183g of caesium chloride were dissolved in 10ml of TBS so that the refractive index was 1.3812.

Purification by Isopycnic Centrifugation

All tumour material to be purified by isopycnic centrifugation was initially subjected to a single rate zonal separation as described before.

Preformed caesium chloride gradients were made in SW-41 Ultra-Clear tubes (Beckman) by adding solutions of caesium chloride (CsCl) of densities ranging from 1.2g/cc up to 1.5g/cc. 1.5ml of a 1.2g/cc density CsCl solution was added to the centrifuge tube by means of 2mm tubing attached to a syringe, which was displaced upwards by 1.5ml of the 1.3g/cc density CsCl being added to the bottom of the tube. This procedure was repeated until the 1.5g/cc density CsCl solution was in place. The gradients were allowed to form for 24 hours at $4^{o}C$ before using. Before use a 1ml layer of a 1.2D sucrose solution was gently added to the top of the

caesium gradient to prevent disruption of the preformed gradient by the addition of the zonal purified virus suspension. Finally 5.0ml of virus suspension was added to each gradient and centrifuged at 40,000 rpm for 18 hours. The gradients were examined against direct light and if in sufficient concentration, the virions could be visualised as a light-scattering band, which was found at the interface of the caesium chloride and the sucrose. If no light-scattering band could be seen the gradient was then fractionated into 10 drop aliquots by piercing the bottom of the tube with a needle, which were then examined by a refractometer to establish the respective refractive indices of the serial samples. Virus was located in the region of 1.32g/cc. The appropriate virus fraction was dialysed against Tris buffer overnight at $4^{o}C$ with continuous agitation before being examined by transmission electron microscopy.

Caesium Chloride (CsCl)

Caesium chloride solutions were prepared by dissolving the following weights of CsCl salt in 10ml of distilled water.

1.2D - 2.77g
1.3D - 4.02g
1.4D - 5.48g
1.5D - 6.73g

Sucrose - 1.2D

5.2g of sucrose were dissolved in 10mls of distilled water.

Phosphotungstic Acid Negative Staining

Materials:

An aqueous solution of 2% phosphotungstic acid (PTA) at pH 7.2 was freshly prepared. Grids were coated with a very thin parlodion support film. To stabilise the plastic film the grids were coated with a thin film of carbon (approx. 50A^{o}) over the parlodion.

Parlodion solution

A 3% stock solution of parlodion in amyl acetate was made by dissolving 0.3g parlodion pieces in 10ml of amyl acetate and allowed two days to dissolve. 0.6% is used for coating the grids, i.e. 0.2ml stock 3% parlodion solution diluted with 0.8ml amyl acetate.

Method:

1) One drop of the virus material was dispensed on to a grid using a micro-pipette.

2) This was kept at room temperature for 2 minutes before removing the excess fluid with filter paper.

3) One drop of 2% PTA solution was added and kept for 2 minutes at room temperature. The excess fluid was removed with filter paper.

The grid was examined on a Zeiss 109 electron microscope.

If a sample contained any traces of caesium chloride it was rinsed for one minute with 0.15M ammonium acetate.

Conclusion

From repeated purification attempts of bovine papillomavirus from tumour material it would appear that rate zonal centrifugation can produce a relatively high yield of virus without an appreciable loss of infectivity. This has been substantiated by the viruses ability to be experimentally transmitted to other cattle, by the high titre antibody levels obtained after immunising rabbits, and when examined by other immunological techniques (ELISA & Western Blot), details of which are not covered in this thesis. However there are present, in the virion sample purified by rate zonal centrifugation, intermediate fibres from keratinocytes. These filaments obviously have the same buoyant density and as such cannot be eliminated.

Virus samples which contain these contaminants can readily be purified by subsequent isopycnic centrifugation but in our hands the resultant virus is less antigenic and is unsuitable for certain procedures and assays (tissue culture, AGID and raising mono-specific antisera), details of which are not covered in this thesis. At the onset of this virus purification procedure , three criteria were established; a high yield; a high degree of purity; and a regime which will not appreciably affect the infectivity. These criteria appear to be fulfilled, especially by the rate zonal centrifugation method, which has been successfully applied to the majority of the work presented in this thesis (Figure 1).

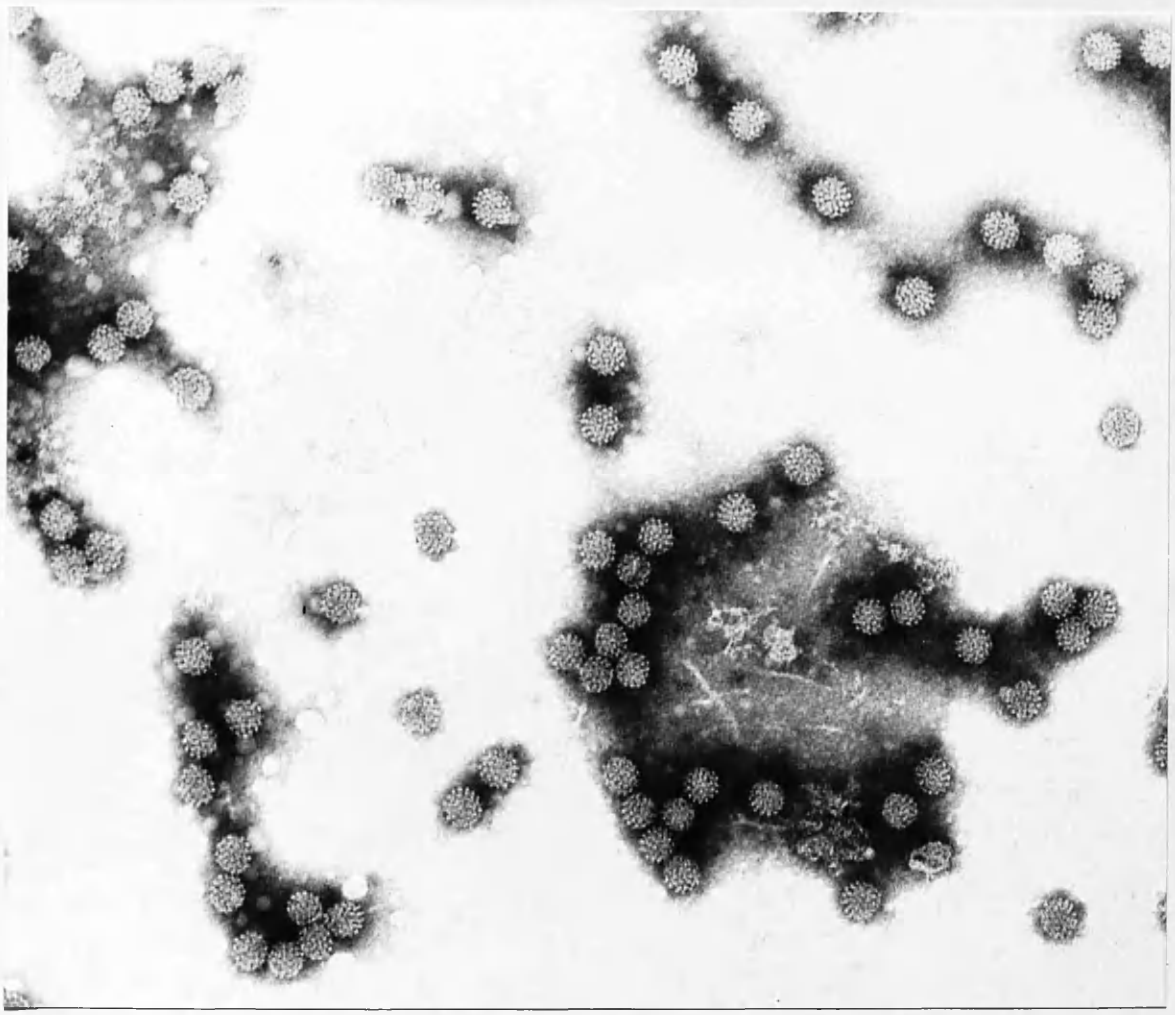

Figure 1: Bovine papillomavirus purified by rate zonal centrifugation and negatively stained by PTA. (x 100,000).

Raising Antisera to Bovine Papillomavirus

As described in chapter 1, 5 BPV's have been found associated with a number of lesions in cattle in the U.K. (Jarrett, 1981). Papillomaviruses from different species are immunologically distinct and antisera raised against intact virions show homologous reactivity but no heterologous cross-reactivity. Shah et al., in 1979 and Jenson et al., in 1980 used BPV-1 and HPV-1 disrupted by sodium dodecyl sulphate (SDS) and demonstrated cross reactivity by immunocytochemistry.

Young, healthy rabbits were selected and 20ml of blood was removed and the serum collected. This pre-immunisation serum was subsequently used as a negative control serum for each specific rabbit antisera produced.

Virus was purified as described by zonal and isopycnic density gradient centrifugation. For practical purposes we have used preparations whose viral content has been estimated by EM negatively stained preparations. The preparations that were used to immunise the rabbits contained approximately 10^{11} to 10^{12} particles per ml.

Before administering the virus plus Freund's adjuvant, it is necessary to determine the integrity of the emulsion by allowing a drop of the suspension to fall onto the surface of cold water. If the droplet remains intact this indicates that the aqueous phase containing the antigen is entirely closed within the oil and it is now fit to use.

Two different sets of antisera were raised ;

1) mono-specific
2) cross-reacting ("split")

The method of injection, site and timing is the same for both types of antisera.

Mono-Specific Antisera

The primary injection consisted of 1ml of purified whole virus suspension with an equal volume of Freund's complete adjuvant (Miles Laboratories Ltd.). There were two further booster injections at weekly intervals with equal volumes of virions and Freund's incomplete adjuvant. The injections were administered deep into the right and left quadricep muscles. Ten days later, the rabbit was bled, by nicking the marginal vein of the ear. Two universals of blood were taken (40ml) and the serum obtained provided the initial batch of rabbit anti-bovine papillomavirus antisera. This was aliquoted, labelled and stored at -20^{o}C until required. After a lapse of 12 weeks the rabbits were again boosted with an equal volumes of virions and Freund's incomplete adjuvant.

Cross-Reacting ("Split") Antisera

The purified BPV virions were disrupted with sodium dodecyl sulphate (SDS) and mercaptoethanol (ME) (Favre, et al, 1975). 0.3ml of purified virus was mixed with 30ul of 10% SDS and 5ul ME and boiled for one minute. The suspension was brought up to 1.2ml with phosphate buffered saline and emulsified with an equal volume of Freund's adjuvant. The procedure is the same as for the mono-specific antisera.

Conclusion

Using the PAP technique to evaluate response, it was found that high titre, cross-reactive antisera was consistently being obtained when virions purified by isopycnic

centrifugation and disrupted by SDS were used. On the other hand there was a very poor antibody response when mono-specific antisera were raised using isopycnic centrifuged virions. The caesium chloride obviously had an adverse effect on the infectivity of the viral capsid antigen. Details of this work are not included in this thesis.

After many immunisation attempts it was decided to use a double rate zonal centrifugation purification procedure even although there are contaminants present in the antigen preparation which will produce an antibody response. These contaminating antibodies are easily diluted out when high titre specific antisera was raised. Antibody levels in antisera raised against disrupted virions as determined by the PAP technique were consistently > 1:500,000.

Immunocytochemical Localisation of Bovine Papillomaviruses

Immunocytochemistry is one of the most sensitive research tools available. Its potential for localising various antigens at the light microscopical level provides us with correlation between structure and function which is necessary in biological research.

Antibodies were raised by immunising rabbits with an antigen purified as highly as possible. The resulting antiserum is not directed solely to the desired antigen as a single antigen molecule contains several characteristic determinants or epitopes and will also contain many "natural" antibodies, which may or may not react with tissue components. Since there are many B-cells producing antibodies against each epitope, this is termed a polyclonal (many cells) antibody. In this thesis the research findings are based on the use of polyclonal antibodies only ; they have proved to be accurately defining virus types with the exception of BPV-1 and 2 which share major antigens. Immunocytochemistry requires antibody to be of high affinity for its antigen, in other words that its binding sites fit well with the antigenic sites on its specific antigen and do not attach to other antigens ; as well as the avidity, or binding strength. A high titre antiserum will allow a higher dilution to be made, which will enable the unwanted or non-specific antibodies, which might react with tissue components, to be diluted out.

Whilst the immunoperoxidase techniques have many advantages, some technical difficulties may be encountered with the procedures. Tissue sections require, in most cases, pre-treatment with trypsin to unmask cross linked antigenic determinants. In addition, endogenous peroxidase activity has to be quenched if not completely eliminated. Finally, you

have to include three control sections with every procedure to ensure the specificity of the reaction. However, this is more than compensated for in the final result, as it is now possible to distinguish and localise specific papillomaviruses.

The major advantage of the technique is that many antigens and not only viral antigens can be demonstrated in routine paraffin embedded material which had been formalin fixed (Sternberger, 1979). This facilitates the use of retrospective studies of material which has been stored.

Of the numerous immunocytochemical methods available for visualisation of antigens the peroxidase anti-peroxidase (PAP) technique was found to be the method of choice for all the experimental work detailed in this thesis. This technique was employed on formalin and corrosive formol fixed material, which was then embedded in paraffin wax.

The method is described on the following page.

Immunoperoxidase Technique (PAP) Adapted from Sternberger, 1974

1) The sections were deparaffinised in xylene and rehydrated in alcohol.
2) They were then immersed in Lugol's iodine followed by immersion in 5% sodium thiosulphate to remove mercuric chloride artifact, followed by a wash in running tap water.
3) Endogenous peroxidase was inhibited by treating with freshly prepared 0.5% hydrogen peroxide (H^2O^2) in methanol for 30 minutes.
4) The sections were then washed well in distilled water.
5) The temperature of the slides was equilibrated in distilled water at 37°C for ten minutes.
6) Sections were treated with 0.1% trypsin in 0.1% calcium chloride (adjusted to pH 7.8 with N/10 NaOH) for 30 minutes at 37°C.
7) The sections were transferred to cold distilled water with agitation for 2-3 minutes.
8) Sections were washed in two baths of Tris buffered saline (TBS) pH 7.6 containing 1% normal swine serum (TBS/NSS) for 5-10 minutes each (to reduce the staining of non-specific tissue binding sites).
9) The primary antiserum, Rab/BPV, at optimal dilution was applied The diluent used was TBS containing 0.1% bovine serum albumin (BSA) and 0.01% sodium azide (this solution prevents the proteins from adhering to the glass) - 1 hour. Overnight incubation was used with higher dilutions.
10) The sections were washed in two baths of TBS/NSS for 10 minutes each.
11) The bridging antibody ; swine anti-rabbit IgG at 1/20 dilution was applied for 60 minutes.
12) Sections were washed in two baths of TBS/NSS for 10 minutes each.

13) The PAP (peroxidase/rabbit anti-peroxidase) at 1/20 dilution was applied for 30 minutes.
14) The sections were then washed in two baths of TBS for 10 minutes each.
15) The peroxidase reaction was developed with DAB (Graham and Karnovsky, 1966) for up to 5 minutes before ;
16) Washing in TBS followed by washing in distilled water.
17) The preparation was counterstained with Mayer's haematoxylin and differentiated in 1% hydrochloric acid in alcohol before blueing in Scott's tap water substitute (STWS).
18) The sections were dehydrated through meth. spirit and alcohol before ;
19) Clearing in xylene and mounted with a resinous medium.

Result : Peroxidase activity stained brown.

Controls :

The following were routinely employed when performing the technique.

1) Known positive control tissue was included to eliminate technical error.
2) Primary antibody was omitted to check endogenous peroxidase activity and the non-specific binding of the secondary antibody.
3) Both the primary and the bridging antibody were omitted to ensure there was no cross-reaction between the rabbit peroxidase anti-peroxidase and the antigens on the tissue section.
4) The primary antibody was omitted but was substituted by the pre-immunisation serum from the rabbit.

10% Buffered formaldehyde (pH 7.0).

