STUDIES ON PAPILLOMA VIRUS:

EXISTENCE OF VIRAL HETEROGENEITY

by

John P. Kosiuk

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Department of Microbiology and Immunology

McGill University,

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ABSTRACT

Human papilloma viruses were isolated from individual sources (non-pooled) and characterized using the techniques of immune electron microscopy and restriction enzyme analysis of the DNA. Using these techniques the existence of virus heterogeneity was demonstrated. When low quantities of wart tissue prevented virus isolation, papilloma virus DNA was shown to be present and suitable for analysis. Virions (HPV) purified from the common warts of compromised individuals (a renal transplant and a leukemic patient) were antigenically distinct and shown to differ in DNA composition based on sensitivity to restriction endonucleases. These HPV's were also shown to be distinct from papilloma virus isolated from plantar warts and bovine papilloma virus.

Papilloma-sized DNA could be selectively extracted from a specimen of genital warts (where virions could not be demonstrated). HPV DNA's were also selectively extracted from 3 common and 2 plantar wart isolates. Agarose gel electrophoresis of the DNA from two of the common wart specimens revealed the presence of size heterogeneity within an individual tumour. Restriction enzyme analysis of the DNA isolated established genetic heterogeneity.
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L'hétérogénéité chez les virions des papillomes, isolés de sources individuelles, fut déterminée en utilisant la microscopie électronique immunitaire et une analyse par des enzymes de restrictions. Même avec des quantités de tissus inappropriées à la récolte de virions, le génome du virus du papillome a pu être décelé en quantité suffisante pour son analyse. Les virions issus d'un patient ayant subi une transplantation rénale et d'un fermier leucémique sont immunologiquement distinct et leurs génomes diffèrent par leur sensibilité aux enzymes de restrictions. De plus, les virions du fermier diffèrent de ceux de souches bovines. Les virions d'un individu atteint de verrue plantaire sont différents de tous ceux déjà mentionnés.

On peut extraire de façon sélective l'ADN provenant de 3 types de verrues soit génitale, commune ou plantaire et ceci même en absence de virions. L'électrophorese sur gel d'agarose d'ADN provenant de 2 spécimens de verrue commune chez un même individu révèle une hétérogénéité dans la grandeur des molécules. L'analyse par des enzymes de restrictions de l'ADN isolé, démontre une hétérogénéité génétique.
Dedicated to my future wife Carol and to my parents,
Mr. and Mrs. Michael Kosiuk.
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The papilloma viruses, members of the papova virus group, have not been intensively investigated. Investigations of these viral agents has been hindered due to the absence of a cell system for their replication in vitro. Originally, it was thought that common warts (verrucae vulgares), flat warts and genital warts (condylomata acuminata) were caused by the same virus. However, current biochemical and serological data have shown the possible existence of several types of human and most recently bovine papilloma viruses.

Our study was undertaken to characterize virus isolated from wart-like lesions in compromised and normal humans and cattle. Serological studies involved the technique of immune electron microscopy; viral DNA was characterized by both contour length measurements and by restriction endonuclease digestion with subsequent examination of the cleavage products by agarose gel electrophoresis.
A. Physical Properties of the Papilloma Virus

1. Classification

The papilloma virus belongs to the papovaviridae family of viruses, but is distinct from polyoma virus by virtue of its genome size, capsid diameter, and the molecular weight of the main structural polypeptide (Crawford 1963a; Wildy 1971; Favre et al 1975a). Papilloma viruses induce benign epithelial tumours, commonly known as warts, in different animal species and in man, which are highly host and tissue specific.

2. Morphology

Particles believed to be the human papilloma virus (HPV) were first observed in aqueous extracts of papilloma tissue removed from children by biopsy, and then prepared for electron microscopy by chromium shadow casting (Straus and Shaw 1949). Spherical particles were observed in clusters, often in crystalline array, ranging in diameter from 50 to 53 nm. This was the first demonstration of crystal formation by an animal virus. The development of negative staining allowed for a more detailed visualization of papilloma virus structure (Brenner and Horne 1959). The viruses of human and animal papilloma viruses were observed to be spherical particles with a diameter of 50-55 nm, composed of 72 morphological units or capsomeres, arranged with cubical symmetry (Crawford and Crawford 1963; Klug and Finch 1965). Since then, typical papilloma virus particles have been detected by electron microscopy in papilloma sections and tumour extracts, and in the nuclei of keratinizing cells and in keratinized cells often in crystalline array (Almeida et al 1962;
1. Characteristics of the Nucleic Acid

The papilloma virus contains a covalently closed circular
duplex DNA, with a molecular weight of about $4.9 \times 10^6$ daltons (Crawford
1965; Fellet and Crawford 1967; Favre et al 1975b; Gissmann and Zur Hausen
1976). The papilloma virus was DNA was originally shown to have an unusual
structure by Watson and Littlefield (1960). On examining papilloma DNA
isolated from Shope Papilloma Virus, they observed two sedimentation coef-
ficients. The structure and molecular weight of papilloma virus DNA
isolated from human warts was first examined by Crawford (1965b). Crawford
showed that the double stranded DNA extracted from HPV had two sedimentation
velocity components when observed in an analytical ultracentrifuge at pH 7.5.
Component 1 had a sedimentation coefficient of 28s, while component 2 had
one of 20s. Furthermore under the action of pancreatic deoxyribonuclease
three distinct components were found on band centrifugation which had sedi-
mentation coefficients of 28s, 20s and 18s. The molecules of component 1
were shown to have a twisted superhelical structure as a result of their behaviour in neutral and alkaline solution. Component 2 represented the relaxed circular form which originated from component 1 by one or more single stranded breaks, while component 3 (18s) represented a linear form. Rowson and Mahy (1967) suggest that the naturally occurring linear molecules are not viral DNA but fragments of host DNA which has been incorporated into viral capsid. This linear DNA occurs at very low frequency in papilloma DNA preparations; not more than 3% in HPV DNA and 6% in Shope Rabbit Papilloma virus DNA. Crawford calculated the molecular weight (M.W.) of the papilloma viral DNA from the observed sedimentation coefficients and found it to be 5.3 x 10^6. Most recent molecular weight determinations have been based on agarose gel electrophoresis and contour length measurement of DNA, revealing a M.W. of about 4.9 x 10^6 (Favre et al 1975b; Gissmann and Zur Hausen 1976).

The base composition of both human and animal papilloma viruses has been examined. The Shope papilloma virus DNA was the first tumour virus DNA to be analyzed in detail (Watson and Littlefield 1960). The composition of this DNA was analyzed by thermal denaturation, chemical analysis and buoyant density. The results obtained indicated that the DNA did not contain significant amounts of unusual bases. Based on buoyant density in Cesium chloride equilibrium density gradient relative to marker DNA from Escherichia coli, the base compositions of human, canine, bovine and rabbit papilloma virus DNA were 41, 43, 45.5 and 47 per cent quanine + cytosine respectively (Crawford 1969).

4. Viral Polypeptides

Papilloma virus DNA with a M.W. of 4.9 x 10^6 daltons would have a coding capacity for proteins with a total M.W. of about 270,000 daltons,
assuming a non-overlapping triplet code. Studies by Favre et al (1975a) on the structural polypeptides of rabbit, bovine and human viruses using sodium dodecyl sulfate polyacrylamide gel electrophoresis have found up to 10 structural polypeptides in highly purified virus particles of each of the above viruses. They have estimated that the total molecular weight of the structural polypeptides is about $25 \times 10^6$, if the M.W. of the genome is $4.9 \times 10^6$ and the percentage DNA per virion is 17.5%. They found that the main components of the three papilloma viruses was a polypeptide with M.W. of 54,000 daltons and 3 polypeptides with M.W. of 16,500, 15,500 and 12,500 daltons. The latter three polypeptides were not found in empty capsids and were assumed to be histone-like proteins. Examination of the M.W. of Bovine papilloma virus (BPV) and HPV polypeptides revealed a close similarity.

Most of the early studies on the proteins of HPV have used pooled wart material and as a result there is the risk of mixtures of different types of HPV which would result in poor characterization (Pass and Maizel 1973; Pass and Marcus 1973; Spira et al 1974; Favre et al 1975a).

B. Morphology and Histology of Human Warts

Warts or papillomata of several different morphological and clinical types are caused by a HPV. These benign tumours result from hyperplasia of papilla and epidermis. The long dermal papillae which project up into the wart give rise to the medical name papilloma for this lesion (Rowson and Mahy 1967). Other pathological features include thickening of the epidermis and increased keratin content of the superficial epidermis. In warts, mitotic figures are seen several layers above the basal cell layer, whereas in normal epidermis such figures are seen only in the basal layer. Inclusion bodies have also been observed in these infections (Blank et al
1951; Almeida et al 1962). These inclusions are of two types, eosinophilic and basophilic. Electron microscopy studies have shown the presence of basophilic inclusions in the stratum spinosum and stratum granulosum and these have been associated with virus development. The nuclear eosinophilic inclusion bodies in the stratum spinosum were not found to be associated with virus development, but seemed to occur as a result of abnormal keratinization (Almeida et al 1962). However inclusion bodies and virus particles cannot be demonstrated in all warts. Another characteristic feature of human warts is vacuolization or death of granular cells, resulting in the formation of clear cells (Laurent et al 1975). Other histological features include alteration in keratohyalin granules and retention of melanosomes in granular and horny cells. There is speculation that this arises because of the inhibitory effect of virus on protein synthesis during epidermal keratinization (Epstein and Fukuyama 1975).