Sodium dihydrogen orthophosphate	4.0g
Disodium hydrogen orthophosphate	6.5g

The above chemicals were dissolved in 900ml of distilled water and 100ml of formaldehyde added.

Corrosive formol.

100ml of formaldehyde was added to 900ml of saturated mercuric chloride.

Tris/HCl buffered saline (TBS) pH 7.6.

The following chemicals were dissolved in 10 litres of distilled water :

Sodium chloride	80.0g
Tris (Tris hydroxymethyl methylamine)	6.05g

Finally 38ml of N hydrochloric acid was added.

DAB (Graham and Karnovsky)

25mg of 3,3′ diaminobenzidine tetrahydrochloride was dissolved in 50ml of 0.05M, pH 7.6 Tris/HCl buffer and 0.15ml of 3% hydrogen peroxide was added immediately before immersion.

Tris/HCl buffer for DAB

12ml of 0.2M Tris (24.228gm/litre) and 19ml of 0.1N HCl was added to 19ml of distilled water. The pH was adjusted to 7.6.

Mayer's haematoxylin.

The following were dissolved in 1 litre of distilled water :

Haematoxylin	1.0g
Sodium iodate	0.2g
Potassium alum	50 g
Citric acid	1.0g
Chloral hydrate	50 g

Scott's tap water substitute (STWS).

The following chemicals were dissolved in 1 litre of distilled water :

Sodium bicarbonate	3.5g
Magnesium sulphate	20 g

The swine anti-rabbit bridging antibody and the PAP (rabbit) were both supplied by Dakopatts Ltd.

Conclusion

The PAP technique has many advantages over previous immunocytochemical methods and has replaced fluorescein as the antibody label of choice in many circumstances. From the results obtained in the experimental material detailed later in this thesis, it is evident that the PAP technique has three major features. The first is its permanence; where sections may be prepared and stored for retrospective studies. The second feature is that excellent histological definition is possible since formalin fixed material is suitable for this technique, which greatly facilitates the identification of the virus containing cells and its

location. Thirdly and most importantly is the sensitivity of this technique, which enables the recognition in fixed tissue of antigenic sites.

Having developed the immunoperoxidase technique for the detection of papillomavirus infections in cattle, it was now possible to locate precisely the antigen distribution and its relationship to the development of the lesion.

CHAPTER 3

EXPERIMENTAL TRANSMISSION OF BOVINE PAPILLOMAVIRUS INFECTIONS

Introduction

It is well established in the case of HPV and the BPV systems that there are numerous virus types (i.e. at least 7 bovine associated viruses and 51 human associated viruses).

It was of major importance to establish whether the immune and protective phenomena were induced by **group specific** or **type specific** antigens and whether there was any degree of cross-immunity between types within a species.

Obviously this would be extremely difficult to investigate in humans and the bovine is the only animal species in which this could be attempted at the moment as several manifestations are common to the two systems; multiple virus types, specificity of lesions, malignant progression of some papillomas and the involvement of co-factors.

This is of major importance in putative vaccination for the future. It was therefore decided that the aims of this thesis were to be three-fold;

1) To study the development of BPV induced tumours and their virus cycle.

2) To determine whether the resistance to bovine papillomavirus induced infections is by group or type specific antigens.

3) To determine the efficacy of several prophylactic vaccination regimes.

Experiment 1.

Experimental Transmission of Bovine Cutaneous Fibropapilloma by BPV-2

BPV-2 is associated with cutaneous fibropapilloma found on all parts of the body but mainly on the forehead, neck, upper thorax and back of cattle. These infections can last for anything up to one year before spontaneously regressing but occasionally some animals are unable to reject the infection and have to be destroyed because of the widespread nature of the lesions. In the following sets of experiments we have the opportunity to study both the tumour cycle; infection -> tumour induction -> tumour regression -> reinfection; and the immunity to papillomavirus infections.

Materials and Methods

Calves

Two calves were purchased from a reputable dealer who previously clinically examined both the calf and the dam and were selected as being free of any obvious papillomavirus infection. They were 12 week old, male, Ayrshire cross calves and on arrival they were immediately housed in a clean, well ventilated pen in the isolation unit. They were then bled for both haematological analysis and for serum, the latter being stored at -20^{o}C for future studies. Three weeks later, when the calves were 15 weeks of age and the maternal antibodies were assumed to have disappeared, the experiment was initiated. These calves (49 & 50) were originally involved in the cell membrane vaccine experiment which will be discussed later. Serial biopsies were used to detail the development of the lesions.

BPV-2 Virus Preparation

Virus was purified from a fully developed cutaneous fibropapilloma of the upper body of a cow (76119/10) as described on page 33 using rate zonal centrifugation. A sample of the resultant virus was sent to Dr S. Campo for restriction mapping and hybridization and was subsequently confirmed as containing only BPV-2 genomic DNA. A sample of virus was also sent to Dr H. Laird for quantitative estimation by TEM using phosphotungstic acid negative staining. The virus content was estimated to be approximately 10^{12} particles per ml. This calculation is based on the finding that when 1 or 2 particles are found per grid square in a neat virus preparation, this represents approximately 10^{9} particles per ml. This virus, with 6 grids for each dilution, still had particles in the 10^{-3} dilution thus giving a total of 10^{12} particles per ml.

BPV-2 Transmission Site

The animals were anaesthetised using 3ml of Rompun (Bayer) intramuscularly (IM), before shaving the left side of the neck. The skin was then cleansed with ether before 3 x 4 inch vertical scarification lines were made with the aid of a needle. The depth of the wound is important, as discussed in the literature review and it was found to be necessary to draw blood when scarifying. The neat virus was run into the resultant grooves.

Surgical and Histological Procedures

The calves were anaesthetised with Rompun before surgically removing a small representative piece of tissue for histological examination. All biopsy material was fixed in 10% buffered neutral formalin (BNF) followed by post fixation in corrosive formol. The material was then processed for

in corrosive formol. The material was then processed for embedding in paraffin wax. The sections were initially stained by haematoxylin and eosin (H & E). Further studies using peroxidase anti-peroxidase (PAP) technique were carried out with "split" (cross-reacting) BPV-2 antisera.

Results

In both calves tumour development was first identified macroscopically at 7 **weeks** after challenge. There was a linear reaction on all lines of scarification, measuring approximately 1cm wide and raised about 0.5cm above the adjoining skin.

The biopsy showed that the lesion consisted of a fibroma, which was invading between the already existing adnexi. Among the fibromatous tissue could be seen hair follicles. It was surrounded by normal tissue and dilated lymphatics. The epithelium was thin and showed no rete-peg formation or cytopathic changes (Figure 2). This appears as **Stage 1** in the development of a fibropapilloma.

Using antisera raised to disrupted BPV-2 virions, no viral antigen could be detected at this stage by the peroxidase anti-peroxidase (PAP) technique. (Raising antisera and the PAP technique are described fully in chapter 2).

When examined at 8 **weeks** post challenge, the depilated lesions were easily seen and were uniformly linear. The epithelium appeared to be rough but no obvious papillomatosis. These fibroma-like lesions were approximately 1cm wide and projected 2 to 3mm from the surface. The lesions appeared to be hyperkeratotic.

Histologically this was a fibropapilloma. There was

thickening of the keratinocyte layer of the epithelium plus the formation of a downgrowth of this epithelium into the fibromatous tissue, which was termed plexiform acanthosis. There was also disruption of the continuity of the basal cell layer and the presence of melanin containing cells throughout the epithelium. Over the lesion there was hyperkeratosis and parakeratosis. There was no marked cytopathology but there were indications of viral proliferation in a small area of the lesion (Figure 3). This indicated a late **Stage 2** or early **Stage 3** fibropapilloma.

When examined by PAP, using cross-reactive antisera, there were a few cells expressing viral antigen but they were not heavily laden. These viral antigens were seen in the uppermost cells of the granular layer.

At **11 weeks** post challenge stage there were prominent tumours on all three lines of scarification. They were depilated and markedly papillomatous and measured approximately 2cm wide.

Microscopically the tumour was a well developed fibropapilloma. It had a very active and cellular fibromatous component, overlayen by a hyperkeratotic epithelium which was showing papillomatosis. There was a marked thickening of the keratinocyte layer extending in long pegs into the fibroma and showing some degree of plexiform acanthosis. Both the basal cells and the keratinocytes showed a fairly high mitotic rate. In the upper keratinocyte layer of the epithelium there was cytopathic changes occurring. This was seen as vacuolation of the cytoplasm, margination of the chromatin and diminution in size of the nucleoli. The keratohyalin granules were highly irregular and appeared scattered over the cells and in the incorporation zones they had increased in size to approximately a quarter of the nuclei size. The underlying fibroma was composed both of collagen-producing cells and active fibroblasts. There was

marked parakeratosis.

When examined by the PAP technique there was no staining in any fibroma cells, basal cells or in the keratinocytes which formed the rete-pegs. However positivity did appear at about 5 cells from the keratinising layer in the granular layer. This was very light staining but above these cells where the cytopathic changes were distinct, the nuclei were relatively devoid of structure and there was heavy antigen deposition. There was also positive antigen reaction in the long thin cells of the parakeratotic horny layer (Figures 4 & 5).

In calf 49 after 13 weeks post challenge the lesions were clearly papillomatous, raised and depilated.

Histologically this was a well developed fibropapilloma with no remaining adnexal elements in it. The fibromatous component was not as active with fewer fibroblasts present but had markedly more collagen-producing cells. There was still a markedly thickened keratinocyte layer. Obvious cytopathology was present throughout the length of the biopsy and viral antigen was demonstrated by the PAP technique. The virus was located in the upper granular layer of the tumour and was in abundance.

However in calf 50 a different stage of events was apparent. The lesions were depilated and papillomatous but were not as protruding as calf 49.

Microscopically the lesion was also different. There was no thickening of the squamous epithelium, no plexiform acanthosis and the basal cells were regular and uniform in appearance. Remnants of cytopathic cells were found in the upper granular layer but there was no parakeratosis or hyperkeratosis present. In the sub-epithelial tissue the remaining fibroblasts were active but fewer in number. There

was obvious deposition of collagen which in areas was undergoing hyalinization. Also present in the fibroma was a massive proliferation of lymphocytes around the vessels. Numerous hair follicles were present in both the epidermis and dermis. This lesion was obviously in a regression state. When examined by the PAP technique no viral antigen was detected.

When examined at **17 weeks** post challenge, the lesions in both calves appeared to be regressing markedly, especially calf 50. The surface was hyperkeratotic and the fibromatous component smaller in content.

Histologically the lesions differed dramatically. In calf 50, this selected biopsy showed no linear thickening of the granular layer; however there were several acanthotic areas and many papillomatous protrusions. There were also numerous degenerative, cytopathic cells. The lesion was hyperkeratotic but had no evidence of parakeratosis. Sub-epithelially the fibroma had been largely replaced by collagen and there was a return of the adnexal elements. There was a proliferation of lymphocytes and macrophages in the perivascular connective tissue but not as marked as in the 13 week biopsy. This biopsy appeared to show the final stages of regression. No viral antigen could be detected by the PAP technique.

On histological examination, the biopsy from calf 49 revealed a **Stage 3**, fully developed, cutaneous fibropapilloma. There were present both in the epithelium and the sub-epithelial tissue, hair follicles and aggregates of lymphocytes. Throughout the length of the tumour there were aggregates of irregular shaped keratohyalin granules present in the upper granular layer as well as cytopathological changes. Immunocytochemical analysis revealed a virally productive tumour with no obvious diminution in yield of antigen.

At the **19th week** the lesions were depilated, hyperkeratotic and smaller in size.

Microscopically calf 50 biopsy consisted of a hyperkeratotic lesion with no fibromatous component. There were remnants of lymphocytes and macrophages but no thickening of the epithelium and the lesion appeared to be in a degenerative state. No viral antigen could be seen when examined by the PAP technique.

In contrast the biopsy from calf 49 still appeared as an active fibropapilloma but the epithelium was not so markedly thickened. There was lymphocyte infiltration in the epithelium but the cytopathic changes still persisted. The lesion was hyperkeratotic. Immunoperoxidase still revealed active viral production but the intensity of the reaction and the abundance of antigen was apparently decreased and was obviously related to the sequence of regression.

On macroscopic examination at **week 25**, calf 50 had no visible lesions. Hair growth was restored and the site was completely healed. No further biopsies were taken.

Calf 49 still had prominent ridges on the lines of scarification, which were 2cm broad and raised. They were depilated, hyperkeratotic, fully developed papillomas.

Histologically the epithelium was still markedly thickened but there was an intense proliferation of lymphocytes and macrophages as well (Figures 6 & 7). There was also the re-emergence of skin adnexal elements in one area of the squamous epithelium. In the sub-epithelial tissue there was a large infiltration of lymphocytes alongside highly active fibroblasts as well as the appearance of numerous hair follicles. Although this lesion was obviously in a regressive state, when examined by the PAP technique it was still a

virally productive one.

At the **35th week** there were still three rows of well demarcated, typical, fibropapillomas present. They were depilated, hard and very scaly when touched.

Histologically it appeared as a hyperkeratotic, papillomatous lesion with no fibromatous component except for the connective tissue papillae. Although there were cytopathic cells present there was no viral antigen demonstrated by the PAP technique.

By the **40th week** calf 49 still had three lines of papillomas which were 2cm high x 2cm wide. They had become markedly papillomatous with thick fronds which were easily broken off.

On histological examination it presented itself as a hyperkeratotic, filliform lesion with little or no connective tissue. Where there was a remnant of sub-epithelial tissue it contained masses of lymphocytes and macrophages and the lesion appeared degenerate. There was no viral antigen detected by the PAP technique.

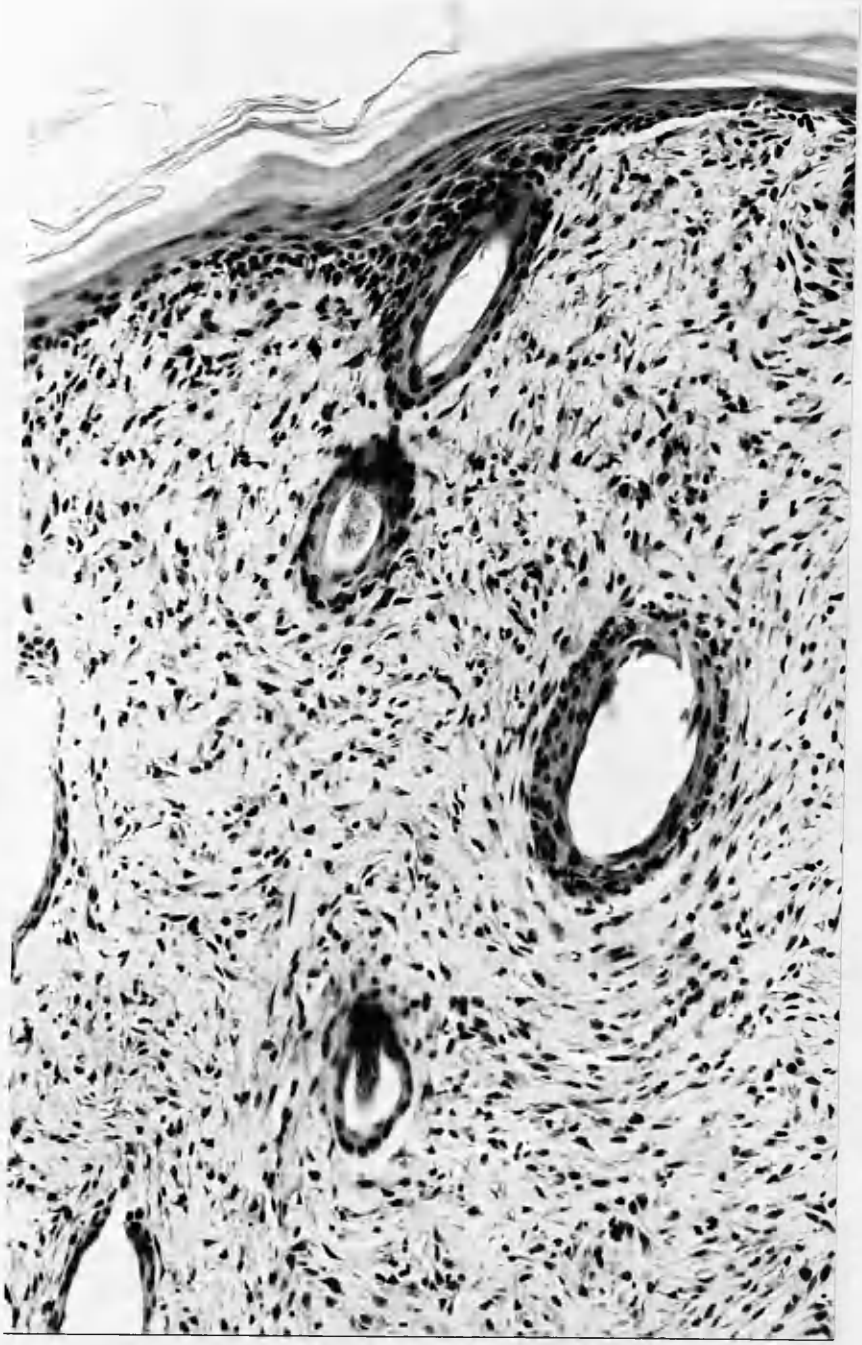

Figure 2: Early stage development of a fibropapilloma induced by BPV-2. Prominent proliferation of fibroblasts and minimal effect on the squamous epithelium. H & E.