C. Replication of Papilloma Virus in Human Skin

Sections of papilloma tissue stained with fluorescin labeled anti-papilloma virus antibody have detected viral antigen only in the granular layer and in the lower portion of the cornified layer, in cells where keratin synthesis is about to begin or has started (Walter et al 1965; Shirodaria and Mathews 1975). Viral antigens are not detected in basal cells in the germinative layer.

The localization of viral DNA replication in wart tissue has also been examined. Nucleic acid hybridization studies with complementary RNA (cRNA) of the respective viruses has been used for the detection of viral DNA persistence in tumour cells believed to be transformed by virus (Green et al 1970). Using tritiated cRNA (to papilloma viral DNA), DNA
synthesis in papilloma tumours was detected by autoradiography in the nuclei of keratinizing granular cells just above the stratum spinosum and the upper Malphigian layers but not in the basal cell. The number of viral genomes in granular cells increased as the cells progressed toward the horny layer. In normal skin mitoses are limited to only the deepest regions of the epidermis (basal or parabasal regions), therefore the uptake of $^3\text{H}$ thymidine by the more superficial nuclei would infer papilloma DNA synthesis (Rashad 1969; Orth et al 1971; Orth et al 1977b).

The exact mechanism of papilloma formation remains to be determined. However it is believed that a single basal epidermal cell undergoes an abortive infection, whereby there may be persistence or partial expression of viral DNA which may be either in the integrated or autonomous state. Evidence for papilloma formation from infection of a single basal cell comes from experiments involving glucose-6-phosphate dehydrogenase (G6PD) typing in heterozygote female patients with warts (with two-X chromosomes coding for different isoenzymes). Murray et al (1971) found only one G6PD in each of 6 warts which they tested and thus proposed that warts arise from a single cell clone.

In summary, the infected basal cell is probably stimulated to divide by the virus. Viral replication may be totally repressed in the proliferative germinal layer but might be turned on during the expression of the differentiation of the host cells (keratinization), where virion production would begin with a resulting cytopathic effect.

D. Attempts to Cultivate Papilloma Viruses in Vitro

1. Human Papilloma Viruses and in Vitro Systems

Many attempts have been made to grow the papilloma virus in an
in vitro culture system, but most have been unsuccessful or irreproducible (Mendelson and Kligman 1961; Orozlan and Rich 1964; Rowson and Mahy 1967; Eisinger et al 1976). Multiplication has rarely been observed and there have been very few reports of the production of a cytopathic effect (CPE) using different human and animal cell culture lines. Butel (1972) was unable to show the production of any viral antigen, CPE, or demonstrate any viral replication, using a variety of culture techniques, but was able to show an increase in cellular DNA synthesis in contact inhibited embryonic human kidney cells after wart virus infection. There have also been recent reports by Lancaster and Meinke (1975) of an increase in cellular DNA synthesis after infection of a confluent monolayer of a cell line derived from foetal human foreskin (NHP cells) by human wart virus. An increase in cellular DNA synthesis was previously shown to be necessary for viral DNA integration (Sheinen 1966; Lehman and Defendi 1970). Therefore the possible persistence of papilloma virus genomes in host cells was examined for using DNA-DNA hybridization studies. Lancaster and Meinke were able to show papilloma DNA persistence in cells up until 11 months after infection, but without any observable CPE. They postulated that under the right conditions the viral DNA would be derepressed, with resulting viral production and CPE. The persistence of viral DNA in host cells was also demonstrated by Nieuwema et al (1975) when they were able to detect viral DNA sequences in DNA extracted from epithelial outgrowths obtained after culture of common wart explants, but were not able to detect viral antigens in these cells. Using an epithelial cell line (Bean epithelial cells) Eisinger et al (1975) have observed CPE infection with human wart virus. They were able to detect $10^7$ to $10^8$ particles per ml in cells which were enlarged compared to normal cells, had enlarged nucleoli and eosinophilic inclusions. They report the
occurrence of CPE after 4-5 days post infection and successful passage of the virus 14 times. However there have been no further reports on the success of this culture system and we were unable to obtain Bean epithelial cells from that laboratory.


There have been reports on the activity of Bovine papilloma virus (BPV) in tissue culture. BPV was shown to transform bovine and mouse cells (Black et al 1963; Thomas et al 1964). Transformed cells were morphologically different from control cells and had an indefinite life span. Lancaster et al (1976) have also demonstrated by nucleic acid hybridization the existence of BPV DNA in tumours induced by BPV, but where no virus could be detected.

The interaction of Shope papilloma virus (SPV) with rabbit and primate cells in tissue culture has also been examined (Chardonnet et al 1978). However they were unable to show production of infectious SPV or demonstrate the presence of detectable viral antigens.

E. Types of Human and Animal Papillomas

1. Human Papillomas Associated with a HPV

The human papilloma virus (HPV) has been associated with different types of skin and mucosal benign tumours which are distinguished by their morphological and histological features as well as their anatomical sites.

a) Verrucae vulgares (Common Warts): The most common type of skin wart is the type known as verruca vulgaris. This wart may occur singly or in multiple form, that is often in groups. These warts show various
degrees of papillary hyperplasia and may appear as sharply circumscribed mass of 1 to 5 mm. in diameter, with a hard roughed verrucous surface. They may occur anywhere on the skin but are most often found on the hands. Typical skin warts rarely spread to the genital areas.

b) Verrucae Plantares (Plantar Warts): These warts are considered topographic variants of verruca vulgares. These warts usually occur on the plantar surface of the foot and do not project outward but lie deep in the skin surrounded and covered by hyperkeratotic tissue. It is thought that these warts are driven into the skin by the pressure of walking and are imprisoned by a thick layer of keratinized cells on the surface.

c) Flat Warts: Another topographic variant of verruca vulgares. There is thickening of epidermis without papillary hyperplasia. Clear cells are seen to replace in part the stratum spinosum and stratum granulosum (Laurent et al. 1975; Epstein and Fukayama 1975).

d) Condylomata Acuminata (Venereal Warts): These warts affect the moist and soft skin of external genitalis and the perianal regions. These warts may become fungating masses. Such lesions usually occur with little keratinization. These warts are believed to be venereally transmitted (Barrett et al. 1954; Oriel 1971a; Oriel 1971b), and malignant conversion has been observed in rare instances. Thus the hypothesis has been advanced that the virus found in genital warts may be involved in the aetiology of human genital cancer (Zur Hausen et al. 1975; Zur Hausen 1976). Their possible role in human cancer will be examined later in (section I). Viral particles resembling typical papilloma virus have been observed irregularly in these lesions (Dunn and Oglivie 1968; Almeida et al. 1969; Oriel and Almeida 1970).

e) Epidermodysplasia Verruciformis (EV): EV is a rare disease characterized by disseminated skin lesions which resemble flat warts both
morphologically and histologically or are pityriasis like in appearance (Lutzner 1978). These lesions usually develop in childhood and may become generalized and persist throughout life. This disease has a familial occurrence which suggests that it is a recessively inherited genetic disease (Jablonska et al 1972). It has also recently been postulated that EV may involve a defect in immune functions resulting in life-long persistence of warts (Prawer et al 1977). Humoral immunity has been shown to be normal in most patients but cell mediated immunity is depressed. In addition to the typical EV lesions, these patients have been reported to have common warts, plantar warts and genital warts to a greater extent than the normal population. Twenty-five percent of EV patients may develop intraepidermal carcinomas or squamous cell carcinomas and such transformations demonstrate clearly that a human papilloma virus may be oncogenic (Orth et al 1977b; Orth et al 1978d; Orth et al 1979). Typical papilloma particles are observed regularly in the benign lesions (Aaronson and Lutzner 1967; Ruieter and Van Mullem 1970), but have not been detected in advanced malignant lesions (Aaronson and Lutzner 1967; Jablonska et al 1970; Jablonska et al 1972).

f) Laryngeal Papillomas: Most authors subdivide laryngeal papillomas into juvenile and adult types. In juvenile laryngeal papillomatosis, multiple squamous papillomas of the larynx are found. They behave like skin warts and occur most frequently in children between 1 and 5 years of age (Zur Hausen et al 1975). It is believed that genital warts at delivery may predispose the baby to this infection (Duff 1971; Cook et al 1973). Typical virus particles have been observed in these papillomas by electron microscopy (Boyle et al 1971; Boyle et al 1973).

Adult type laryngeal papillomas are usually solitary and tend more frequently to become malignant. They are believed to have a
different viral etiology (Zur Hausen 1977).

Some of the properties of the HPV's are summarized in Table 1 on the following page.
<table>
<thead>
<tr>
<th>TYPE OF LESION</th>
<th>TYPE OF VIRUS</th>
<th>VIRAL CONTENT</th>
<th>AGE DISTRIBUTION</th>
<th>MALIGNANT TRANSFORMATION</th>
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<tr>
<td>COMMON WART</td>
<td>HPV 2</td>
<td>low to moderate</td>
<td>mostly children some adults</td>
<td>none reported</td>
</tr>
<tr>
<td>PLANTAR WART</td>
<td>HPV 1</td>
<td>high</td>
<td>mostly children some adults</td>
<td>none</td>
</tr>
<tr>
<td>EPIDERMODYPLASIA VERRUCIFORMIS</td>
<td>HPV 3, HPV 4</td>
<td>moderate, moderate</td>
<td>lifelong</td>
<td>yes</td>
</tr>
<tr>
<td>CONDYLOMATA ACUMINATA</td>
<td>?</td>
<td>low</td>
<td>young adults (venereal trans-</td>
<td>yes (rare)</td>
</tr>
<tr>
<td>FLAT WART</td>
<td>HPV 3</td>
<td>moderate</td>
<td>children and adults</td>
<td></td>
</tr>
<tr>
<td>JUVENILE LARYNGEAL PAPILLOMA</td>
<td>?</td>
<td>very low</td>
<td>infants and children</td>
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1Based on Orth's and Favre's classification scheme (see section J. Existence of Heterogeneity Within Papilloma Viruses.