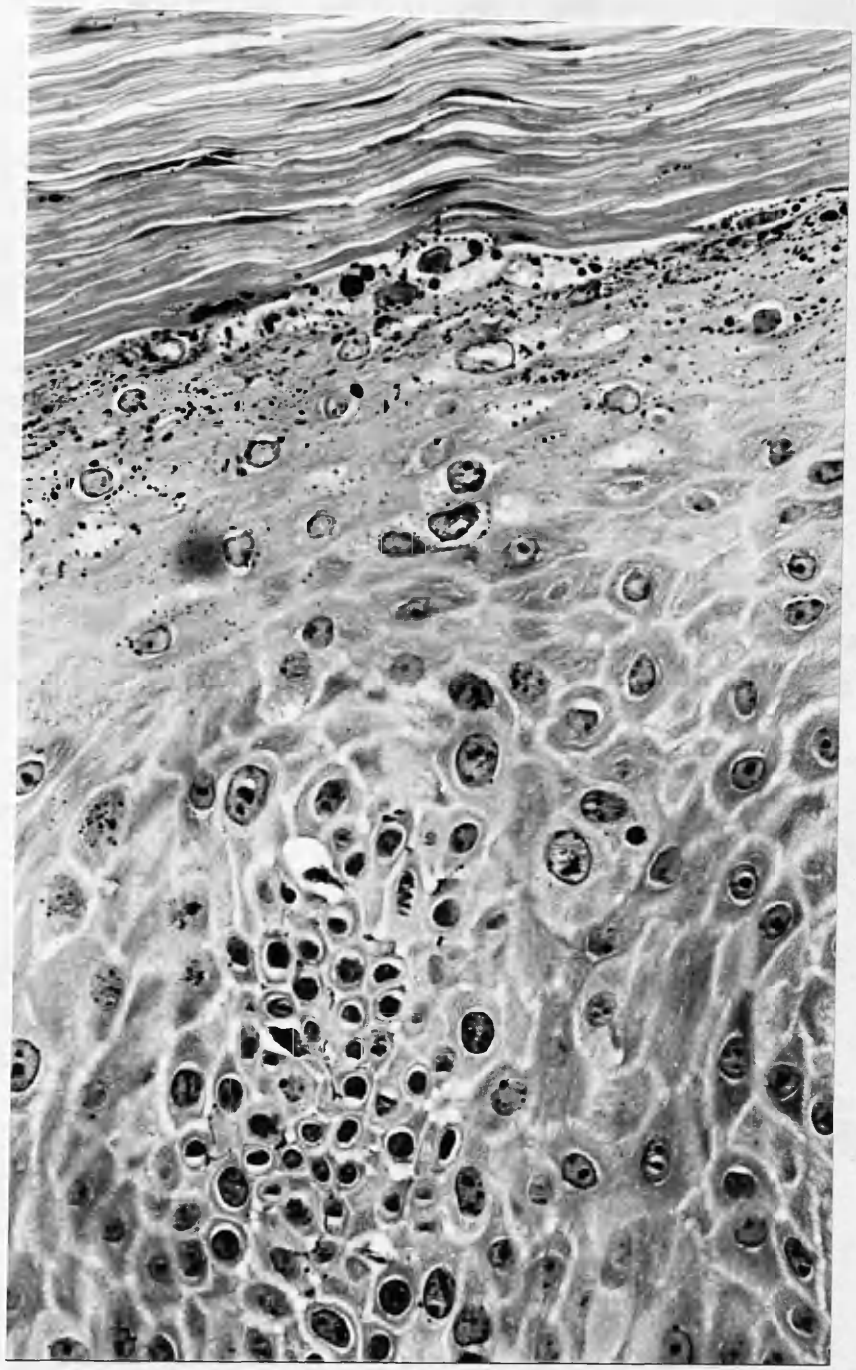

Figure 3: A later stage in development of the tumour in Figure 2 showing the interface of Stages 2 & 3. Keratinocyte proliferation with thickening and cytopathic changes in the granular layer. Obvious hyperkeratosis present. H & E.

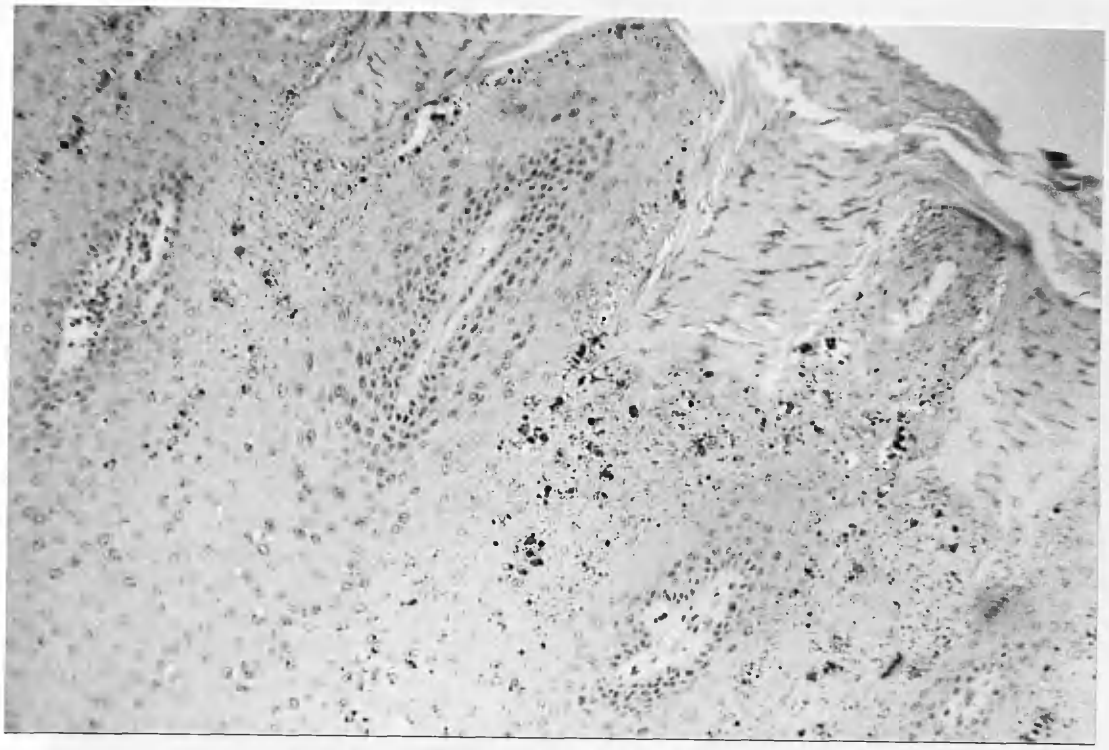

Figure 4: Immunocytochemical staining of a BPV-2 papilloma at Stage 3, showing the restriction of the viral antigen to the granular layer. PAP.

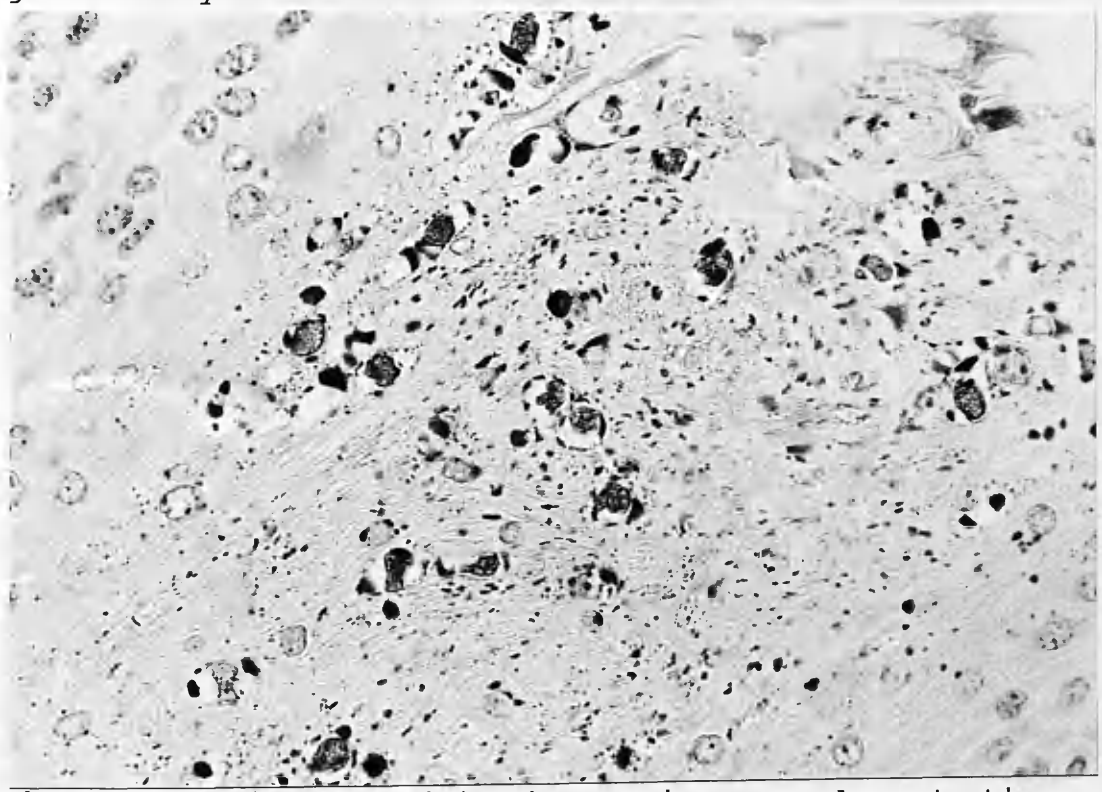

Figure 5: A higher magnification of Figure 4, demonstrating the restriction of antigen to the granular layer nuclei. PAP.

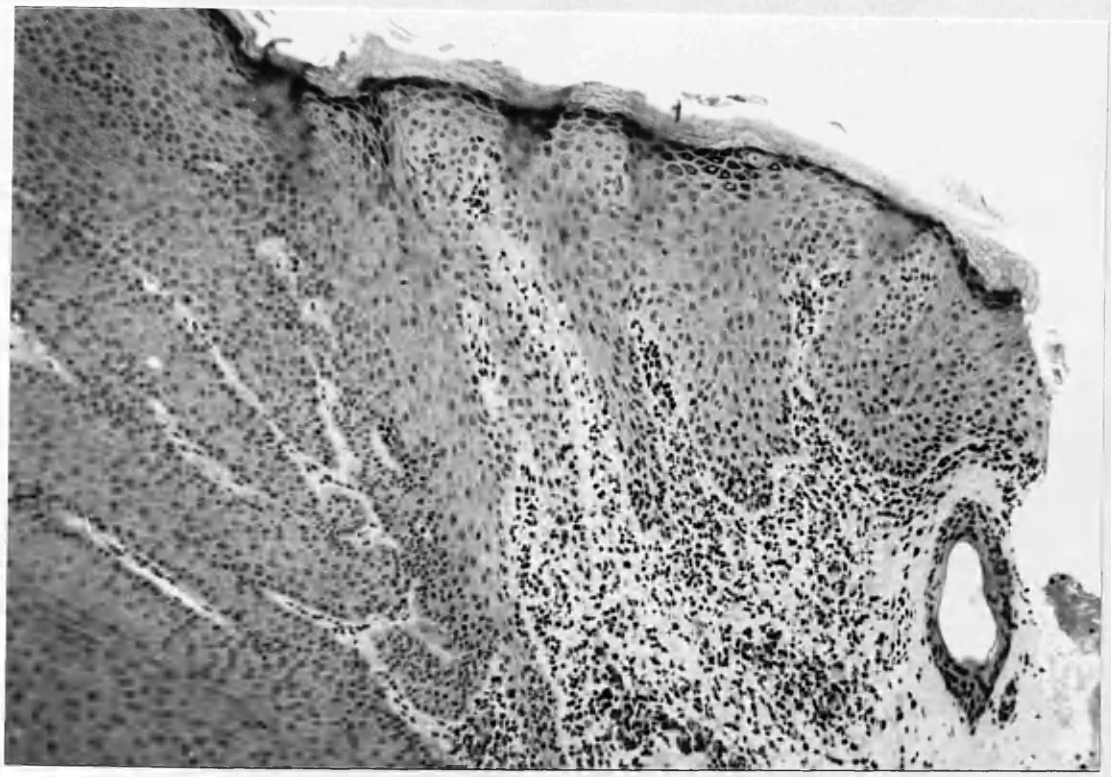

Figure 6: The start of the rejection phase. Heavy mononuclear infiltrate of the previous fibroma below the acanthotic epithelium. H & E.

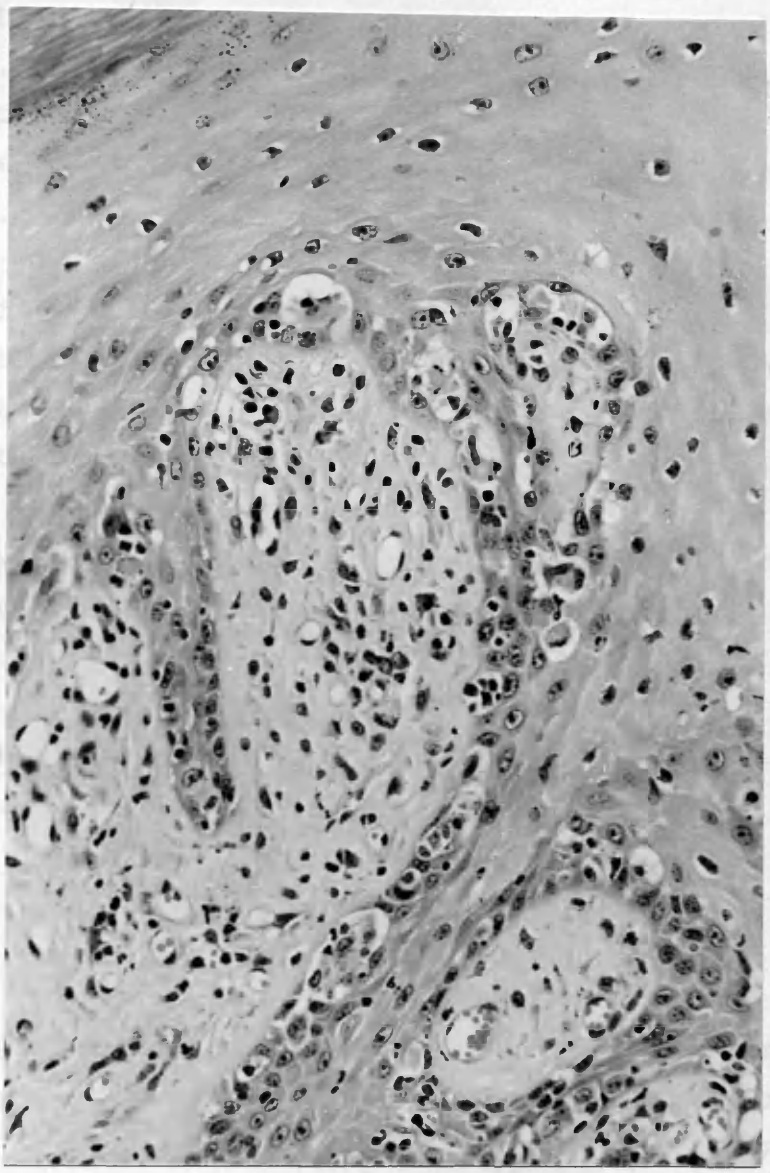

Figure 7: A higher magnification of Figure 6, showing invasion of the proliferated epithelium by macrophages & lymphocytes. H & E.

Experiment 2

Experimental Transmission of Bovine Alimentary Papilloma by BPV-4

BPV-4 is associated with epithelial papillomas of the alimentary tract in cattle. These lesions are of the frond type with no fibromatous component and no plexiform acanthosis. They were found in association with alimentary squamous carcinomas in high incidence cancer areas. It is this synergistic mechanism which is of interest to scientists.

When purifying virus from tumours of unknown content, it is important to study the development of the lesion to ascertain the differentiation process in relation to virus production and to determine if this was related to ageing. This small experiment allowed the study of an experimentally induced BPV-4 tumour cycle without the use of co-factors (bracken or immunosuppression).

Material and Methods

Calf

This calf (72) was purchased from the same dealer as the previous experiment and was selected by clinical examination as being free of any obvious papillomavirus infection. The calf was a 13 week old, male, Friesian and on arrival was immediately housed in a clean, well ventilated pen in the isolation unit. It was then bled for both haematological analysis and for serum, the latter being stored at -20^{o}C for future studies. Two weeks later the experiment was started.

BPV-4 Virus Preparation

The BPV-4 transmission virus was purified from a pool of bovine oesophageal papillomas obtained from the Glasgow abattoir. These tumours were stored in 50% PBS/Glycerol at $4^{o}C$ until required for purification.

BPV-4 tumours (4g) were homogenised in a Silverson grinder, using 20ml of PBS as diluent. The suspension was clarified at 2,000 and 10,000 rpm for 10 minutes each. The supernatant was purified by rate zonal centrifugation as described on page 33 and the resultant virus content was estimated by phosphotungstic acid negative staining on the TEM and quantal evaluation.

BPV-4 Transmission Site

The calf was anaesthetised with 3ml of Rompun (IM) and immediately before administering the virus, 2ml of Brietal Sodium was given (IV). 5ml of the purified BPV-4 virus was inoculated into the soft and hard palate by numerous intra-mucosal injections. At regular intervals, determined by the development of the tumours, the calf was clinically examined and bled for both haematological analysis and serum.

Surgical and Histological Procedures

The calf was anaesthetised with Rompun and Brietal before surgically removing a lesion with the aid of a 3mm biopsy punch. The biopsy was fixed in 10% BNF followed by post fixation in corrosive formol. The material was then processed for embedding in paraffin wax. The sections were initially stained by H & E. Immunocytochemical localisation of the viral antigen was demonstrated by the PAP technique using type specific antisera.

Results

A mouth examination was carried out 2 **weeks** after the virus was administered and found numerous, small, opalescent lesions at the sites of injection. No biopsy was taken.

At 5 **weeks** there were numerous small, translucent, projectile lesions on the palate and pharynx - all injection sites. No biopsy was taken.

Under anaesthetic, at 6 **weeks** post transmission, a mouth examination revealed the presence of distinct, multi-fronded papillomas at the sites of injection. A biopsy was taken for histological examination.

Microscopically this biopsy showed the earliest developmental stage of an alimentary papilloma. There was obvious keratinocyte proliferation with dyskeratosis and the early formation of the fronds from the base of the papilloma. There was also parakeratosis present at the tips of the frond but not at the incorporation zones or at the keratinocyte / keratin border (Figures 8 & 9). In the incorporation area there was however evidence of the formation of inclusion bodies, which were indicative of virus presence (Figure 10). This was confirmed by the PAP technique using antisera raised to disrupted BPV-4 virions. There were many cells in the sub-keratin layer and the incorporation areas were expressing viral antigen but not strongly as indicated by the light nature of the reaction.

At 9 **weeks** calf 72 was subjected to another mouth examination. This revealed numerous, small but distinct, multi-fronded papillomas on the palate. A punch biopsy was taken for histological examination.

Histologically this biopsy appeared to be bigger in size in

comparison to week 6, with the base broader and the fronds longer. The fronds were well developed and had fully keratinised tips instead of the parakeratosis seen in the previous biopsy. There were marked incorporation zones present coupled with parakeratosis. The PAP technique localised the associated viral antigen in the many flattened nuclei in the keratin and the sub-keratin layer. There was a positive increase in the number of virally active cells present compared to week 6.

The mouth examination at **week 12** was very similar to the previous one except there was a wide range of papilloma sizes covering the palate.

Microscopically the lesions appeared as well developed epithelial papillomas (Figure 11). On the H & E section, inclusion bodies were easily identified in the cytopathic cells present in the incorporation areas. Immunocytochemically viral antigen was confirmed in these cells as well as well as the sub-keratin areas of the fronds.

When examined at **16 weeks** post transmission, there were still present numerous, filliform papillomas on the palate.

Histologically these lesions appeared as well developed papillomas but were larger than previous biopsies. There was a marked mitotic activity in the basal and parabasal layer which gave rise to a large number of keratinocytes in the immediately overlying area. On the H & E stained section there appeared to be large numbers of cytopathic cells present in the incorporation areas. This observation was supported when the PAP technique was applied except that the cells which were positive only gave a faint signal.

The **21 week** mouth examination revealed numerous, blunted, filliform papillomas on the palate.

Histologically, including the immunocytochemical examination, a similar finding to the 16 week biopsy was seen.

At the **26 week** mouth examination there were still numerous, blunted, typical BPV-4 induced papillomas on the palate.

Microscopically these tumours are larger with many more fronds arising from the base. There are still many cytopathic cells present in both the sub-keratin and the incorporation areas. However it was mainly the cells in the keratin and sub-keratin areas which showed any strong positivity although it was not as abundant as in previous biopsies. In the incorporation areas there was a faint reaction which probably represented the remains of a viral infection as against an active infection.

This calf was subjected to further examinations at **30, 35, 41** and **52 weeks** and there was no diminution in the numbers of papillomas, nor was there any change in the histological structure of the lesions. When examined by the PAP technique no viral antigen was expressed in any of the biopsies examined.

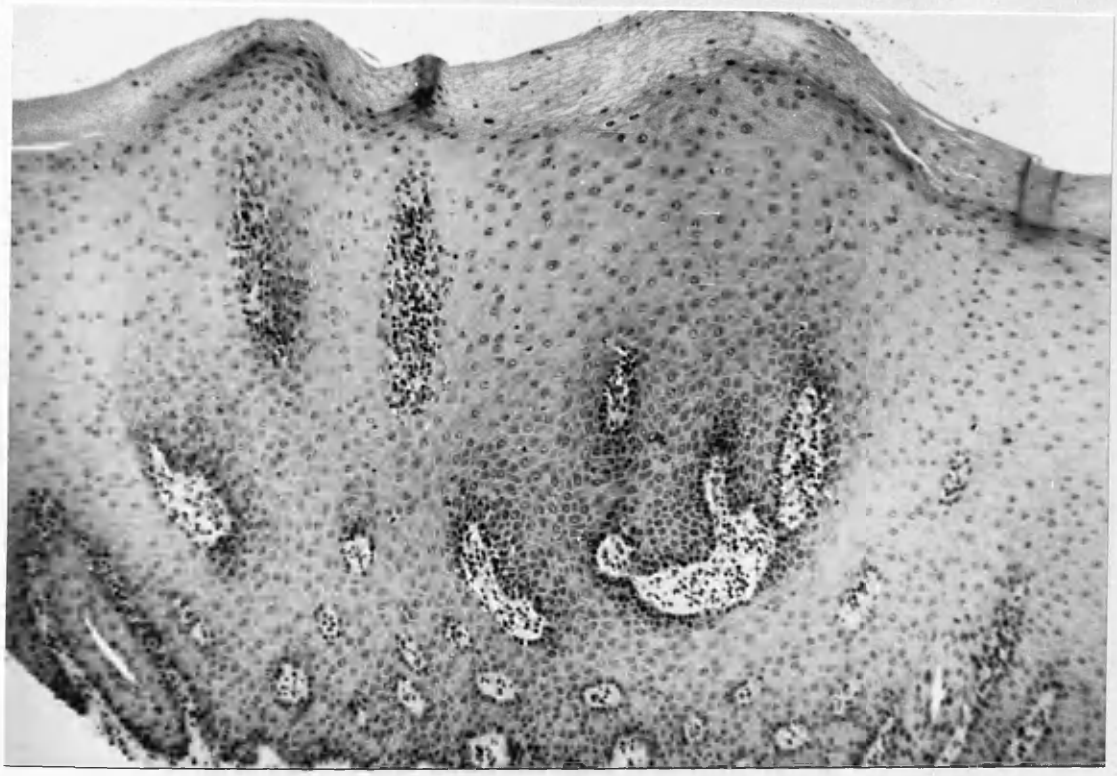

Figure 8: Earliest development stage of a BPV-4 lesion. Plaque of proliferated keratinocytes with only the earliest signs of papilloma development. H & E.

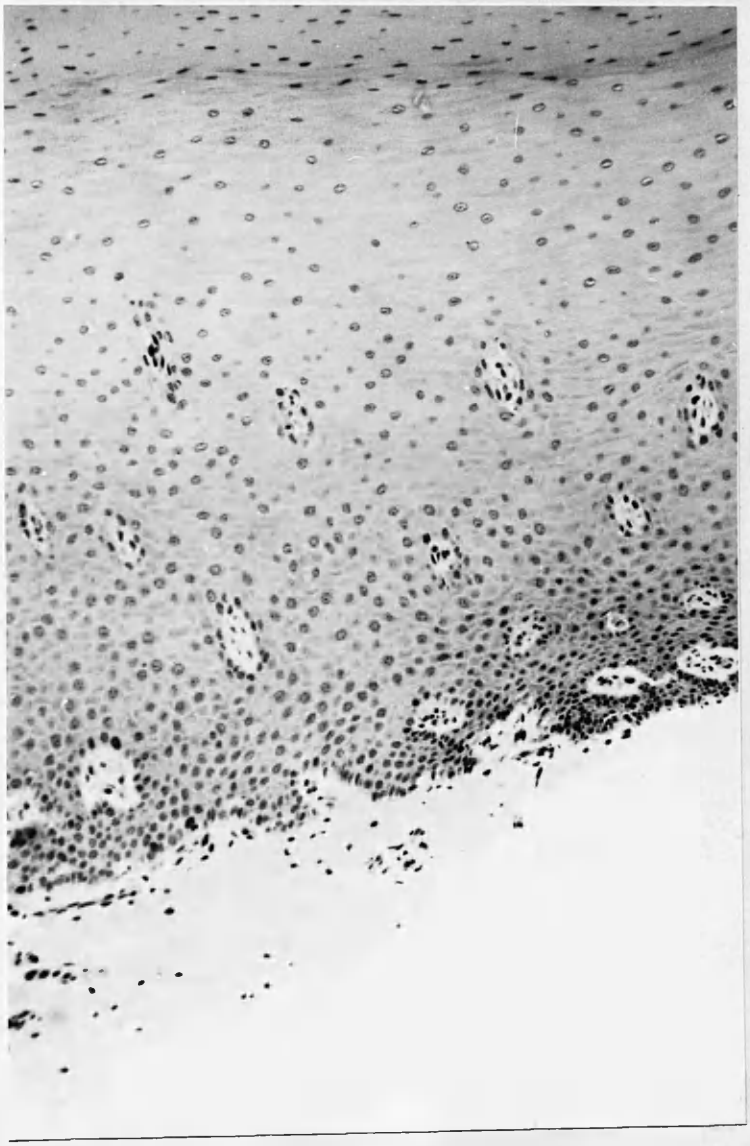

Figure 9: A higher magnification of Figure 8, showing thickening of the alimentary-specific granular layer. H & E.

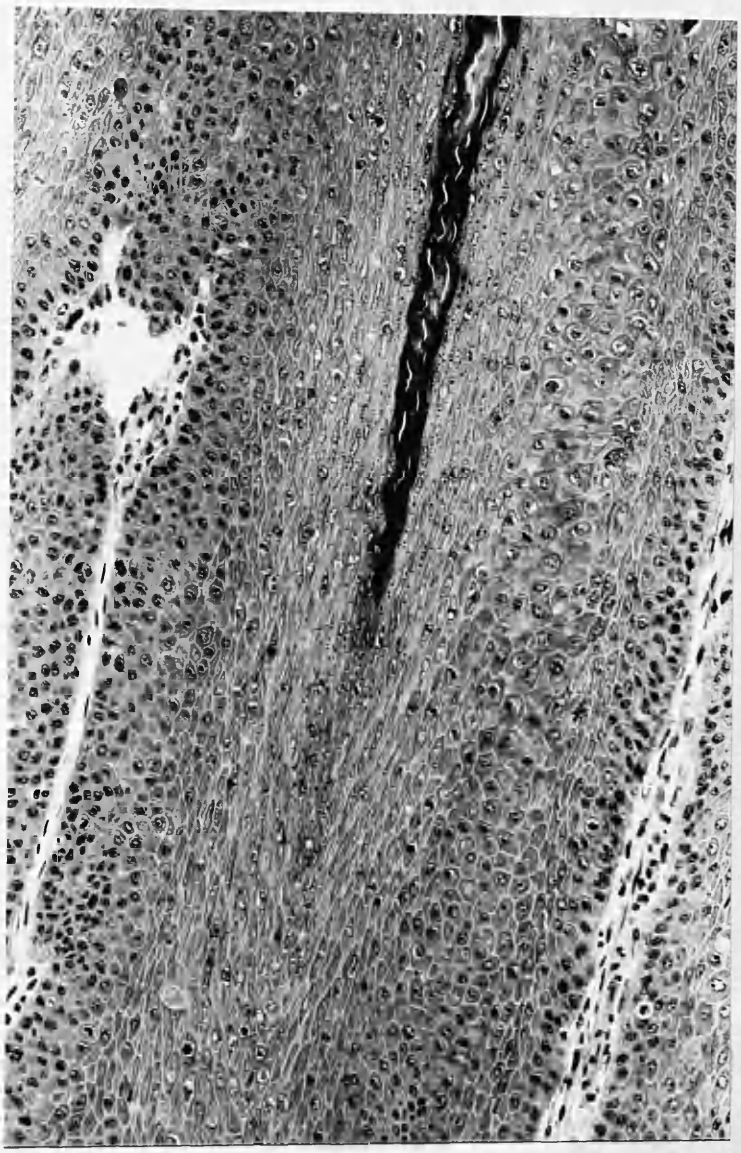

Figure 10: A developed BPV-4 papilloma showing the virus-producing incorporation zone (bottom, centre) and the typical frond structure. H & E.

Experiment 3

Experimental Transmission of BPV-5 and BPV-6 Tumours by the Type Specific Virus

BPV-5 and BPV-6 are associated with the rice-grain fibropapilloma and the squamous papilloma respectively, found on the teats and surrounding skin of cattle. They are very common lesions and often appear as multiple infections with each other and also with fibropapilloma induced by BPV-1. Due to the small size of the rice-grain tumour and the mode of development it has proved to be very difficult to obtain sufficient virus for biological studies. There was also a numerical lack of BPV-6 lesions to facilitate adequate studies and as a result, the rice-grain lesions and the squamous papillomas had to be pooled (separately) in order to overcome this problem.