TABLE 1. COMPARISON OF THE HUMAN PAPILLOMA VIRUSES
2. Animal Papilloma Viruses

The papilloma virus induces skin or mucosal benign epithelial tumours under natural conditions in several animal species including cotton-tail rabbit, dog, bovine and horse (Rowson and Mahy 1967). However the most frequently studied animal papilloma viruses are the Bovine papilloma virus (BPV) and the Shope papilloma virus (SPV).

The BPV is probably the most intensively investigated animal papilloma virus. There appears to exist several different types of BPV (Lancaster and Olson 1978). These viruses can induce bovine fibropapillomatosis (Olson et al. 1965), as well as penile (Olson et al. 1968), and esophageal papillomatosis (Jarrett et al. 1978). The BPV has been implicated as the agent in atypical bovine papillomatosis, as virus morphologically indistinguishable from BPV can be isolated from these papillomas. There appears however to be no dermal fibroplasia involved in this disease.

The SPV occurs naturally in the cottontail rabbit where it multiplies in the keratinizing epidermal cells and produces typical papillomas (Stone et al. 1959). Under experimental conditions the SPV can infect domestic rabbits and produce papillomas which contain little or no virus but which develop into malignant carcinomas in up to 75% of the cases (Rous and Beard 1935; Syverton 1952; Orth et al. 1978b), the incidence of carcinoma in the cottontail rabbit is around 25%.

F. Transmission of Warts

The papilloma virus was the first tumour inducing virus shown to be active in cell free preparations (Rowson and Mahy 1967). However relatively little is known about transmission of papilloma virus except for that of Condylomata Acuminata which is thought to be venerealy transmitted.
Virus appears to enter the skin directly and minor injuries and irritation of the skin appear to aid in this infection. There appears to be no evidence for the existence of papilloma virus in the environment outside the actual wart tissue. Rowson states that warts appear to be transmitted from person to person both directly (contact with wart tissue) and indirectly by virus contaminated objects (Rowson and Mahy 1967).

There are several reports in the literature of attempts to propagate papilloma virus in individuals. The earliest report is that, of Licht (1894) innoculating wart material, isolated from another person, into his own skin with a resulting production of a wart (Rowson and Mahy 1967). Other early studies showed that warts could be transmitted by bacterial free filtrates. However innoculation of wart material into individuals did not always produce warts. This suggested to some investigators that HPV had a low probability of establishing a successful infection or that certain host factors were responsible.

Wart infections seem to be prevalent in children and young adults with a decline in later years indicating an increase in resistance with age. Their prevalence in children may be due to factors associated with contact developed during physical recreation (Coles 1958).

There is no real evidence for the transmission of warts between different animal species. There has been one early report in the literature of warts being transmitted from cattle to man (Rowson and Mahy 1967) but this has never been further substantiated. The inability to show inter-species transmission of bovine and human wart viruses might seem strange in light of the fact that one epidemiological survey had showed that veterinarians and butchers had a significantly higher incidence of warts than workers in other professions (Rowson and Mahy 1967). However the BPV
has been shown to have a broader host range in that inoculation into the brain of calves resulted in meningiomas (Gordon and Olson 1968) and intradermal injections in horses resulted in fibroblastic neoplasia. Despite these exceptions, animal papilloma viruses show a high degree of host and tissue specificity, that is, only those epithelia of those species which are infected in natural conditions are susceptible.

6. Species Specificity

There appears to be no detectable antigenic cross reaction between RPV, domestic rabbit oral papilloma virus, canine papilloma virus, BPV, and human plantar papilloma virus (Le Bouvier et al 1966; Koller and Olson 1972; Favre et al 1974). Different $G + C\%$ content have also been reported for each of the above viruses (see pg. 4). Furthermore when in vitro transcribed RNA's were used in molecular hybridization studies, no sequence homology was detected between the different animal papilloma viruses or HPV. However despite this evidence, there has been a recent report of an antigenic relationship between the structural polypeptides of RPV and plantar wart virus (Orth, et al 1978b). The common antigenic determinants were detected in immunodiffusion and immunofluorescence using sera of rabbits bearing a transplantable carcinoma - a tumour derived from a skin papilloma induced in a domestic rabbit by a recoverable strain of RPV. Two types of antigenic determinants were found to be shared by RPV and HPV (plantar). One type was shown to be masked in intact particles while the other was located on the surface but was unable to elicit antibody formation. These results seem to provide evidence for an evolutionary relationship between papilloma viruses of different species and suggest that they derive from a common ancestor. This is mere speculation and further studies are needed to
clarify these points.

H. Immunology of Wart Virus Infections

1. Cell Mediated Immunity

Cell mediated immunity (CMI) seems to play an important role in wart regression, evidenced by increased papilloma proliferation in patients with renal allotransplants or with long-lasting cell-mediated immune deficiencies (leukemia) (Spencer and Anderson 1970; Morison 1975b; Reid et al 1976; Viac et al 1977). A study by Touraine et al (1975) on the incidence of warts showed that warts occur at a higher frequency (28%) in immunosuppressed patients than in control subjects (8%). However, the incidence of warts in patients with humoral immunodeficiencies was not greater. The warts of renal transplants usually appear after 1 year or more of immunosuppressive therapy, and the number of warts is usually great. Wart regression in such patients is not common and there is a tendency for reoccurrence after treatment.

Lee and Olson (1968) showed that existing bovine papillomas continue to grow after humoral antibodies have developed and regress simultaneously with lymphocyte infiltration. Other clinical investigators have reported regression of warts after inflammation (Tagami et al 1974). Therefore, it seems that CMI is important in preventing the dissemination and persistence of warts or wart-like lesions.

2. Humoral Antibody Response

Almeida et al (1965) were the first group of investigators to report on humoral immune responses to structural antigens of HPV. They demonstrated IgM antibodies in the sera of patients by particle agglutination and precipitin tests. This was later confirmed by other workers. Shifodaria
and Matthews (1975) reported the presence of IgM (100%) IgG (97%) and IgA (80%) in the sera of patients with regressing warts. However other authors have not been able to correlate antibody response to wart regression (Oglivie 1970).

Pyrhonen and Johansson (1975) were able to show an inverse relationship between the duration and number of warts and the antibody titre. However clarification is still needed on the relative role of humoral response in wart regression.

3. Non-Structural Antigens

The presence of a T antigen, a non-viral marker of unknown function which indicates integration of viral DNA into the cellular genome and which has been found in other papova viruses, has not been demonstrated in human warts. However Pass and Marcus (1973) describe the presence of two non-structural antigens in wart tissue homogenates. Neither antigen appeared to be a structural component as evidenced by their different migration rates in acrylamide gel electrophoresis compared to proteins isolated from purified HPV. They detected these nuclear and cell surface antigens by immunofluorescence in warts, squamous cell carcinomas and normal skin. However these antigens were detected in concentrated extracts of normal skin by agar gel diffusion but in much lesser quantities than in wart tissue. The existence and role of these antigens in wart tumours has yet to be confirmed by other workers.

I. Papilloma Viruses: Possible Role in Cancer

1. The Animal Papilloma Viruses

Tumours produced by the animal papilloma viruses are usually
self-limiting and regress, however, malignant transformation has been observed in tumours caused by the cottontail rabbit (Shope) papilloma virus, bovine fibropapilloma virus both in natural and experimental conditions (Rous and Beard 1935; Syverton 1952; Olson et al 1969; Jarrett et al 1978). Cottontail rabbit papillomas have been reported to be transformed into invading and frequently metastasing squamous cell carcinomas in 25% of the cases (Kidd et al 1940; Syverton 1952). Moreover when this same virus (Shope papilloma) is experimentally injected into domestic rabbits the incidence of malignant transformation is three times higher providing evidence for the oncogenic potential of a papilloma virus and the role of genetic constitution in such malignancy.

The BPV has also been implicated as a causative agent of malignant alimentary tract papillomas in cattle in the upland areas of Scotland (Jarrett 1978). It is believed that the BPV acts synergistically with bracken fern, which is consumed by the cattle, to produce a marked increase in papillomas and later in life carcinomas, at least some of which are directly derived from pre-existing papillomas. BPV has also been isolated from urinary bladder tumours in cattle fed bracken fern further suggesting a co-carcinogenic effect (Olson et al 1969). The presence of intranuclear inclusion bodies characteristic of papilloma virus and the direct visualization of virus by electron microscopy in suspensions of tumour tissue were both observed in these studies. The co-carcinogenic action of polycyclic hydrocarbons and virus had been previously shown by Rodger and Rous (1951) when epidermal cells exposed concurrently to both agents were shown to convert to maligncy long before and more frequently than controls. Thus it seems that environmental factors (dietary in cattle) can influence the oncogenic potential of a virus. Further evidence for the
role of BPV in production of malignant tumours could come from nucleic acid hybridization experiments using probes for papilloma DNA sequences.