The object of these experiments was to induce numerous rice-grain and squamous papillomas with purified BPV-5 and BPV-6 virions to study the tumour cycle and provide a source of material for future vaccine studies.

Material and Methods

Calves

Four calves were purchased from the same source as the previous experiments. These calves were not in contact with other animals and were selected as being free of any obvious papillomavirus infection by clinical examination. The calves were 10 week old, male, Friesians and on arrival were randomly selected into 2 groups, which were then housed in separate, clean, well ventilated pens in the isolation unit.

They were immediately bled for both haematological analysis and for serum, the latter being stored at -20°C for future studies. The experiment was initiated when the calves were 15 weeks old.

Group 1) BPV-5 transmission calves - 61 and 62
Group 2) BPV-6 transmission calves - 53 and 59

BPV-5 Virus Preparation

The virus was purified from a pool of teat rice-grain fibropapillomas, which had previously been histologically screened and typed (Figure 12). The lesions were obtained from the Glasgow abattoir and stored in 50% PBS/Glycerol at 4°C until required for purification.

Teat rice-grain papillomas (1.74g) were homogenised in a Silverson grinder, using 17.4ml of 0.5% sodium deoxycholate and 0.5% NP40 as diluent. The suspension was clarified at 2,000 and 10,000 rpm for 10 minutes each. The supernatant was purified by rate zonal centrifugation as described on page 33. The virus was visualised by TEM phosphotungstic acid negative staining and confirmed as positive with numerous particles present. A sample was also sent for restriction mapping and hybridization and was subsequently confirmed as containing only BPV-5 genome.

BPV-6 Virus Preparation

The virus was purified from a pool of teat frond squamous papillomas obtained from the Glasgow abattoir. These tumours were stored in 50% PBS/Glycerol at 4°C until required for purification.

Teat frond papillomas (15.57g) were homogenised in a Silverson grinder, using 60ml of 0.5% sodium deoxycholate and

0.5% NP40 as diluent. The suspension was clarified at 2,000 and 10,000 rpm for 10 minutes each. The supernatant was purified by rate zonal centrifugation as described on page 33. The virus was visualised by TEM phosphotungstic acid negative staining and confirmed as positive with numerous particles present. A sample was also sent for restriction mapping and hybridization and was subsequently confirmed as containing only BPV-6 genome.

BPV-5 and -6 Transmission Site

All 4 calves were anaesthetised with 3ml of Rompun (IM). The abdomen between the teats and the teats themselves were cleansed with ether prior to inoculation.

Each group of calves were deeply scarified on the teats and the adjacent skin of the udder with the aid of a needle and 3ml of the type specific virus was run into the resultant groove.

The calves were bled for haematological analysis and serum sampling on a fortnightly basis and were clinically examined weekly.

Surgical and Histological Procedures

The calves were anaesthetised with Rompun before surgically removing a representative piece of tissue for histological examination. All biopsy material was fixed in 10% BNF, followed by post fixation in corrosive formol. The material was then processed for embedding in paraffin wax. The sections were initially stained by H & E . Localisation of the viral antigen was carried out using type specific antisera in the PAP technique.

Results of BPV-5 Transmission

In both calves 61 and 62 tumour development was first seen at **26 weeks** and was a raised, depilated line 2mm wide on the site of previous scarification. The biopsy showed a typical plaque like lesion with a marked epithelial thickening and a continuous keratinising edge with cytopathic changes in the nuclei. There was also well established plexiform acanthosis present but with no involvement of the sub-epithelial tissue. This is a BPV-5 lesion.

At **28 weeks** the biopsy consisted of an epithelial plaque with no papillomatosis and no fibromatous component. There was an amplification of the granular layer with some evidence of viral inclusion bodies in the nuclei of the upper most cells of the granular layer.

By **week 33** the biopsy was still basically a plaque like lesion with a thickened granular layer, large keratohyalin granules and cytopathology evident and widespread under the superficial keratin layer. There was still no fibromatous component and the lesion had an acanthotic base.

The biopsy at **37 weeks** revealed a plaque lesion with a markedly thickened granular layer and plexiform acanthosis. There was however a mild degree of papillomatosis protruding from this plaque (Figure 13).

When biopsied at **46 weeks** the tumour was still an identifiable BPV-5 lesion. The base was acanthotic of a plexiform type and the granular layer was similar. On the outside edge of the tumour there was mild papillomatosis but the cytopathic changes were confined to the length of the sub-keratin layer. In this particular biopsy there was an adjacent fibroma. There was now evidence of a marked mononuclear reaction around the vascular spaces, which may

have indicated the onset of the rejection process.

By 52 **weeks** the lesions are very similar to the previous biopsy. There were lymphoid and macrophage accumulations around the vascular spaces and at one edge of the lesion there was invasion and destruction of the basal cells by this mononuclear reaction.

Results of BPV-6 Transmission

Tumour development was first noted at 4 **weeks** as a raised epithelial plaque on the site of scarification. When examined by H & E, the lesion consisted of a small fibromatous nodule with the skin adnexal elements remaining. Overlying this sub-epithelial lesion was a slightly thickened squamous epithelium due to keratinocyte proliferation and there was also the first signs of rete-peg formation. There was no cytopathology and no viral antigen localised by immunocytochemistry.

By **6 weeks** the fibroblast involvement in the sub-epithelial tissue was drastically reduced and replaced with collagen. There was a mild thickening of the epithelium along the length of the tumour due to keratinocyte proliferation. Keratohyalin granules were now evident in the sub-keratin layer and the areas of plexiform acanthosis were more established. No obvious cytopathology was seen nor was there any demonstrable viral antigen by the PAP technique.

At **10 weeks** post infection, two thirds of the squamous epithelium was moderately thickened with a degree of parakeratosis. The plexiform acanthotic areas were well developed and definitive fronds formed. There were cytopathic cell changes in the sub-keratin layer but no viral antigen was localised by immunocytochemistry. In the sub-epithelial

tissue a few lymphatic capillaries re-emerged.

The **18 week** biopsy revealed a distinguishable papillomatous lesion. The squamous epithelium was markedly thickened and in the upper keratinocyte layer there were a number of cytopathic cells present. These cells were found to contain viral antigen by the PAP technique using type specific antisera. In the sub-epithelial tissue there was virtually a full return of the skin adnexal elements.

By the **23rd week** the biopsy appeared as a well developed epithelial papilloma with cytopathic cells in association with large aggregates of keratohyalin granules in both the sub-keratin layer and the incorporation areas. Virus containing cells were localised in these areas, when examined by the PAP technique.

At **25 weeks** the fronds of the papilloma were more numerous and elongated. (see Figure 14 for gross picture). This was still a virally productive tumour.

The **29 week** biopsy was a well developed epithelial papilloma with obvious cytopathology. Viral antigen was visualised in both the sub-keratin and incorporation zones by immunocytochemistry and electron microscopy.

The calves were observed for a further 6 months, with biopsies taken at monthly intervals. Only at 12 months did the lesions finally lose their viral antigen staining by the PAP technique.

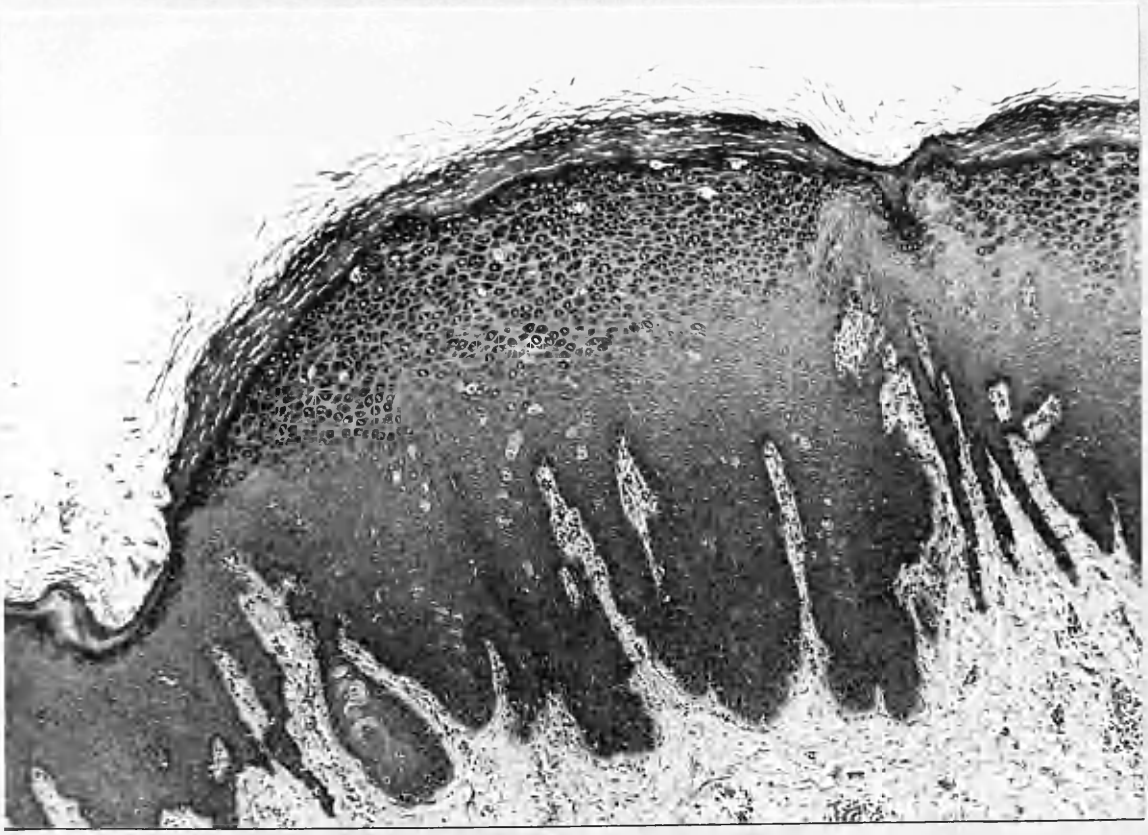

Figure 12: A BPV-5 papilloma from a field case showing the marked granular cell proliferation. H & E.

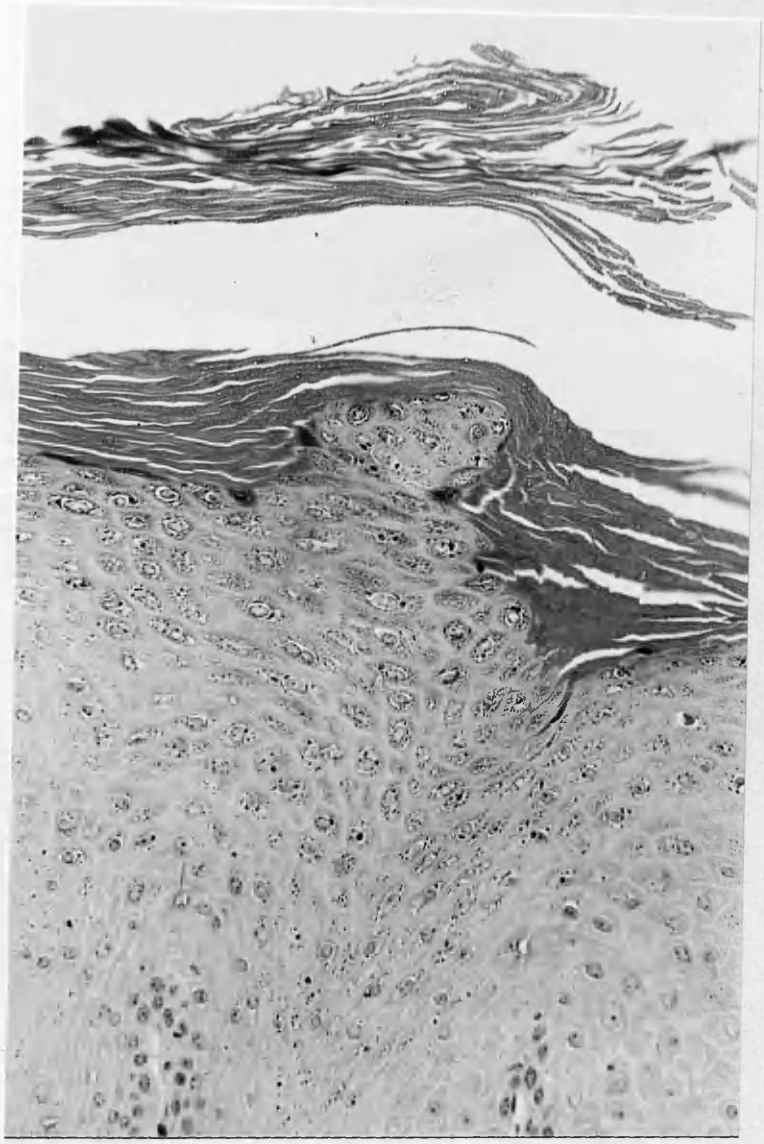

Figure 13: An experimentally induced BPV-5 papilloma showing the marked proliferation of the granular layer. H & E.

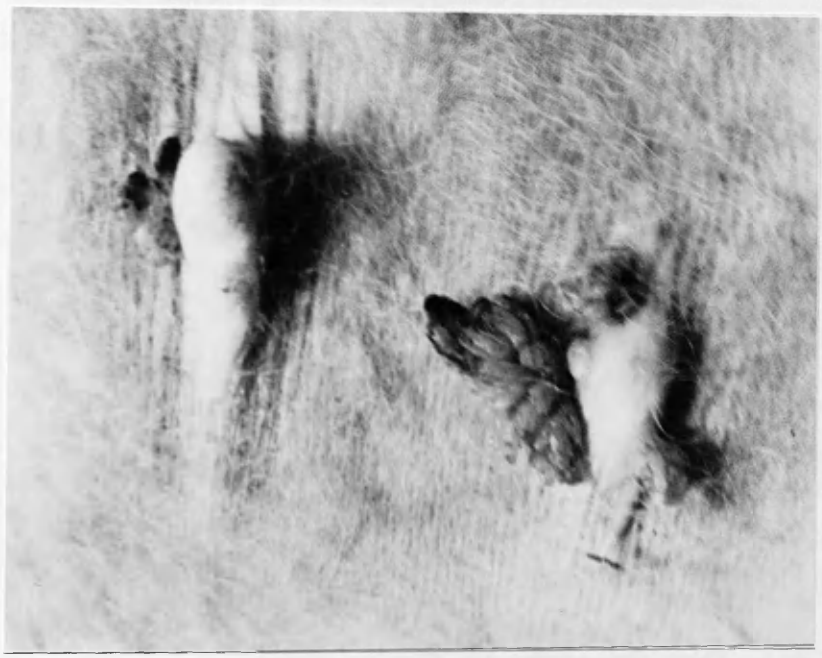

Figure 14: An experimentally induced BPV-6 papilloma on the teats and udder skin.

Discussion

The objective of these experiments were to study the timing of the development of the stages of tumours induced by BPV-2, 4, 5 and 6 and to verify that the lesion and site specificity for each virus corresponded to what had previously been found in naturally occurring outbreaks or had been demonstrated experimentally (Jarrett et al, 1985). As the main work of this thesis is concerned with immune protection and rejection, it was necessary to have a clear idea of the timing of the above phases.

In BPV-2 infections, the sequence was found to be as follows; Stage 1 in the development of the fibropapilloma was first recognised at 7 weeks after challenge. This lesion consisted of a fibroma with no thickening of the overlying epithelium and no expression of viral antigen. However 1 week later the lesions had shown marked signs of development. There was now thickening of the squamous epithelium and the establishment of plexiform acanthosis. There was no marked cytopathology but when examined by immunocytochemical techniques there was a small focus of cells expressing viral antigen. By 11 weeks the lesions were recognised as Stage 3 fibropapillomas with viral antigen in abundance in the upper granular layer of the epithelium. After this stage the animals differed dramatically; one calf (50), on histological examination, was obviously undergoing regression. This was apparent as there was no thickened epithelium, no plexiform acanthosis, no para -or hyperkeratosis and no viral antigen expressed. In the sub-epithelial tissue there was a massive proliferation of lymphocytes around the vessels and normal skin adnexal elements were also present. In contrast, calf 49 still appeared as a virally active fibropapilloma and it wasn't until 25 weeks after challenge that the first signs of regression were recognised; large infiltration of lymphocytes around the capillaries and the return of numerous hair

follicles. Although the lesion was undergoing regression, it was still a virally active one. The process of regression was a slow one and it was not until nearly a year after challenge that calf 49's skin returned to it's normal state.

In the BPV-4 transmission experiment, 2 weeks after the virus was injected, there were numerous, small, opalescent lesions seen in the mouth. By week 6 there was a fully formed epithelial papilloma, which had cells containing viral antigen, as demonstrated by the PAP technique. In the following weeks the papilloma did not change in structure but there was a significant increase in the overall size of the lesion and in the amount of viral antigen being expressed. However by week 21 the number of cells containing viral antigen diminished appreciably and was accompanied by a corresponding decrease in the positivity of the reaction. There was also a marked mitotic activity in the basal and parabasal layers. This stage remained constant until week 30, when no viral antigen was expressed. The epithelial papillomas remained on the palate, pharynx and oropharynx for more than 1 year but subsequent biopsies failed to detect any cells containing viral antigen.

In the BPV-5 infections, tumour development was first noted at 26 weeks after infection. This biopsy revealed a plaque like lesion with a marked epithelial thickening and cytopathic cells in the sub-keratin layer. This was an extremely slowly developing tumour and it was not until week 37 that the classical `rice grain' lesion was identified. However the biopsy taken at week 46 showed the first evidence of a sub-epithelial fibroma component. This might indicate that the fibromatous part occurs at a later stage of development of the BPV-5 lesion in contradistinction to that of BPV-1 and 2 where it occurs as the first stage. There was also a marked mononuclear reaction radiating from the perivascular spaces, which might indicate the first stage of

a rejection reaction. By week 52 the basal cells had been invaded and in parts destroyed by accumulations of lymphocytes and macrophages. This rejection reaction had no obvious effect on the squamous epithelium. Immunohistochemical examination of these biopsies is not yet completed and therefore it is not possible to comment on the viral expression cycle at this moment.

In the experimentally induced BPV-6 infection, the first sign of tumour development was noted at 4 weeks after challenge. This biopsy revealed a plaque-like lesion consisting of a small fibromatous nodule with a slightly thickened overlying epithelium. There was no obvious cytopathology and no viral localisation. This lesion continued to develop but at a slow rate and it was not until week 18 that a fully developed papilloma was seen. There were cytopathic cells in the upper keratinocyte layer and these were confirmed to be virus-containing cells when examined by the PAP technique and by transmission electron microscopy. In subsequent biopsies over 8 months, virus-containing cells were demonstrated by the PAP technique using type specific antiserum. Only after 12 months did the lesions finally lose their viral antigen staining, when examined immunologically.

From the transmission experiments, the nature and the timing of the developmental stages have been found. The periods when active viral replication is taking place have been defined. With the technology described in chapter 2, it is now very simple and quick to determine the type of virus causing an infection, which would be beneficial to clinicians and scientists if any therapeutic regimes were to be considered in the future. These experiments allow the diagnosis of tumours caused by the different viruses and indicate the times at which virus production is present and maximal. This is valuable in choosing source material for any given virus isolation. From the above data it is imperative

to determine as far as possible the stage and age of the lesion. This is necessary if virus purification is to be undertaken, as there is no value in taking old, well established lesions because they are not prolifigate in virus.

Introduction

BPV-4 is the causative agent of papillomatosis of the upper alimentary canal in cattle (Jarrett et al, 1978). Some of these papillomas may progress to cancer (Jarrett, 1981) in animals which are exposed to continuous ingestion of bracken fern and are chronically immunosuppressed (Jarrett et al, 1978b).

One of the main reasons for carrying out this series of experiments was to re-investigate the whole status of immunity to bovine papillomavirus infections, since it became obvious that different types and subgroups existed. It was apparent that many anomalies in the past such as the irregular success of crude vaccines could be due to antigenic heterogeneity. The experiments were therefore designed to examine the status of a papillomavirus infection and reinfection among different virus types.

CHAPTER 4

REINFECTION OF TUMOUR CALVES WITH BOVINE PAPILLOMAVIRUS TYPE-4

Experiment 4

BPV-4 Reinfection of BPV-2 Infected Calves

Two calves (49 & 50) from the previous BPV-2 cell membrane prophylactic vaccine experiment were used. These had been through a full cycle of BPV-2 induced fibropapilloma and the susceptibility of these animals to an infection from a different sub-group, BPV-4 was investigated.

Material and Methods

BPV-4 Virus Challenge Preparation

The virus for this transmission experiment was purified from a pool of oesophageal epithelial papillomas, which are associated with BPV-4. These tumours were obtained from the Glasgow abattoir and were stored in 50% PBS/Glycerol at $4^{o}C$ until required for purification.

Oesophageal papillomas (8.3g) were homogenised in a Silverson grinder, using 41.5ml of 0.5% sodium deoxycholate and 0.5% NP40 as diluent. The suspension was clarified at 2,000 and 10,000 rpm for 10 minutes each. The supernatant was purified by rate zonal centrifugation as described on page 33 and the resultant virus was examined by TEM phosphotungstic acid negative staining, which confirmed the presence of numerous particles.

BPV-4 Challenge Site

The two calves were anaesthetised with 3ml Rompun (IM) and immediately before injection of the virus 2ml of Brietal Sodium (Lilly) was given intravenously (IV). 3ml of the

purified BPV-4 virus was given by numerous intra-dermal inoculations into the soft and hard palate. At fortnightly intervals the calves were clinically examined and bled for both haematological analysis and serum sampling.

Surgical and Histological Procedures

The calves were anaesthetised with Rompun and Brietal before surgically removing a lesion with the aid of a 3mm biopsy punch. The biopsy material was fixed in 10% BNF followed by post fixation in corrosive formol. The material was then processed for embedding in paraffin wax. The sections were initially stained by H & E. Immunocytochemical localisation of the viral antigen was demonstrated by the PAP technique using type specific antisera.

Results

Calves 49 and 50 mouths were examined 2 **weeks** after injection and there was no obvious lesions seen.

The first obvious lesions were noted at 4 **weeks** post injection where both calves had distinct tumours present over the inoculation sites on the palate. These lesions were slightly raised, white, projectile plaques 1 to 2mm in diameter. Histologically the biopsy contained a small developing tubule which would ultimately form a papilloma but there was no evidence of a papilloma present. There was a marked mitotic activity of the basal and parabasal cells. No parakeratosis or dyskeratosis present in this lesion. When examined by the PAP technique, using antisera raised to disrupted BPV-4 virions, there was no evidence of a viral infection.

The 6 **week** examination revealed more than 30 small, white

papillomatous like lesions projecting 2mm from the palate. No biopsy was taken.

Both calves were examined at **8 weeks**, when it was decided to destroy calf 50 a) to obtain early stage lesions for immunocytochemical studies and b) to obtain early transformed cells in an attempt to grow in tissue culture. There were >25 palatine lesions which were 1 to 2mm raised, white plaques, the most protuberant being 1mm high. Histologically they were plaque like lesions with varying degrees of epithelial thickening. Most exhibited a marked mitotic activity and thickening of the basal and parabasal layers but the cells could not be differentiated. There was a slight frond formation at this stage and parakeratosis was present. The PAP technique, using rabbit anti-split BPV-4 antisera, demonstrated a significant number of cells containing viral antigen. This was localised in the sub-keratin layer.

At 10 weeks calf 49 had numerous true papillomas covering the palate and one was biopsied. Microscopically this was confirmed as a well developed epithelial papilloma. Overlying the fronds and in the incorporation areas there was parakeratosis. The cells within these areas had obvious cytopathic changes and inclusion bodies were easily identified in the H & E section. Viral antigen was found in abundance, by the PAP technique, at the keratinocyte keratin junction and in the incorporation areas.

At 18 weeks there were still present numerous well developed papillomas at the sites of injection. Histologically the papillomas were well developed and fully fronded in appearance. The fronds were longer and had keratinised tips and there were marked incorporation zones present with evidence of parakeratosis at these points as well as overlying the fronds. Large numbers of virus containing cells were present in the sub-keratin layer and the incorporation

areas. This was confirmed by the PAP technique.

After 37 weeks post injection, the papillomas had not diminished in numbers but were smaller and broader in size. They still retained their filliform structure and were apparently not spreading to the adjacent non-injection sites. Microscopically it was a well developed papilloma but with a marked hyperkeratosis present. The keratinocytes and the basal and parabasal cells appeared not as active as in previous biopsies. Immunocytochemically there was virtually a negative reaction. There were cytopathic cells, which were degenerating and had a speckled positivity confirming a previous viral infection.

This calf was monitored at monthly intervals and at the 59th week there was still present a few blunted, filliform papillomas.

Experiment 5

BPV-4 Reinfection of BPV-5 and BPV-6 Infected Cattle

Using the calves from previous BPV-5 and BPV-6 transmission experiments, which had successfully completed the tumour cycle, it was decided to observe the susceptibility of these animals to a BPV-4 infection.

Material and Methods

BPV-4 Virus Challenge Preparation

The virus for this transmission experiment was purified from a pool of oesophageal epithelial papillomas, which are associated with BPV-4. These tumours were obtained from the Glasgow abattoir and were stored in 50% PBS/Glycerol at $4^{o}C$ until required for purification.

BPV-4 tumours (5g) from a pooled source were homogenised in a Silverson grinder, using 25ml of 0.5% sodium deoxycholate and 0.5% NP40 as diluent. The suspension was clarified at 2,000 and 10,000 rpm for 10 minutes each. The supernatant was purified by rate zonal centrifugation as described in chapter 2 and the resultant virus was visualised by TEM phosphotungstic acid negative staining. This confirmed the presence of numerous viral particles.

BPV-4 Challenge Site

All animals were anaesthetised with 3ml of Rompun (IM) and immediately before injection of the virus 2ml of Brietal Sodium was given IV. 3.5ml of the purified BPV-4 virus was given by numerous intra-dermal inoculations into the soft and

hard palate. At fortnightly intervals the calves were clinically examined and bled for both haematological analysis and serum sampling.

Results

The four calves (53, 54, 55 & 56) were subjected to a mouth examination **4 weeks** after the injection of BPV-4 into the palate.

Calves 53 & 54, which had previously undergone a tumour cycle induced by BPV-6, had numerous small, translucent plaque like lesions on the palate. There was also two small well formed papillomas. Calves 55 & 56, which had previously undergone a tumour cycle induced by BPV-5 and had a secondary spread of BPV-1 induced lesions, had clusters of small, refractile tumours on the palate as well as one papillomatous-like lesion.

As the previous reinfection experimental calves were subjected to detailed histological examination by surgical biopsying of the developing tumours, it was decided to clinically examine these animals to avoid unnecessary trauma.

At **6 weeks** post infection all four calves had >20 small, projectile lesions on the palate as well as the odd one or two frond papillomas.

By **8 weeks**, the 20 or more lesions were typical squamous papillomas albeit small in projection.

After the 8 week examination the calves mouths were clinically examined at monthly intervals.

The **31 week** mouth examination showed that all the calves had

masses of well developed papillomas on the palate at the sites of injection. Microscopically they were well developed papillomas, with numerous fronds and a marked hyperkeratosis present. There were large numbers of virus containing cells in both the incorporation zones and the sub-keratin layer. The PAP technique confirmed the presence of type specific viral antigen in these sites and that the cells were heavily laden.

The final mouth examination was carried out at **40 weeks** and the mass of epithelial papillomas were still present on the palate. Many of the individual papillomas were merged together to form a solid plaque.

Discussion

As stated earlier, it was of major importance to establish whether the immune and protective phenomena were induced by **group** or **type specific** antigens and whether there was any degree of cross-immunity between types within a species. The object of the above experiments were to answer these questions. The animals selected had all completed the cycles of oncogenesis from infection to rejection for BPV-1, 2, 5 & 6.

From the above data, all the calves developed lesions within the first 4 weeks of the virus application. These were slightly raised, white, projectile lesions found over the inoculation sites. At this stage there were no cells containing viral antigen. By 8 weeks there were numerous, small papillomas on the palate. There were a significant number of cells containing viral antigen as demonstrated by the PAP technique. These calves were examined at regular intervals over 1 year and still had papillomas present in their mouths. Positive staining of the viral antigen by

immunocytochemistry was not seen after the 40th week.

These two experiments show that infection with one type of BPV does not confer protective immunity against another type.

CHAPTER 5

VACCINATION STUDIES OF CATTLE AGAINST PAPILLOMAVIRUS INFECTIONS

Prophylactic and Therapeutic Treatments for Papillomavirus Infections

Papillomavirus infections are common in cattle but they still pose difficult therapeutic problems because of their persistent and recurrent nature. This subsequently results in serious reproductive as well as economic problems. There are numerous clinical treatments for human cutaneous or mucosal papillomas (Rees, 1985; Leventhal et al, 1985), which include surgical removal (incorporating CO_2 laser and cryosurgery), chemotherapy (using podophyllin, bleomycin etc.), antiviral agents (interferons etc.) and immunotherapy (eg. vaccines).

Surgery is frequently required in patients with laryngeal papillomatosis, possibly every few weeks, to allow an open airway but the chances of remission with this technique are relatively low (Steinberg et al, 1987). It has been shown that podophyllin may have significant toxicity and malignancy risks when used as a therapeutic agent (Stoehr et al, 1978; Chamberlin et al, 1972). However recent data in the review by Weck and Whisnant (1987) is giving hope in the use of interferon with its antiviral and antiproliferative effects. This therapeutic agent has consistently shown in major clinical trials its ability to induce regression and effective control of HPV infections of the respiratory tract, genitalia and the skin, but not without side effects. Although some studies using autologous vaccines have reported a high success rate, there is no direct evidence that the responses are specific for HPV antigens as Malison et al, (1982) showed that a vaccine prepared from warts was not significantly different from one prepared from the patients skin. Therefore further studies are required to determine the function of PV proteins, which may determine the identification of common, protective antigenic determinants.

All the above treatments have had initial success but there is no evidence of a sustained effective therapy due to the high recurrence rate. It is also evident that the current therapeutic regimes do not selectively inhibit the viral process but only destroy the infected epithelial cells.

Cattle provide us with an excellent animal model system for papillomavirus infections in humans, as several manifestations are common in the two systems; multiple virus types, specificity of lesions, malignant progression of some papillomas and the involvement of cofactors.

With these considerations it was decided, using cattle as a model system, to develop and examine the efficacy of prophylactic immunisation regimes using i) purified membranes from transformed bovine cells; ii) purified virus; iii) papillomatous tumour extracts.

Vaccination Procedures Against PV Infections in Cattle

Experiment 6

Prophylactic Vaccination Studies of Cattle Using In Vitro Transformed Bovine Cells

It has been established that BPV's contain all of the genetic information necessary for the transformation of bovine fibroblasts **in vitro** (Jarrett et al, 1988 in press). It was therefore a natural progression to ascertain the efficacy of membrane preparations from BPV transformed bovine cells as immunogens.

An experiment was designed, using 4 calves, in a pilot study to determine the feasibility of using bovine cells grown in a tissue culture system as a protective agent in BPV infections.

Material and Methods

Calves

The four calves were purchased from a reputable dealer who previously examined both the calf and the dam to ensure there was no evidence of a papillomavirus infection. The calves were all 12 week old, male, Ayrshire cross and on arrival were immediately housed in the isolation unit. This experiment consisted of 2 vaccinates and 2 non-vaccinates (controls) and these were housed in separate isolation pens. They were then bled for both haematological analysis and for serum, the latter being stored at -20^{o}C for future studies. Three weeks later, when the calves were 15 weeks of age the

experiment was initiated.