2. HPV: Role in Cancer

The malignant conversion of laryngeal papillomas, condylomata acuminata and the lesions of epidermodysplasia verruciformis has been reported, suggesting a role of HPV in cancer (Orth et al 1977b).

It is believed that 5% of the cases of carcinoma of the vulva arise from pre-existing genital warts (Woodruff and Novack 1962; Underwood and Hester 1971) and penile warts have been reported to give rise or be associated with up to 15% of penile carcinomas (Rhatigan and Saffos 1972). However in all reported cases of malignant transition virus particles were not observed in the cancerous lesions. Most of the studies on the malignant conversion of condylomata acuminata have been epidemiological and stronger evidence for the role of virus as a carcinogenic agent would come from biochemical studies involving nucleic acid hybridization, but this remains to be done.

Presently there is no evidence for the role of common wart virus in human malignant tumours despite the fact that these viruses are epitheliotropic which might favor production of skin carcinomas.

It is believed that Epidermodysplasia Verruciformis (EV) is the human counterpart of the Shope Papilloma System. The malignant conversion of the lesions of EV has been observed in 25% of the patients (Jablonska ef al 1972). The clinical aspects of the lesions and the malignant conversion in EV are related to the type of HPV responsible for the infection HPV4 being associated with a much high frequency of malignancy than HPV3 (Orth et al 1979) (See section on viral heterogeneity...
for definition of HPV3 and HPV4). Like in the Shope papilloma virus system, virus is only found in benign lesions but not in those having undergone malignant transformation (Orth et al 1977b). The preferential localization of the carcinomas in sun exposed regions suggest a promoting role of sunlight (Glinski et al 1976; Hammer et al 1976). Like the Shope papilloma system, the course of the disease seem to be influenced by the genetic constitution of the host (Rajogopalan et al 1972), which may result in greater susceptibility of the host keratinocytes to HPV3 or HPV4 or in an inability to repair U.V. damaged DNA. Congenital predisposition is also supported by the results obtained after heteroinoculation of papilloma virus isolated from EV lesions into patients induced typical flat warts which were indistinguishable from juvenile flat warts and regressed spontaneously after several months without any malignant changes (Lutzner 1978).

Studies on the role of papilloma virus in cancer have probably been hindered due to the lack of a reliable in vitro culture system and the reliance on viral isolation from tumours with variable (often very low) virus content. Detection of a T antigen and nucleic acid hybridization studies using DNA or cRNA probes would certainly be beneficial in determining whether the malignancy observed developed from warts or from new de novo lesions. The mechanism of DNA integration and persistence in host cells needs to be examined more carefully as well as the role of environmental carcinogens on the rapidly proliferating epidermal cells which are found in warty lesions. Further biochemical characterization of the different papilloma virus isolates will allow us to determine the oncogenic potential of the viruses involved.
J. Existence of Heterogeneity Within Papilloma Viruses

1. Basis of Techniques Used in Examination of Viral Heterogeneity
   a) Immune Electron Microscopy: This technique allows for visualization of antibody-antigen reactions in the electron microscope, after negative straining. The procedure was developed simultaneously by two independent groups of workers. Lafferty and Oertelis (1961) using influenza viruses as their antigen, were able to show distinctive changes in the morphology of the virus when it was incubated with homologous antibody and then examined by electron microscopy. Anderson et al (1961) used mixed preparations of polio virus and bacteriophages to show that these viruses, which are morphologically distinguishable, were aggregated only by their specific antisera.

   Examination of wart virus antigen by IEM was first applied by Almeida et al (1969). Incubating rabbit antisera prepared against purified virus extracted from human wart tissue with purified HPV, for 1 hour at 37°C, they were able to observe specific changes in the appearance of the virus. On examination of grids prepared they saw virus particles that were aggregated and also obscured by what appeared to be a halo of antibody molecules, making it no longer possible to delineate the overall outline and surface detail of the virus. In the untreated control (pre-immune sera), however, the particles displayed sharp outlines and were randomly distributed. When HPV was mixed with homologous antisera a tenfold increase in particle number could be observed when compared to control antisera. Very few particles remained single, and aggregates, ranging from those containing a few virus particles to others which were so large that no proper resolution could be obtained, were observed.

   One of the advantages of IEM is the detection of clumps of
viruses when no virus is visible in the original suspension because of low concentration. As little as $10^8$ virus particles are sufficient to act as suitable specimens, whereas the formation of precipitin lines in gel diffusion tests requires a considerable amount of virus antigen, being of the order of $10^{12}$ particles per ml.

Another advantage is that it is possible to recognize the individual antibody molecules, surrounding the virus particles, at higher magnification.

We ourselves used this technique to examine the antigenic relationship of different papilloma viruses (human and bovine).

b) Restriction Enzyme Analysis: Restriction enzymes recognize specific nucleotide sequences in double stranded native unmodified DNA and cleave both strands of the duplex. These enzymes have been recovered from several bacteria including a variety of strains of E. coli and from some strains of Haemophilus (Meselson et al 1972; Gromkova and Goodgal 1972; Sack and Nathans 1973). Because of their specificity, that is, their ability to cleave at certain well-defined base sequences, usually palindromic in nature, they have been used by many investigators in the study of DNA structure.

The cleavage patterns obtained after endonuclease treatment have been examined by velocity sedimentation through sucrose gradients but this method is imprecise. Electrophoresis through polyacrylamide gels proved to be more accurate but is perhaps more suitable for electrophoresis of smaller molecular weight compounds, such as proteins, as the pores in such gels are quite small. However, electrophoresis through polyacrylamide gels have certain disadvantages, in that the method is sensitive to the ionic strength of the loading buffer, requires lengthy staining and
destaining procedures, and tedious slicing of gels or autoradiographic procedures (Sharp et al 1973).

In order to overcome some of the problems associated with polyacrylamide gel electrophoresis, Sharp et al (1973) developed the rapid technique of analytical agarose - Ethidium Bromide electrophoresis. This method eliminates lengthy staining and destaining procedures and can resolve DNA fragments which are less than $7 \times 10^6$ daltons, without being sensitive to the ionic strength of the buffer. In this procedure very low concentrations of ethidium bromide are used to stain the gels. The ethidium bromide can either be incorporated in the gel and running buffer or the gel may be stained for 30 minutes in a solution of .5 ug per ml of ethidium bromide after completion of the run. The dye binds to the DNA and on exposure to ultraviolet light fluoresces and bands containing as little as .5 ug of DNA can be visualized (Sharp et al 1973). By changing the gel matrix and running time, a wide range of molecular weights can be resolved. The method of restriction enzyme analysis followed by agarose gel electrophoresis is quite suitable for studies of viral heterogeneity in that the cleavage patterns obtained may be used to identify viral sub-types, and has been employed in our studies.

2. Characterization of MPVs

Initially it was thought that the different clinical types of papilloma lesions were caused by the same virus and variation in morphology was due to the local conditions at the site of infection. This idea was based on the results obtained from transfer experiments where innoculation of virus isolated from laryngeal papillomae or penile condylomata acuminata into the skin of volunteers resulted in typical common warts
being produced (Rowson and Mahy 1967). As the virus could not be grown in tissue culture, and the methodology for biochemical characterizations had not yet been developed, all that was left was the detection of viral particle in sections of warts by electron microscopy, which was insufficient for distinguishing different sub-types of morphologically identical viruses. However through the use of newly developed serological and biochemical techniques it has recently been shown that there exists several sub-types of papilloma viruses in man.

Heterogeneity of HPV was first detected through the use of nucleic acid hybridization studies. This was based on the criterion that virally induced tumour cells harbor DNA sequences of the transforming agent which are covalently linked to host DNA (Sambrook et al 1968; Green 1970; Adams et al 1973). Using this technique a radioactive complementary RNA (cRNA) was transcribed in vitro, with the aid of Ecoli polymerase, from DNA isolated from plantar wart virions. This cRNA was then used as a probe to detect homologous DNA sequences in DNA isolated from laryngeal papillomas and condylomata acuminata, the latter of which does not contain significant quantities of virions. The results obtained showed that there was no homologous sequences in laryngeal papillomas and condylomata acuminata which suggested viral heterogeneity (Zur Hausen et al 1974). The level of human papilloma sequences in DNA isolated from condylomata acuminata was also examined for by Delap et al (1976), by measuring the effect of adding known amounts of unlabeled, denatured fragmented condyloma DNA on the rate of re-association of 125I labelled human papilloma DNA from a pool of common warts. The results they obtained showed that there were no homologous DNA sequences in condylomata tissue, even though papilloma-sized DNA could be detected in tumours.
Serological, as well as epidemiological evidence, supported the existence of a distinct type of papilloma virus as the aetiological agent of condylomata acuminata. Epidemiological studies showed that condylomata acuminata was venereally transmitted (Oriel and Almeida 1970). Using the technique of immune electron microscopy (see pg. 22) Almeida et al (1969) showed a one way cross reactivity between the viruses isolated from common warts and those of condylomata acuminata (C.A.). It was shown that the antisera to common wart virus reacted with both skin and genital wart viruses but that the antisera to genital wart virus reacted only with genital wart virus. As mentioned previously there had been reports of production of typical common warts after injections of extracts prepared from penile C.A. (Rowson and Mahy 1967). This might be explained if the morphology of C.A. lesions are closely examined. There seems to be several types of anogenital papillomas including "keratin rich (hard papilloma of the skin, as well as softer often larger cauliflower-like lobulated papillomas of the mucous membranes and or skin" (Delap 1977). Therefore it is conceivable that these lesions may be caused by different types of papilloma virus, one of which may cause typical skin warts when injected at other skin sites. However, genital warts contain only small amounts of papilloma virus which makes their characterization difficult.