Group 1) Cell membrane vaccine calves - 49 & 50
Group 2) Vaccine controls - 51 & 52

BPV-2 Cell Membrane Vaccine

This was an established bovine conjunctival cell line which had been transformed with BPV-2 and was termed 16CON/BCPV-16. This procedure was carried out by a colleague Dr B. Watt.

The conjunctiva was obtained from a late term foetus and was cut up finely with scissors before being subjected to trypsinisation. The cells were then plated on a 25mm plastic petri dish at a density of 5×10^5 cells per plate. 0.5ml of bovine cutaneous papillomavirus (BCPV) C16 at 1/50 dilution (for purification see isopycnic centrifugation in chapter 2) was added to each plate along with 2ml of Dulbecco's Minimum Essential Medium (MEM) containing 10% foetal bovine serum (FBS) and incubated overnight at 37^oC. The cultures were then washed three times with fresh medium and incubated at 37^oC. After 24 hours the cells were given another wash with medium containing FBS. The cultures were split when they were confluent, which was approximately twice a week and transformation assessed. These particular cells were stored in liquid nitrogen at passage 20 until they were required. The cells were thawed and washed 3 times in serum free MEM before a viability count was established. They were then resuspended in 2 x 2ml aliquots containing 2.45×10^7 cells per aliquot.

Vaccine Site

The two vaccinate calves, 49 and 50 were injected with 1ml of transformed cells into the left quadriceps muscle and 1ml of transformed cells plus 1ml of Alhydrogel (aluminium

hydroxide) adjuvant into the right quadricep muscle. This procedure was repeated 14 days later.

BPV-2 Vaccine Challenge

Virus was purified from a fully developed cutaneous fibropapilloma of the upper body of a cow (76119/10) as described on page 33, using rate zonal centrifugation. A sample of the resultant virus was sent to Dr S. Campo for restriction mapping and hybridization and was subsequently confirmed as containing only BPV-2 genome. A sample of virus was also sent to Dr H. Laird for quantitative estimation by TEM using phosphotungstic acid negative staining. The virus content was estimated to be approximately 10^{12} particles per ml. This calculation is based on comparing protein estimations, a method prolifigate in virus wastage and haemagglutination, that when 1 or 2 particles are found per grid square in a neat virus preparation, this represents approximately 10^{9} particles per ml. This virus, with 6 grids for each dilution, still had particles in the 10^{-3} dilution thus giving a total of 10^{12} particles per ml.

BPV-2 Challenge Site on Vaccinated Calves

Three weeks after the last vaccine injection the animals were anaesthetised using 3ml of Rompun (Bayer) intramuscularly (IM), before shaving the left side of the neck. The skin was then cleansed with ether before 3 x 4 inch vertical scarification lines were made with the aid of a scarification needle. The depth of the wound is important, as discussed in the literature review, and it was found to be necessary to draw blood when scarifying. Into the resultant grooves was run the neat virus.

Control Calves

The two control calves 51 and 52, were challenged with the same virus preparation and at the same time as the vaccinated calves, but the site and mode were varied as described below.

BPV-2 Challenge Site on Control Calves .

The animals were anaesthetised as described for the vaccinated calves. Both sides of their necks were shaved and treated with ether immediately before inoculation. On the right side of the neck 3 x 4 inch vertical scarification lines were made and the technique followed as described for the vaccinated calves. However, on the left side of the neck a grid pattern was drawn on the shaved patch with 5 replicates of dilutions, ranging from 10^{-1} to 10^{-6} of the same virus. The virus was diluted in TBS. On the caudal side of the grid, 5 "neat" intradermal inoculations of 0.1ml purified BPV-2 virus was given, followed by the series of virus dilutions, again by intradermal inoculation.

The calves were examined weekly with regular bleeding on a fortnightly basis.

Surgical and Histological Procedures

The calves were anaesthetised with Rompun before surgically removing a small representative piece of tissue for histological examination. All biopsy material was fixed in 10% BNF followed by post fixation in corrosive formol. The material was then processed for embedding in paraffin wax. The sections were initially stained by H & E. Immunological studies were carried out where applicable by the PAP technique using the "split" BPV-2 antisera.

Results

Tumour development was first noticed at 7 **weeks** post challenge in both the unvaccinated and the vaccinated calves. This was seen as a smooth surfaced, fibromatous like lesion on the site of scarification. Histologically this was confirmed as a **Stage** 1 fibropapilloma with no evidence of replicating virus present.

(A detailed histological description of all the biopsy material in this experiment is given in chapter 3).

By 8 **weeks** these lesions had developed into a **Stage** 2 fibro-papillomas and again no viral antigen could be detected by the PAP technique.

At 11 **weeks** on both the vaccinated and unvaccinated calves, prominent, depilated and markedly papillomatous lesions were seen on the site of previous scarification. Histologically, the tumour was a **Stage** 3, well developed fibropapilloma with a marked thickening of the keratinocyte layer and showing a degree of plexiform acanthosis. In the upper keratinocyte layer there was cytopathic changes present and when examined by immunocytochemistry (PAP) the presence of nuclei containing virus was confirmed.

The calves were examined for a further 7 months during which regular biopsies were taken for histological examination. The biopsy at 25 **weeks** still showed a virally productive fibropapilloma but by the 35th **week** although there was still present a confluent line of typical fibropapillomas, no viral antigen could be demonstrated. Histologically it appeared as a hyperkeratotic lesion with no fibromatous component. The last biopsy was taken on the 40th **week** and the lesions were hyperkeratotic and easily broken off.

Experiment 7

Comparison of Prophylactic Vaccination Studies in Cattle Between BPV-2 Tumour Suspension and Purified Virions

BPV-2 is associated with cutaneous fibropapilloma found on all parts of the body but mainly on the forehead, neck, upper thorax and back. These infections can last for anything up to one year before spontaneously regressing but occasionally some animals are unable to reject the infection and are destroyed because of the widespread involvement of these lesions.

This experiment was designed to test and compare the efficacy of specific tumour suspension and purified virions, to induce rejection of a papillomavirus infection.

Material and Methods

Calves

Nine calves were purchased from the same source as the previous experiment and were examined along with the dam for any evidence of a papillomavirus infection. The calves were 10 week old, male, Friesians and on arrival were randomly selected into three groups, which were housed in separate, clean, well ventilated pens in the isolation unit. The experiment was designed as follows;

Group 1) BPV-2 Tumour vaccine calves - No's 64, 65 & 66
Group 2) BPV-2 Virus vaccine calves - No's 69, 70 & 71
Group 3) Vaccine control calves - No's 74, 75 & 76

All were bled on arrival for both haematological analysis and for serum sampling, which was labelled and stored at -20°C for future studies. Six weeks later, when the calves were 16 weeks of age the experiment was initiated.

BPV-2 Tumour and Virus Vaccine Preparation

Both BPV-2 tumour cells and virions were obtained from a fully developed cutaneous fibropapilloma of the upper body of a cow (76119/12), which had been previously confirmed by restriction mapping and hybridization as containing only BPV-2 genome. Viral content and quantitation was confirmed by phosphotungstic acid negative staining on the TEM. The virus content was estimated to be approximately 10^{12} particles per ml.

BPV-2 Tumour Extract Preparation

24g of diced cutaneous papilloma epithelium (76119/12) was homogenised in a Silverson grinder for 20 minutes at 4°C, using 48ml of Tris buffer as diluent. The material was divided into two equal amounts ie. 37ml each. The tumour cell extract was made up to 65ml with Tris buffer, mixed well and then aliquoted into 6 x 10ml, each therefore containing approximately 2gm equivalent of BPV-2 tumour. 3 x 10ml aliquots were stored at -70°C for use in the second inoculation.

BPV-2 Virus Preparation

The 37ml from the tumour extract preparation was spun at 2,000rpm
for 5 minutes and the supernatant stored at 4°C. The cellular debris was further homogenised using 29ml of 0.5% sodium deoxycholate and 0.5% NP40. When the homogenising was complete the original stored supernatant was added to the

homogenate and thoroughly mixed. The material was then purified as described on page33by rate zonal centrifugation. There was 27ml of virus realised. After dialysis the virus was concentrated to 20ml with Carbowax and then aliquoted into 6 x 3ml amounts, each containing approximately 2gm equivalent of tumour virus. 3 x 3ml aliquots were stored at -70^{o}C for use in the second inoculation.

Vaccine Sites

The three tumour cell vaccinate calves, 64, 65 and 66 were injected with 10ml of tumour suspension deep into the right and left quadricep muscles. The booster was given 21 days later by the same material and by the same procedure.

The three virus vaccinate calves, 69, 70 and 71 were injected with 3ml of purified BPV-2 virions into the right quadricep muscle. The booster was given 21 days later with the same material and by the same procedure.

BPV-2 Vaccine Challenge

The virus was purified from the same source as the vaccines ie bovine cutaneous fibropapilloma (76119/12); confirmed by restriction mapping and hybridization as BPV-2 only. 7gms of 76119/12 was homogenised using 35ml of 0.5% sodium deoxycholate & 0.5% NP40 as diluent. The virus was purified as described on page 33 by rate zonal centrifugation. 10ml of purified BPV-2 was obtained and this was used for the challenge on the vaccinated calves as well as the control calves.

BPV-2 Challenge Site for Vaccinated and Control Calves

Three weeks after the last vaccine injection all the animals

were anaesthetised using 3ml of Rompun (IM), before shaving a patch on the right side of the neck. The skin was then cleansed with ether before 4 x 4 inch vertical scarification lines were made with the aid of a scarification needle. 1ml of purified BPV-2 virus was run into the resultant groove. The calves were examined and bled for haematological analysis and serum sampling on a two weekly basis.

Surgical and Histological Procedures

The calves were anaesthetised with Rompun before surgically removing a small representative piece of tissue for histological examination. All biopsy material was fixed in 10% BNF followed by post fixation in corrosive formol. The material was then processed for embedding in paraffin wax. The sections were initially stained by H & E. Immunological studies were carried out using cross-reactive antisera in the PAP technique.

Results

At 4 weeks, tumour development was first recorded in the **unvaccinated group** (74, 75 & 76). This was a small, raised, fibromatous like lesion. These lesions developed until the **6th week**, when they were seen as typical cutaneous fibropapillomas.

By the **9th week** post infection, all animals were clinically examined. In the **virus vaccination group**, only calf 70 had a slight thickening of the epithelium in one of the lines of scarification. Histologically, there was no thickening of the squamous epithelium but underlying there was a small fibromatous nodule. There was no cytopathology and when examined by the PAP technique there was no viral antigen

expressed.

Calf 65 in the **tumour vaccination group** was the only calf to have
any visible lesions. It had a few, smooth surfaced nodules on one line of scarification. On examination by H & E the lesion was very similar to the biopsy in calf 70. Again there was no viral antigen detected by the PAP technique.

All the **unvaccinated calves** (74, 75 & 76) had prominent and markedly papillomatous lesions on all the lines of scarification. The tumours were depilated and hyperkeratotic. Histologically the tumour was a well developed, hyperkeratotic fibropapilloma with a marked plexiform acanthosis. There was no remaining adnexal elements. The keratinocyte layer was markedly thickened and in the upper layer there was numerous cytopathic cells surrounded and partially masked by highly irregular and dense keratohyalin granules. The viral antigen was positively located in this area by the PAP technique.

When examined at **14 weeks** calf 70 of the **virus vaccine group** had no lesions on the scarification sites and the former biopsy site had completely healed. Calves 69 and 71 still had no lesions on the sites of scarification.

In the **tumour vaccine group**, calf 65 still had obvious lesions on one of the scarification lines. There were 4 flattened, 12 x 10 mm, fibromatous like lesions with very little overlying keratin. Histologically, it was a **Stage 1** fibropapilloma. The lesion had a slight thickening of the keratinocyte layer in one small area which slightly penetrated into the fibromatous tissue. Overlying a small area of the fibromatous component was an ulcerated epithelium. There was some evidence of cytopathic changes but there was no virus visualised in the cell nuclei by the PAP

technique. Calves 64 and 66 had no lesions on the sites of scarification.

The **unvaccinated group** (74, 75 & 76) had numerous, depilated, hyperkeratotic fibropapillomas on all lines of scarification. Microscopically, they were well developed, hyperkeratotic fibropapillomas with a marked plexiform acanthosis. Cytopathic changes were still evident and virus containing cell nuclei were positively demonstrated in the upper granular layer by the PAP technique.

At **18 weeks** post infection calf 65 from the **tumour vaccine group** still had 4 fibromatous lesions. Whereas in previous examinations these lesions were of single entities they had now merged together to form a linear lesion. Histologically, this was a **Stage** 2 fibropapilloma. It still had slight thickening of the epithelium but had a more obvious plexiform acanthotic form. There was still an area of ulcerated epithelium present. Again some evidence of cytopathic changes but no replicating virus could be demonstrated by the PAP technique. This calf was examined finally at the **29th week** and there was no epithelial lesions or thickening visible.

Experiment 8

Prophylactic Vaccination Studies in Cattle Using Purified BPV-4 Virions as the Immunogen

BPV-4 is associated with alimentary squamous papillomas in cattle and is a member of subgroup B of the BPV's. This group, which includes BPV-3, 4 and 6 transform only squamous epithelial cells. These frond type warts are purely epithelial lesions and were found in 20% of cattle usually as a single tumour. However some animals were found to have several hundred papillomas in the mouth, tongue, pharynx and oesophagus. These animals were ultimately destroyed due to their severe clinical disease. Under experimental conditions the course of a primary infection lasts approximately 12 months.

Another very interesting finding was the presence of upper alimentary carcinomas at the same sites as these squamous papillomas. The relationship, if any, between this virus induced papilloma and the carcinoma is still not resolved.

This experiment was designed to test the efficacy of specific tumour virions to protect cattle against a homologous papillomavirus infection.

Material and Methods

Calves

Four calves were purchased from the same source as the previous experiments to ensure that the calves were not in contact with animals which might have a papillomavirus

infection. The calves were 10 week old, male, Friesians and on arrival were randomly selected into 2 groups, which were then housed in separate, clean, well ventilated pens in the isolation unit. This experiment consisted of 2 vaccinates and 2 non-vaccinates (controls). The calves were bled on arrival for both haematological analysis and for serum, the latter being stored at -20^{o}C for future studies. Six weeks later, when the calves were 16 weeks of age the experiment was initiated.

Group 1) BPV-4 virus vaccine calves - 115 & 116
Group 2) BPV-4 vaccine control calves - 117 & 118

BPV-4 Virus Vaccine and Challenge Preparation

Both the virus vaccine and the challenge preparation were purified from a single case of bovine oesophageal papillomatosis, which as stated earlier associated with BPV-4. These tumours were obtained from the Glasgow abattoir, which is a large automated and modern slaughterhouse. The oesophaguses were examined and selection was based on single cases where there were numerous epithelial papillomas present. These tumours were stored in 50% PBS/Glycerol at 4^{o}C until required for purification.

BPV-4 tumours (20g) from a single case were homogenised in a Silverson grinder, using 60ml of PBS as diluent. The suspension was clarified at 2,000 and 10,000 rpm for 10 minutes each. The supernatant was aliquoted into 2gm equivalents ie 6.6ml. To estimate viral content, 6.6ml of the supernatant was purified by rate zonal centrifugation as described in chapter 2 and the resultant virus visualised by TEM phosphotungstic acid negative staining. The virus content was estimated to be approximately 10^{12} particles per ml. The doses for the vaccine animals were aliquoted into 4 x 6.6mls.

The virus for the challenge dose was aliquoted in to 4 x 1ml.

BPV-4 Vaccine Sites

The two prophylactic vaccine calves, 115 and 116 were injected with 6.6ml of the clarified viral suspension plus 6.6ml of Freund's complete adjuvant. After 14 days had elapsed another injection of 6.6ml of virus plus 6.6ml of Freund's incomplete adjuvant was administered. Both injections were given in equal volumes to the right and left quadricep muscles.

BPV-4 Challenge Site for Vaccinated and Control Calves

Two weeks after the last vaccine injection all the animals were anaesthetised with 3ml of Rompun (IM) and immediately before injection of the virus, 2ml of Brietal Sodium was given (IV). 1ml of the purified BPV-4 virus was inoculated into the soft and hard palate by approximately 20 intra-dermal injections. At fortnightly intervals the calves were examined in detail and bled for both haematological analysis and serum sampling.

Surgical and Histological Procedures

The calves were anaesthetised with Rompun and Brietal before surgically removing a lesion with the aid of a 3mm biopsy punch. The biopsy was fixed in 10% BNF followed by post fixation in corrosive formol. The material was then processed for embedding in paraffin wax. The sections were initially stained by H & E. Immunocytochemical localisation of the viral antigen was demonstrated by the PAP technique using type specific antisera.

Results

At **4 weeks** post infection, the **unvaccinated calves**(117 & 118) had numerous small, white, projectile lesions on the palate. Histologically they were plaque like lesions with epithelial thickening due to the proliferation of the basal and parabasal cells. There was also parakeratosis present overlying the epithelium. In a small area of the biopsy there was evidence of a slight projection, which in the future would have developed into a frond. Cytopathic changes were evident in the sub-keratin layer. The PAP technique, using type-specific antisera, located positive cells in the incorporation areas confirming the presence of a virus-induced tumour.

The **vaccinated calves** (115 & 116) had no visible lesions in the mouth.

By **6 weeks** both **unvaccinated calves** had numerous true papillomas covering the palate. Immunocytochemically the viral antigen was demonstrated in many cells, both in the sub-keratin layer and the incorporation areas. These cells were heavily stained. Again the vaccinated calves showed no evidence of a papilloma infection.

The calves were observed for a further 3 months and biopsies were taken at **8, 10, 12 & 17 weeks** post injection for histological and immunocytochemical examination. Histologically, they were all true epithelial papillomas of the alimentary tract and the viral antigen was immunologically positive by the PAP technique throughout the duration of the experiment.

Throughout this experiment there were no lesions seen in the mouths of the **vaccinated calves.**

Experiment 9

Prophylactic Vaccination Studies of Cattle Using BPV-6 Virions as the Immunogen

BPV-6 is the virus associated with the frond epithelial papilloma on the skin of the teats and udder of cattle. It is a common infection in both beef and dairy cattle where it often occurs in association with lesions produced by BPV-1 and 5 (Lindholm et al, 1984). Although BPV-6 induced tumours are the most common of the paragenital lesions, it also presents itself as a serious clinical problem. A severe infection can easily spread throughout a herd of cattle resulting in interference with milking or secondary infections of the teats, both of which can cause serious economic problems.

This experiment was designed to test the efficacy of specific tumour virions to protect against a homologous papillomavirus infection.

Material and Methods

Calves

Six calves were purchased from the same source as the previous experiments. These calves were not in contact with other animals and were selected as being free of any obvious papillomavirus infection by clinical examination. The calves were 10 week old, male, Friesians and on arrival were randomly selected into 2 groups, which were then housed in separate, clean, well ventilated pens in the isolation unit. This experiment consisted of 3 vaccinates and 3 non-

vaccinates (controls) The calves were bled on arrival for both haematological analysis and serum sampling, which was stored at -20°C for future studies. The experiment was initiated when the calves were 20 weeks old.

Group 1) BPV-6 virus vaccine calves - 101, 103 & 105
Group 2) BPV-6 vaccine control calves - 102, 104 & 108

BPV-6 Virus Vaccine Preparation

Both the virus vaccine and the challenge preparation were purified from a pool of teat frond papillomas obtained from the Glasgow abattoir. These tumours were stored in 50% PBS/Glycerol at 4°C until required for purification.

Teat frond papillomas (19g) were diced by scissors and homogenised in a Silverson grinder using 95ml of 0.5% sodium deoxycholate and 0.5% NP40 as diluent. The suspension was clarified at 2,000 and 10,000 rpm for 10 minutes each. The supernatant was purified by rate zonal purification as described on page 33 and the resultant virus was quantified by TEM phosphotungstic acid negative staining. The virus content was estimated to be approximately 10^{11} particles per ml. A sample was also sent for restriction mapping and hybridization and was subsequently confirmed as containing only BPV-6 genome.

Vaccine Sites

The three prophylactic vaccine calves, 101, 103 and 105 were injected with 1.5ml of purified BPV-6 virions plus 1.5ml of FIA. After 14 days had elapsed another injection of the same preparation was administered. Both injections were given in equal volumes to the right and left quadricep muscles.

BPV-6 Challenge Preparation

A similar preparation to the BPV-6 virus vaccine was made using 9.5g of pooled teat frond papillomas and 45ml of diluent. The same procedures and checks were made as described above.

BPV-6 Challenge Site for Vaccinated and Control Calves

Two weeks after the last vaccine injection all the animals were anaesthetised using 3ml of Rompun (IM). The abdomen between the teats was cleansed with ether before a linear scarification line was made with the aid of a scarification needle to the skin between the anterior and posterior teats on the left side. 1ml of purified BPV-6 virus was run into the resultant groove. The calves were bled for haematological analysis and serum sampling on a fortnightly basis and were clinically examined weekly.

Surgical and Histological Procedures

The calves were anaesthetised with Rompun before surgically removing a small representative piece of tissue for histological examination. The biopsy was fixed in 10% BNF followed by post fixation in corrosive formol. The material was then processed for embedding in paraffin wax. The sections were initially stained by H & E. Immunocytochemical localisation of the viral antigen was demonstrated by the PAP technique using type specific antisera.

Results

At 10 weeks on the **unvaccinated calves** (102, 104 & 108), raised epithelial plaques were seen on the site of previous

scarification. The **vaccinated calves** (101, 103 & 105) had no obvious lesions present on the line of scarification.

By **14 weeks** the plaques had a roughened surface and the scarification line was now confluent with these lesions. Histologically the epithelium was thickened in places due to keratinocyte proliferation and there was the beginning of the formation of plexiform acanthosis. There were no obvious cytopathic cells and there was no viral antigen detected by the PAP technique, using type specific antisera. Again no lesions were seen on any of the **vaccinated animals.**

When biopsied at **20 weeks** the lesions on the **unvaccinated calves** were circumscribed with a filliform surface. Microscopically the lesions were true epithelial papillomas of specific type. The stratum granulosum was thickened, especially in the areas between the papillomatous projections. In the upper keratinocyte layer there were many virus containing nuclei when examined by H & E and this finding was confirmed by the PAP technique.

Two of the **vaccinated calves** were not however free from lesions at this examination. On the line of scarification in calves 101 and 105 there were small but prominent, depilated lesions which did not resemble the BPV-6 tumours induced in the unvaccinated calves and were obviously fibropapillomas. This was confirmed by histological examination as a typical cutaneous fibropapilloma and viral antigen was positively demonstrated by the PAP technique, using a cross-reactive antisera.

In the **unvaccinated calves** at **26 weeks** there appeared to be a secondary spread of these tumours. Histologically the tumours appeared as before except there was more fronds, some of which were hyperkeratotic. There was a dramatic increase in the number of cytopathic cells and in the appearance of

masses of irregular keratohyalin granules. Virus containing cells were easily found by the PAP technique, using type specific antisera.

In the **vaccinated group** 103 still had no lesions but the fibropapillomas on calves 101 and 105 continued to develop. When examined by H & E they were fully developed, hyperkeratotic fibropapillomas with marked plexiform acanthosis. There was an abundance of cytopathic cells which were immunologically positive by immunocytochemistry (PAP).

At **31 weeks** the biopsy appeared similar to the previous except the lesion was completely hyperkeratotic. There was an chronic inflammatory reaction in the sub-epidermal tissue and in several areas there was interruption of the epidermo-dermal junctions. Cytopathic cells were still evident at this stage.

The fibropapillomas on the **vaccinated group** had to be surgically removed due to the marked increase in size of the lesion and also because it was ulcerating. There was still an abundance of viral antigen in the upper granular layer when examined by the PAP technique. This was confirmed by restriction mapping and hybridization as a BPV-1 infection.

Discussion

The rationale of using bovine cells transformed **in vitro** as a protective agent was that the protein products of the 'early' genes, involved in transformation, could be present on the surface of the cells and be effective in immunising against tumour induction by BPV-2.

Experiment 6 was designed to test this theory. However, 7

weeks after the application of the viral challenge preparation, a Stage 1 fibropapilloma developed on both the vaccinated and non-vaccinated calves. At week 8 they had developed into Stage 2 tumours and by the 11th week the lesions were fully developed Stage 3 fibropapillomas with viral containing cells present. For over 6 months these fibropapillomas were virally active but at week 35, although there was still a confluent line of typical fibropapillomas, there was no viral antigen to be detected by immunocytochemistry. The lesions finally sloughed off after 40 weeks had elapsed.

The attempts to protect by immunising against infection by BPV-2 using **in vitro** transformed bovine fibroblast cells was not successful. This may be due to a low level of the appropriate antigen.

The next experiment was designed to test and compare the efficacy of a BPV-2 tumour suspension and purified virions, to induce rejection of a papillomavirus infection.

Before this experiment was done, it was unknown whether protection depended on structural antigens of the virus, early antigens in the viral replicative cycle or neo-antigens of tumours. The experiment was therefore structured to make an exact comparison by taking 2 aliquots of tumour, making a homogenate of one and a virus purification from the other. The second preparation should only contain structural virion antigens while the first contained all three.

Tumour development was first recorded in the **unvaccinated calves** 4 weeks after application of the viral challenge preparation. By the 9th week all the **unvaccinated calves** had prominent fibropapillomas with cells containing viral antigen. There was also at this stage one animal from each of the **virus vaccine group** and the **tumour vaccine group**, which

had a small fibromatous nodule present on the line of scarification. Histologically, these lesions showed no thickening of the squamous epithelium or any cytopathology and when examined by the PAP technique, there was no viral antigen detected. At week 14 there was no visible lesion seen on calf 70 from the **virus vaccine group** but in the **tumour vaccine group**, calf 65 had a Stage 1 fibropapilloma with an overlying ulcerated epithelium. All the other **vaccinated calves** were still free of a papillomavirus infection. The **unvaccinated animals** had well developed fibropapillomas with numerous cells containing viral antigen. Calf 65 from the **tumour vaccine group** did develop a Stage 2 fibropapilloma by week 18, with no virus containing cells but this lesion suddenly regressed until week 29, when there was no visible lesion to be seen at the previous site of tumour development.

This experiment has demonstrated that animals could be immunised against a homologous infection and oncogenesis by vaccines prepared from virus and tumour extracts. In the final assessment there was no difference between the two preparations. The common feature of both vaccines was the presence of virus and their structural proteins.

With the success of therapeutic regimes in protecting against a member of Group A virus, it was logical to examine the efficacy of a therapeutic vaccine preparation from Group B.

The first experiment in this group was using purified BPV-4 virions as the immunogen. The first mouth examination at 4 weeks revealed that there were numerous, small projectile lesions on the palate of the **unvaccinated calves.** Although the lesions were small, they contained several cells with viral antigen in the sub-keratin layer of the incorporation areas. The **vaccinated animals** had no visible lesions. All the calves were subsequently subjected fortnightly, over a six month period, to a clinical examination and the appropriate

biopsies taken. In the **unvaccinated animals**, the projecting lesions developed into true epithelial papillomas by week 8. The lesions were typical of a BPV-4 infection and the upper keratinocyte layer showed the presence of virus containing nuclei, which were immunologically positive by the PAP technique. No lesions were observed at any time in the mouth of the immunised animals.

Again this experiment demonstrated that cattle can be protected against a homologous infection and oncogenesis by parenteral inoculation of purified virus.

Finally, it was decided to examine a prophylactic vaccine regime using BPV-6 as the immunogen. BPV-6 tumours are the most common of the paragenital lesions and present themselves as a serious clinical and economic problem.

The first lesions were observed at 10 weeks after the challenge, on the **unvaccinated animals.** These were plaque-like lesions on the site of scarification application but no lesions were visible on the **vaccinated animals.** By week 20 these plaque lesions had developed into typical frond-like BPV-6 tumours. In the upper keratinocyte layer there were many virus containing cells. Again no BPV-6 induced lesions were seen on the **vaccinated calves** but there were small, depilated lesions lesions which did not resemble a BPV-6 infection on two of the calves. These were morphologically fibropapillomas of a quite different structure than the BPV-6 lesions and they contained large amounts of BPV-1.

In chapter 2 the development and timing of the various papillomatous lesions was discussed in detail. However in this particular experiment and subsequent studies, which will not be dealt with in this thesis, it was found that the latency period of this BPV-6 infection was much longer than

that for BPV-2 and -4; in this instance full development was not completed until four months after challenge. It was also noticeable, in contra-distinction to BPV-2 and -4, that secondary spread of tumours from the infecting site to surrounding skin took place.

Virus for this experiment had to be obtained from naturally occurring tumours of the udders of cattle from commercial farms. Co-infection of the teat with individual discrete BPV-1 and -6 tumours occurs in 23% of cattle cases (Lindholm et al, 1984). Large numbers of cases were examined in an attempt to obtain teats showing only BPV-6 frond-type papillomas for virus purification. Examination of the purified virus preparation by Southern blot hybridization technique, using cloned DNA probes of all the BPV types showed only the signals for BPV-6. This is a very sensitive technique. However, in this experiment a BPV-1 fibropapilloma arose.

Two possibilities had to be examined; one, the more obvious, is that the challenge material was contaminated by skin containing small amounts of BPV-1 virus or viral DNA. The small amount would be reflected in the long latent period before the tumours were recognised. The second possibility was that the recipient animals had latent non-clinical BPV-1 infections. This has been shown to occur in a variety of circumstances (Jarrett, 1987). There is at present no knowledge of whether the development of overt tumours of one type can lead to the activation of latent virus of another type. This phenomena has not been seen at other BPV infection sites other than on the teats.

This experiment has shown that BPV-6 induces immunity to reinfection against homologous virus, but it does not protect against a BPV-1 infection.

In chapter 4 it was shown that papillomavirus infection with

tumour induction and regression leads to only type-specific immunity. This also stands true for vaccination against BPV-2, 4 & 6 infection by intra-muscular injection of virus and adjuvant.

CHAPTER 6

FINAL CONCLUSIONS

The studies described in the preceding chapters of this thesis have resulted in the reproduction of papillomavirus infections in cattle. This was crucial in order to widen our understanding of the pathogenesis of the infection and to attempt to fulfill the objectives of this research project.

The methodology, which was developed for purifying papillomaviruses, for both transmission and immunisation studies appeared satisfactory for the aims of this study. The immunocytochemical techniques used for the demonstration of BPV antigens was found to be sensitive enough to locate precisely the viral distribution and its relationship to the development of the lesion.

The final conclusions of this thesis can be summarised as follows;

1) Following the isolation of different BPV types and their classification by DNA restriction and hybridization, immunocytochemical techniques were developed to identify each of the viruses.

2) This peroxidase anti-peroxidase method demonstrated that each virus had specific lesion types and life cycle as had been inferred from morphological and molecular biological studies of spontaneous field cases of the different tumours.

3) The pathogenesis study revealed the nature and timing of virus replication and shedding for a range of virus infections.

4) Immunity to reinfection was produced by the tumour induction and regression cycle. It appeared to be strictly type-specific as judged by BPV-2, 4, 5 and 6 cases.

5) Prophylactic vaccination was possible by the intramuscular injection of purified virus only. This demonstrated that protection is specified by virus structural proteins and not `early' virus gene expression. No obvious advantage was seen in administering the virus along with a suspension of papilloma cells. Immunity to reinfection was not produced by injection of bovine fibroblasts transformed **in vitro** by BPV-2 and expressing only products of the `early' genes.

6) This work paved the way to choosing genetically-engineered products of the structural genes, L1 and L2, which form the capsomeres of the virus. It was then found that the product of the L1, but not the L2, conferred immunity to reinfection of the same nature described here for purified virus.

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