Viral heterogeneity in other skin papillomas has only been recently demonstrated (Gissmann et al 1977; Orth et al 1977a; Orth et al 1978d). It had been assumed that the properties of virus isolated from lesions of high virus content, in this case the plantar wart, were those of the human wart virus, and as a result virus found in lesions containing smaller amounts may have been overlooked. Furthermore, the difficulty of obtaining sufficient amounts of wart specimens from the same individual led

many investigators to use pooled wart material, which might have obscured detection of viral heterogeneity.

Through the use of restriction endonuclease mapping (see pg. 23), nucleic acid hybridization techniques, and characterization of viral proteins by SDS polyacrylamide gel electrophoresis several sub-types have been demonstrated. Gissmann et al. (1977) characterized 50 individual wart isolates using the above techniques and in doing so they identified 4 distinct types of human wart virus which they named HPV1, HPV2, HPV3, HPV4. However they were not able to show differences in the biological activity of their isolates as HPV1-4 were found both in plantar and common warts. HPV1 was found in the majority of their isolates and these usually yielded high amounts of virus. This was opposed to HPV4 which was found in lesions with low virus content. In nucleic acid hybridization studies it was found that the cRNA from HPV1 annealed to the same extent with HPV2 and HPV3, whereas no base homology could be detected with HPV4. Using several different restriction endonucleases and examining the cleavage products on polyacrylamide gels it was shown that HPV1-3 were quite similar while HPV4 was shown to be entirely different. There were only slight variations in the cleavage patterns of HPV1-3 and it was assumed that HPV2 and HPV3 were the more rarely occurring sub-types of HPV1. The protein patterns of HPV1-4 were also examined on SDS polyacrylamide gels. HPV1-3 had identical protein patterns but differed from HPV4 in the molecular weight of their major proteins. HPV1 and HPV4 did not cross react in complement fixation assays. The age distribution of patients with warts induced by HPV1-3 and HPV4 also differed. HPV1-3 was seen to predominate in individuals 5-15 years of age while HPV4 was found more often in those 20-25 years of age (Pfister and Zur Hausen 1978a). It was later shown by this same group of workers that
HPV4 could give positive results in immune electron microscopy when reacted with serum from a patient with epidermodysplasia verruciformis. Recently Zur Hausen and Pfister (1978) have shown that warts isolated from two different sites on the body (hand and foot) in the same patient contained the same virus. Using serological characterization they showed that HPV1 and HPV4 appear in the ratio 3:1 with HPV1 being more prevalent in plantar warts 4:1 than in verrucae vulgares 2:1. Several of their isolates however did not react with HPV1 or HPV4 antiserum suggesting the existence of unrelated papilloma virus, but as the particle concentration in these warts was too low, biochemical characterization was impossible (Gissmann et al 1977; Pfister and Zur Hausen 1978b).

The biochemical and immunological characterization of human papilloma isolates has also been undertaken by Orth and co-workers. They too have demonstrated the existence of four completely distinct papilloma viruses which they named HPV1-4 after the chronological order of their isolation (Orth et al 1977b). However their classification scheme differs from that of Gissmann. HPV1 was isolated from plantar warts while HPV2 was isolated from a patient bearing multiple common hand warts; HPV3 and HPV4 were isolated from cases of epidermodysplasia verruciformis.

Despite having DNA's with almost identical molecular weights (5.26 x 10^6 and 5.23 x 10^6) HPV1 and HPV2 DNA's had different cleavage patterns after restriction endonuclease digestions were carried out and also differed in their sensitivity to the enzymes. The two viruses also had distinct polypeptide patterns. The main components of plantar HPV were a major polypeptide with M.W. 54,000, a polypeptide with M.W. 76,000 and 3 polypeptides which co-migrated with cellular histones (Favre et al 1977). The main components of HPV2 were a major polypeptide
with a M.W. of 67,000 and a polypeptide of M.W. 53,000 as well as four low M.W. polypeptides only one of which co-migrated with the histone-like proteins.

DNA-DNA reassociation and cRNA-DNA filter hybridization studies did not detect any homology between these viruses while immunodiffusion and immunofluorescence tests did not detect any antigenic cross reactions.

The association of particular types of HPV with EV has also been demonstrated (Jablonska et al 1972; Pass et al 1977; Orth et al 1979). The morphological features of the lesions found in patients with EV differed according to the type of virus; HPV3 was found in patients showing only flat wart-like lesions, while HPV4 was found in patients showing reddish plaques and or pityriasis versicolor-like lesions (Orth et al 1979). Nucleic acid hybridization studies involving cRNA's detected no sequence homology between HPV3 and HPV4 or HPV1 and HPV2 DNA's.

Examination of DNA isolated from virions in EV lesions revealed that it was 3.3% longer than HPV DNA (Pass et al 1977). No antigenic cross reaction was detected by immunodiffusion, indirect immunofluorescence, or immune electron microscopy, either between EV HPV's and HPV1 or HPV2.

3. Microheterogeneity With HPV Sub-types

The possibility also exists for variation within the individual groups of HPV. Favre et al (1975) showed that in restriction endonuclease digests of pooled plantar warts variations could be detected in the cleavage patterns in some instances. They estimated that a small population of these plantar virions contained either variations in
sequence or were a heterologous population of viruses themselves. Gissmann and Zur Hausen (1976) also reported variation in the number of cleavage sites when the same enzyme was applied to different pooled plantar wart DNA preparations. An additional Bam I site and Hind III site were shown to occur in one out of three DNA preparations. Studies on the sensitivity of plantar wart virus DNA also suggest the existence of viral microheterogeneity. Favre et al (1977) were able to demonstrate the absence of a Hpa II site in 40 to 50% of DNA molecules purified from virions obtained from three different pools of plantar warts. Furthermore this same group were able to detect a greater genetic heterogeneity in common warts (verrucae vulgares). Papilloma DNA isolated from a patient suffering from multiple common warts showed a high proportion of resistant molecules after treatment with the restriction endonuclease EcoRI and Bam and a sequence heterology of 8% was detected by the heteroduplex technique (Orth et al 1977a). Delap et al (1977) were also able to detect atypical fragments after restriction endonuclease mapping was performed on DNA isolated from pooled common warts, further supporting microheterogeneity within individual sub-types.

Studies on EV also report the presence of virus isolated from EV type lesions which are immunologically distinct from HPV3 and HPV4 (see pg. 28), as determined by immunodiffusion and immunofluorescence; molecular hybridization studies performed also confirmed this heterogeneity (Pass et al 1977; Orth et al 1979).

Therefore it seems from the evidence just presented that a number of human papilloma viruses remain yet to be characterized and further investigations in this area are needed.
4. Viral Heterogeneity Within the Animal Papilloma Viruses (BPV)

There have been reports of the existence of different classes of BPV. Lancaster et al. (1978) showed that in 10 cases of bovine fibropapillomatisis, there were two distinct papilloma viruses involved. The two types were distinguished on the basis of nucleic acid hybridization and were shown to share 52 to 58% of their DNA sequences. However polypeptides isolated from these two types of virus had similar electrophoretic mobilities.

Virus has also been isolated from a disease called atypical bovine papillomatosis which has morphologically indistinguishable from BPV but was shown by immunodiffusion to be antigenically unrelated (Barthold et al. 1974).

K. Conclusion

As a result of the lack of a reliable in vitro culture system for papilloma viruses, these viruses have not been intensively investigated. Investigations of these agents have had to rely on extraction of virus from wart tissue. The difficulty in obtaining enough wart tissue from single donors has led many investigators to use pooled wart specimens from several donors (Gissmann et al. 1976; Delap et al. 1977; Favre et al. 1977b). Biochemical and serological characterization of such pooled material would limit the detection of different viral sub-types. This lack of examination of single wart isolates led to our own investigations in the papilloma field.

Epidemiological surveys have shown that compromised individuals such as immunosuppressed renal transplants and leukemics have a higher incidence of warts than the general population (Touraine et al. 1975). We were able to obtain sufficient quantities of wart tissue from such compromised individuals for purpose of virus isolation and characterization
of DNA by restriction endonuclease digestion.

Our interest in warts from compromised individuals was also augmented by earlier reports of isolation of distinct types of papovaviruses from previously characterized papovaviruses in such patients by two groups of independent workers (Gardner et al 1971; Padgett et al 1971). Gardner et al (1971) were able to isolate a virus, which had the size and morphology of a papovavirus of the polyoma genus in the urine of an adult male receiving immunosuppressive therapy following a renal transplant. They were able to detect these viruses which they called BK virus in the nuclei of inclusion bearing epithelial cells that were shed in the urine. The DNA isolated from this virus differed from SV40 and other papovaviruses in their sensitivity to various restriction endonucleases and were also shown to exhibit a high degree of heterogeneity (Howley et al 1975). The heterogeneity was assumed to arise from a high proportion of defective virions. Padgett et al (1971) were also able to isolate a distinct type of papovavirus, the size of polyoma genus, which they named JC virus from the diseased brain tissue of a case of progressive multifocal leukoencephalopathy (PML), and subsequent work has shown that this virus is regularly associated with PML (Narayan et al 1973).

Therefore in characterizing papilloma virus isolates from compromised individuals we were hoping to find "unusual" types of papilloma viruses from those previously characterized (see section on Viral Heterogeneity), as well as demonstrate the possible existence of defectives. At the same time we planned to examine wart virus isolated from plantar and common wart lesions to determine whether they were antigenically or biochemically similar. In doing so we hoped to clarify conflicting reports on the relationship of distinct papilloma viruses with specific anatomical
sites. We also attempted to isolate the viral agent responsible for condylomata acuminata, as the aetiology of this infection is still somewhat circumspect.
III. MATERIALS AND METHODS

A. Abbreviations and Definitions

EDTA: Ethylene diamine tetracetate, sodium salt
PBS: Phosphate buffered saline
Sarkosyl: N-lauroyl Sarcosine, sodium salt
SDS: Sodium dodecyl sulfate
CsCl: Cesium chloride
Tris-HCl: Tris (hydroxymethyl) aminomethane titrated with 2N HCl to the indicated pH
PTA: Phosphotungstic acid (sodium salt)
Pt-Pd: Platinum-palladium
BPV: Bovine Papilloma Virus
HPV: Human Papilloma Virus
Form 1: Covalently closed circular duplex DNA
Form 11: Open circular duplex DNA-DNA containing one or more single stranded nicks
Form III: Linear DNA

B. Isolation of HPV from Single Donors

1. Patients Examined

The donor subjects consisted of a patient suffering from extensive common hand warts, who was also an immuno-suppressed renal transplant; a leukemic farmer covered in typical common warts (who subsequently died); an individual with a large growth of plantar warts on the base of the foot; a female with genital warts (condylomata acuminata).
2. Sample Collection and Storage

Wart specimens were kindly provided through Dr. R. D. Wilkinson of the Dermatological Dept. of the Royal Victoria Hospital. Common and genital warts were surgically excised and then immediately stored at -20°C. Plantar warts were removed by shave biopsy and also stored at -20°C. All samples were stored separately. The wart tissue was excised from each individual over a period of several months (except for the case of condylomata acuminata) from lesions which were morphologically identical and appeared at the same anatomical sites. When at least 5 grams of tissue had accumulated in a single donor, the papilloma virus was then extracted.

3. Virus Purification

The frozen wart material was covered in .15M NaCl solution and allowed to thaw out. The thawing solution was discarded and the wart material was rinsed twice with fresh solution. The wart tissue was then cut with scissors into fine pieces and then ground with sand using a mortar and pestle. A volume of .1M NaCl, .05M sodium phosphate buffer pH 8 was added (approximately 5 ml per gram of wart tissue) (see appendix for composition of all buffers), and the tissue further ground until it was finely macerated. The wart, sand and buffer mixture were then centrifuged at 8000 g for 15 minutes at 4°C in a Sorval RC2-B centrifuge using rotor SS34. The supernatant was collected and stored at 4°C. The pellet was resuspended in fresh sodium phosphate buffer and centrifuged once more at 8000 g (SS34 rotor) for 10 minutes. The supernatants were then combined and trypsin (Difco) added to give a .01% solution. After incubation at 37°C for 1 hour the mixture was centrifuged for 15 minutes at 8000 g (SS34 rotor)
rotor) at 4°C. The supernatant was then centrifuged at 80,000 g for 75 minutes at 4°C using the Beckman L2-65 ultracentrifuge fitted with rotor 50.1. The pellet was resuspended in .05M NaCl, .01M EDTA, .05M phosphate buffer pH 7.4 and the resulting supernatant was centrifuged at 8000 g for 15 minutes. The supernatant was collected and 6.4 grams of cesium chloride (CsCl) was added and the volume made up to 15 ml with phosphate buffer pH 7.4. The virus-CsCl solution (density 1.34g/ml) was then centrifuged using an SW 65 Beckman rotor at 110,000 g for 24 hours at 4°C. After centrifugation two sharply visible bands corresponding to full particles and empty capsids were collected separately after piercing the bottom of the tube with a syringe and using a Cornwall pipetting outfit to separate the fractions. The homologous bands were pooled and dialyzed against PBS pH 7.4.

4. Electron Microscopic Examination of HPV

The homogeneity of the purified wart preparations was checked by examination of viral particles under a Philips 300 Electron Microscope, after negative staining with 2% PTA pH 7.2. The negative staining procedure involved the use of carbon coated formvar grids. A small drop of virus suspension was placed on the grid surface and allowed to stand for 10 to 15 seconds. The excess was then removed by touching the grid to the edge of a piece of filter paper. The grid was then washed with a drop of distilled water, blotted dry and stained immediately with a drop of PTA for about 10 seconds, and once again dried. The grid was then examined in the electron microscope. Micrographs were taken at 60 kV, using Kodak Sheet Film.
5. Determination of Virus Concentration

Viral concentration were estimated from the protein concentration determined by taking the optical density (O.D.) of the purified virus preparation at 260 nm and 280 nm, using PBS as the blank. Protein concentration was estimated from the relationship:

\[ \text{Protein Conc. (mg/ml)} = 1.45 \times \text{OD}_{280} - 0.74 \times \text{OD}_{260} \] (Kalckar, 1947)

A Gilford Model 250 Spectrophotometer was used to take the O.D. measurements. Knowing that a protein concentration of 50 \( \mu \)g corresponds to approximately \( 10^{11} \) particles per ml, the virus concentration could be estimated (Gissmann et al, 1977; Viac et al, 1977).

C. Isolation of BPV from Bovine Papillomas

Bovine papillomas were kindly provided by Dr. Raymond Roy of the Dept. of Veterinary Medicine, University of Montreal, at St. Hyacinthe, P.Q. The papillomas were excised from cattle infected with multiple papilloma lesions or bearing single warts. The tissue was stored at -70°C. Approximately 5-6 grams of tissue was used for virus isolation using the method previously described (Materials and Methods B-3).

D. Detection of Papilloma Virus in Saline Lavages

Desquamations from extensive warts on the hands were collected by having the patients wear surgical gloves filled with 50 ml of saline. The lavages were then stored at -20°C, until used. When required, the lavages were unfrozen and then filtered through .45 um millipore filters. The filtrates were collected and then examined in the electron microscope for papilloma virus particles, using the negative staining procedure described previously (Materials and Methods B-4).
filtrates were then treated as in the procedure for virus purification (Materials and Methods B-3).

E. Immune Electron Microscopy Studies Involving Human and Bovine Papilloma Viruses

1. Types of Viral Antigens Examined

Papilloma viruses purified on Cs-Cl gradients were used as antigens in immune electron microscopy studies. The viruses used consisted of bovine and human wart viruses previously described (Materials and Methods section B-1 and C). These were designated BPV (multiple); BPV (single); HPV Common (renal transplant); HPV Common (leukemic); HPV plantar (normal). Each of the above viruses was injected separately into female New Zealand rabbits.

2. Preparation of Rabbit Antisera

a) Pre-immune Sera: 25 cc. of blood was taken via percutaneous puncture of the ear arteries of the female New Zealand rabbits prior to antigen injection.

b) Preparation of Immune Sera: The rabbits were immunized with .1 ml of the respective virus suspensions (approximately $10^{11}$ particles/ml), diluted to .5 ml with PBS and then mixed with an equal volume of Freund's complete adjuvant. The injections were given subcutaneously in aliquots of .15 ml, into 6 different lymph node drainage areas. One booster injection was given in the same manner three weeks after the primary injection. The antisera were collected at 2-week intervals after the primary injection until maximum titer had been achieved. The blood specimens were drawn with aseptic precautions without anticoagulants. The serum was separated and
3. Determination of Antibody Titer

The antibody titer was determined by indirect immunofluorescence experiments, using pieces of wart tissue obtained from each of the donor subjects. The pre-immune sera served as a control. This was done in the laboratory of Dr. R. D. Wilkinson, Dept. of Dermatology. Staining was achieved using a fluorescein labelled goat antiserum to rabbit gamma globulin and slides were viewed with a Zeis fluorescence microscope.

4. Formation and Detection of Antibody-Antigen Complexes

0.2 ml of purified (diluted) virus suspension (10^{10} particles/ml) was mixed with 0.7 ml of physiological saline and 0.1 ml of antisera, diluted with PBS so that the titer was 1/1000 (Almeida and Waterson 1969). After thorough agitation, the mixture was incubated at 37°C for 1 hour and then stored at 4°C overnight. The mixture was then spun at 8000 g for 45 minutes in the Sorval centrifuge using rotor SS34 at 4°C. The supernatant was discarded and the pellet was resuspended in 0.1 ml of distilled water and a drop of this was used in negative staining using the procedure previously described (Materials and Methods B-4). The grids were examined under the Phillips 300 Electron Microscope, for virus-antibody complexes.

F. Extraction of DNA from Papilloma Virions

Papilloma DNA was extracted from virions previously purified on CsCl gradients, using a modification of the procedure of Tai et al (1972). Sarkosyl (5% stock solution) was added to the virus suspension (1.2-1.9 ml)
so that the final concentration was 1%. The mixture was well shaken and then incubated at 25°C for 30 minutes, after which the NaCl concentration was adjusted to 1M. The mixture was then centrifuged at 10,000 rpm in a Sorval centrifuge using rotor SS34 at 4°C for 15 minutes. The supernatant was collected and to it added 2 mls of Ethidium Bromide (500 ug/ml). The volume was then made up to 5 mls with .05M Tris pH 7.5. CsCl (analytical grade) was then added to bring the solution to a density of 1.55 g/ml. The CsCl-ethidium bromide solution was then centrifuged in an International Ultracentrifuge using (Rotor No 494) at 38,000 rpm for 40 hours. Two fluorescent DNA bands were located under UV radiation and separately collected using a Cornwall pipetting device. The ethidium bromide was then removed by 3 extractions with isopropanol saturated with 2M CsCl. The DNA solutions were then dialyzed extensively against .01M Tris-HCl, .001M EDTA pH 7.9. The papilloma DNA concentration was estimated by taking the OD readings at 260 and 280 nm of the purified dialyzed DNA solutions using a Gilford Model 250 Spectrophotometer. Knowing that an optical density of 1 at 260 nm in a 1 cm. light path corresponds to approximately 45 ug of DNA per milliter (Hotchkiss 1957) the papilloma DNA concentration could be determined.

G. Isolation of Viral DNA from Wart Tissue

Viral DNA was selectively extracted from wart tissue obtained from single donors by modification of the Hirt procedure (Hirt 1967). Wart specimens were obtained through the Depts. of Dermatology of the Royal Victoria Hospital and the Montreal Children's Hospital. Wart parings and condylomata tissue were stored at -20°C until used. The frozen wart material was rinsed in .15M NaCl and then cut into fine pieces.
and minced. The fragments were incubated in 10 ml of extraction buffer which consisted of .01M Tris - .01M NaCl - .01M EDTA - .5% SDS containing 50 ug/ml proteinase K and the mixture was incubated at 37°C for 6 to 10 hours. The NaCl concentration was then adjusted to .1M and the mixture held at 4°C overnight. The mixture was then centrifuged at 18,000 rpm at 4°C (SS34 rotor) and the supernatant was collected and extracted 2-3 times with phenol saturated with .05M Tris pH 8. The aqueous phase was then mixed with two volumes of pure ethanol and NaCl added to make a .1M solution. The mixture was then stored at -20°C overnight and was then spun at 18,000 rpm (SS34) at 4°C for 1 hour. The DNA pellet was resuspended in .05M Tris and Form I and Form II molecules were separated on CsCl-ethidium bromide gradients as previously described.

H. Preparation of DNA for Electron Microscopy

Papilloma DNA was prepared for electron microscopy according to the Kleinschmidt technique (Kleinschmidt et al 1965) DNA (.2-.5 ug/ml) was mixed with .1 ml of 1 mg/ml cytochrome c solution, 10 ul of .1M EDTA and the volume made up to 1 ml with doubly distilled water. (All solutions were previously filtered through .10 μm millipore filters). A drop of the spreading solution was then slowly deposited near the interface of the hypophase (.25M Ammonium acetate) allowed to stabilize for 30-60 seconds and then 3 samples were picked up on carbon coated formvar grids. Each sample was washed in 90% ethanol for 10 seconds, and then in 2 methyl-butane for 10 seconds. The grids were observed and photographed at 40 kV in the Philips 300 Electron Microscope after Pt-Pd shadowing (which was kindly done by the Dept. of Biochemistry, McGill University.
Contour length measurements of papilloma DNA were made using SV40 DNA, kindly provided by Dr. John Hassel, as an internal length marker. The negative of the electron micrographs were enlarged and the images of the DNA molecules were traced onto paper. The contour lengths of covalently closed circular duplex DNA (Form II) were measured using a coordinometer attached to a digital computer which was placed at our disposal by the DATAC laboratories, Dept. of Engineering, McGill University.

I. Restriction Endonuclease Cleavage of Wart Viral DNA

1. Enzymes

The following restriction endonucleases were employed: Ecoli R₁ (Eco R₁), Haemophilus influenza II (Hind II), Haemophilus influenza III (Hind III), Haemophilus parainfluenza II (Hpa II), all of which were purchased from Boeringher Mannheim, and Bacillus amyloliquefaciens (Bam I), which was purchased from Miles Co. All restriction enzymes were stored at -20°C.

2. Enzymatic Digestion

Eco R₁ reaction mixtures (50-60 ul) consisted of .1M Tris-HCl pH 7.5, .05M NaCl, .01M MgCl₂, 1 ul Eco R₁ enzyme (10 units) and .5 to 1 ug of papilloma DNA, which were incubated at 37°C for 90 minutes.

Bam I reaction mixtures (50-60 ul) consisted of .1M Tris-HCl pH 7.5 and .01M MgCl₂, 1 ul Bam enzyme (10 units) and .5 to 1 ug of DNA which was incubated at 37°C for 3 hours.

Hind III reaction mixtures (50-60 ul) consisted of .006M Tris-HCl pH 7.5, .05M MgCl₂, .05M NaCl, 5 ug of bovine serum albumin, 1 ul of Hind III enzyme (10 units) incubated at 37°C for 3 hours.
Hind II reaction mixtures (50-60 ul) consisted of 0.01M Tris-
HCl pH 7.5, 0.01M MgCl₂, 0.05M NaCl, 0.015M dithiothreitol, .5 to 1 ug of DNA, and 2 ul of Hind II enzyme (8 units) incubated at 37°C for 3 hours.

Hpa II reaction mixtures consisted of 0.01M Tris-HCl pH 7.5, 0.01M MgCl₂, 0.015M dithiothreitol, .5 to 1 ug of DNA, and 2 ul of Hpa II enzyme (8 units) incubated at 37°C for 3 hours.

All buffers were filtered through Nygeen filters .45 u, before use and those which did not contain dithiothreitol were autoclaved. The reactions were stopped by adding EDTA to a final concentration of .02M. 10 ul of a solution of 80% sucrose, .25% bromophenol blue were then added and the mixture was applied to the gel.

J. Agarose Gel Electrophoresis

Electrophoresis was carried out in a vertical gel electrophoresis apparatus. Agarose gels were prepared by dissolving agarose (1.2% W/V) (Sigma electrophoresis grade) in a hot solution of 0.04M Tris, .005M Sodium acetate, .001M EDTA pH 7.9 (Buffer E) (Hayward and Smith 1972). The melted agarose was poured between two vertical glass plates, the base of which contained a polyacrylamide plug, which served to prevent the gels from sliding out (see appendix for composition of the polyacrylamide plug). The electrophoresis was carried out at 70 V for 8 to 8.5 hours at room temperature, using Buffer E as the electrophoresis buffer. After the run, the gels were stained in a solution of Buffer E containing .5 ug/ml ethidium bromide, for 30 minutes. The gels were then rinsed in distilled water and examined by direct illumination from a short wave UV light (UV Products, San Gabriel, California). The DNA was seen as fluorescent bands and the gels were photographed with a polaroid land camera using Polaroid Type 55 P/N or
type 57 film and a Kodak 23 A red filter.

K. Calculation of Molecular Weight by Gel Electrophoresis

The molecular weights of the papilloma DNA fragments obtained after restriction endonuclease cleavage were calculated using SV40 DNA (kindly provided by Dr. John Hassell, currently of the Dept. of Microbiology, McGill University), cleaved by Hind III as a length marker. The molecular weights of these fragments are $1.2 \times 10^6$, $0.79 \times 10^6$, $0.74 \times 10^6$, $0.37 \times 10^6$, $0.28 \times 10^6$, and $0.14 \times 10^6$ (Danna et al. 1973).
IV. EXPERIMENTAL RESULTS

A. Virus Isolation

1. Morphology

Papilloma virus was extracted from common, plantar and bovine wart tissue as described in Materials and Methods. The papilloma virus was shown to separate into two well-defined bands on equilibrium centrifugation in CsCl gradients. Electron microscopic examination of the serial gradient fractions showed that the top band, which appeared at a density of 1.32 g/ml as measured in a refractometer consisted mainly of electron lucent particles which are reportedly virions without viral DNA (Rowson and Mahy 1967). The bottom band consisted mainly of electron dense or full particles and appeared at a density of 1.34 g/ml.

In negative stain preparations using 2% PTA, the papilloma virus appeared to be a spherical particle of about 50-55 nm and thus displayed the characteristic morphology of papilloma viruses described by Klug and Finch (1965). Viruses isolated from bovine and human wart tissue were morphologically identical. A negative stain preparation of papilloma virus can be seen in fig. 1.

2. Quantitation of Papilloma Virus

The yield of virus particles varied according to the type of tissue undergoing extraction. Papilloma virus was quantitated from the O.D. readings taken at 260 and 280 nm using the relationship previously described in Materials and Methods (B-5). Papilloma virus appeared in greatest quantities per gram of tissue in multiple bovine tumours, being of the order of $9 \times 10^{11}$ particles per ml. Comparisons are based on the fact
fig. 1. Electron micrograph of bovine papilloma virus particles, purified on CsCl gradients, after staining with 2% PTA. Bar represents 100 nm.

A. full particle. B. empty particle.
that approximately the same wet weight of tissue was used for the various virus extraction processes. Furthermore when the virus was harvested from CsCl gradients the same volumes were always collected. The yield of virus extracted from the common warts of the immunosuppressed renal transplant (R.T.) and leukemic farmer (LEUK.) were lower, being of the order of $2 \times 10^{11}$ and $6.00 \times 10^{11}$ particles per ml respectively. The yield of virus particles from the patient with plantar warts was approximately $2-3 \times 10^{11}$ particles per ml. This value is rather low considering that there have been reports that plantar tissue is high virus yielding (Orth et al 1978d). A summary of the yields of virus particles obtained from the various tissues is shown in Table 2.

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>TISSUE TYPE</th>
<th>OD$_{260}$</th>
<th>OD$_{280}$</th>
<th>PROTEIN CONTENT (ug)</th>
<th>VIRUS CONCENTRATION Particles per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPV</td>
<td>multiple fibro-epithelial</td>
<td>1.44</td>
<td>1.04</td>
<td>448</td>
<td>$9 \times 10^{11}$</td>
</tr>
<tr>
<td>HPV LEUK</td>
<td>common</td>
<td>1.22</td>
<td>.85</td>
<td>330</td>
<td>$6.5 \times 10^{11}$</td>
</tr>
<tr>
<td>HPV R.T</td>
<td>common</td>
<td>.203</td>
<td>.177</td>
<td>106</td>
<td>$2 \times 10^{11}$</td>
</tr>
<tr>
<td>HPV</td>
<td>plantar</td>
<td>.24</td>
<td>.205</td>
<td>119</td>
<td>$2 \times 10^{11}$</td>
</tr>
</tbody>
</table>

**TABLE 2:** Summary of virus yields obtained from human and bovine wart tissue.

3. **Attempts to Isolate Papilloma Virus from Condylomata Tissue**

Condylomata tissue obtained from the genital region of a female, was extracted according to the method previously described for virus isolation. However no detectable bands were observed on CsCl gradients and it would appear that this tissue did not contain significant, if any,
papilloma virus particles. There is still speculation as to whether a papilloma virus is the aetiological agent of this tumour (Zur Hausen et al 1976; Delap et al 1976). Typical papilloma virus particles have been reported to occur irregularly in these lesions (Oriel and Almeida 1970; Orth et al 1978d). Attempts were subsequently made to isolate papilloma viral DNA from this tissue and these results will be examined later.

B. Examination of Saline Lavages

Electron microscopic examination of several saline lavages revealed the presence of papilloma virus particles in certain instances. Visualization of papilloma particles depended on the extent of the number of warty lesions on the patient's hand. Direct examination of the saline lavages of people bearing few warts did not reveal any papilloma virus and attempts to concentrate virus proved negative. However virus particles could be detected in the saline lavages of the leukemic farmer, bearing many typical hand warts. Using the procedure for virus isolation from wart tissue, virus could be concentrated by centrifugation and the virus concentration as estimated by latex counting was approximately $6 \times 10^{10}$ particles per ml. Low yields and the presence of a unknown contaminant prevented purification on CsCl gradients and the study was no longer pursued.

In summary, it appears that this method is useful for detecting papilloma virus in patients, bearing significant numbers of hand warts, who are unwilling to undergo the painful wart excision process.

C. Serological Studies with Papilloma Virus

Rabbit sera was raised separately against virus isolated from bovine multiple, bovine single, human common (leukemic), common (renal

1. See Appendix A pg. ivb.
transplant) and plantar wart viruses. The antigenic relationship of these viruses was examined using the technique of immune electron microscopy.

The antisera raised was mixed with the various virus preparations as described in Materials and Methods. Pre-immune rabbit sera served as controls.

The following criteria were used to demonstrate a positive reaction:

1. Changes in the appearance of the virus: obscuring of virus particles by antibody molecules which appeared around the virus as a "halo" making it no longer possible to observe arrangements of the subunits forming the capsid.

2. Viral aggregation: very few particles remained single and there was at least a 10 fold difference in the number of virus particles in a given field compared to control or heterologous non-reactive antisera.

The various types of reactions observed using this technique are shown in fig. 2.

The results obtained when the common wart virus of the leukemic farmer was incubated with homologous antisera (anti-common leukemic) and antisera raised against bovine, common (renal transplant) and plantar wart viruses are shown in fig. 3. As can be seen the common wart virus only reacted with its homologous antisera. The results obtained after mixing the various wart virus preparations with the different antisera is presented in Table 3.
Electron microscopic examination of papilloma virus - antibody reactions. The grids were stained with 2% PTA pH 7.2.

A. Papilloma virus mixed with homologous antisera. Final magnification of electron micrograph is 257,000 x. Bar represents 100 nm. Virus particles are obscured with antibody and aggregated. Antibody (indicated by arrow) can be seen between virus particles.

B. Positive reaction with obscuring of virus particles with antibody (100,000 x). Bar represents 100 nm.

C. Negative reaction observed when virus was mixed with heterologous antisera. Note that the virus particles remain single.

D. Negative reaction. Virus was mixed with pre-immune sera. Although particles appear aggregated they are not obscured by antibody and some particles still remain single.
fig. 3. Results of incubating common wart virus (Leukemic) with homologous and heterologous antisera. Grids were stained with 2% PTA and observed in the electron microscope. Bar represents 100 nm.

A. mixed with pre-immune sera of the rabbit injected with common wart virus (Leukemic)
B. mixed with anti-common (Renal Transplant) sera
C. mixed with anti-plantar sera
D. mixed with anti-bovine sera
E. mixed with its homologous antisera (anti-common Leukemic)
<table>
<thead>
<tr>
<th>VIRAL ANTIGEN</th>
<th>RABBIT ANTISERA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANTI-BPV                ANTI-COMMON ANTI-COMMON ANTI-PLANTAR</td>
</tr>
<tr>
<td></td>
<td>mult. single (R.T.) (LEUK.)</td>
</tr>
<tr>
<td>BOVINE multiple</td>
<td>+ + - - -</td>
</tr>
<tr>
<td>BOVINE single</td>
<td>+ + - - -</td>
</tr>
<tr>
<td>COMMON R.T.</td>
<td>- - + - -</td>
</tr>
<tr>
<td>COMMON LEUK.</td>
<td>- - - + -</td>
</tr>
<tr>
<td>PLANTAR</td>
<td>- - - - +</td>
</tr>
</tbody>
</table>

TABLE 3: Results obtained after incubating papilloma virus with homologous and heterologous antisera.

The results obtained show that both the bovine single and multiple wart viruses are antigenically similar, but did not react against the antisera raised against the human wart viruses. This is not unusual in that papilloma viruses are highly species specific (Le Bouvier et al 1966; Koller and Olson 1972; Favre et al 1974). The human papilloma viruses in this study were shown to be antigenically different from each other, as well as from the bovine papilloma viruses. It has previously been shown by Orth et al (1977) that plantar and common wart viruses were antigenically different as determined by immunodiffusion and immunofluorescence tests. This study using I.E.M. confirms this result but also shows that common wart viruses from different individual isolates may be antigenically dissimilar. The results which we obtained were confirmed by indirect immunofluorescence and immunodiffusion tests performed in the laboratory of Dr. R. D. Wilkinson, Dept. of Dermatology of the Royal Victoria Hospital.
D. Extraction of Papilloma DNA from Virions

Papilloma DNA was extracted from virions as described in Materials and Methods. The disruption of papilloma virus by detergent (Sarkosyl) treatment was monitored in the electron microscope. Examination of grids, prepared after a 25 minute incubation period, revealed the presence of viral particles which had their capsomere arrangement seemingly intact but were now electron lucent particles, that is virus without DNA. The results of such treatment is shown in fig. 4. Longer incubation periods resulted in the disruption of the majority of virions, but a significant number of virus particles remained intact (as electron dense particles) and thus the efficiency of Sarkosyl treatment seems questionable. The preservation of an "intact" capsomere arrangement might allow for the conduction of genetic engineering experiments whereby papilloma virus could be made to harbor foreign DNA (viral or host) sequences.

The papilloma DNA appeared as two fluorescent bands after centrifugation on CsCl-ethidium bromide gradients. The top band, which appeared at a density of 1.56 g/ml, as measured in a refractometer, represented relaxed circular or form I DNA, while the bottom band represented superhelical or covalently closed circular DNA and appeared at a density of 1.6 g/ml.

The yield of DNA (ug/ml), as determined from the O.D. readings at 260 nm, for the various types of wart virus preparations is presented in Table 4.
fig. 4. Electron microscopic examination of BPV after incubation with Sarkosyl for 25 minutes. Bar represents 100 nm.
TABLE 4: Papilloma virus concentration and yields of DNA obtained after extraction.

<table>
<thead>
<tr>
<th>VIRUS TYPE</th>
<th>CONC.</th>
<th>MLS. VIRUS SUSPENSION EXTRACTED</th>
<th>O.D. 260 nm</th>
<th>DNA CONC. (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPV MULT.</td>
<td>$9 \times 10^{11}$</td>
<td>1.4</td>
<td>.263 .72</td>
<td>11.5 31.7</td>
</tr>
<tr>
<td>COMMON LEUK.</td>
<td>$6.5 \times 10^{11}$</td>
<td>.8</td>
<td>.323 .217</td>
<td>14.2 14.2</td>
</tr>
<tr>
<td>COMMON R.T.</td>
<td>$2 \times 10^{11}$</td>
<td>1.9</td>
<td>.12 .139</td>
<td>5.3 6.1</td>
</tr>
<tr>
<td>PLANTAR</td>
<td>$2 \times 10^{11}$</td>
<td>1.5</td>
<td>.22 .259</td>
<td>9.7 11.4</td>
</tr>
</tbody>
</table>

E. The Appearance of Papilloma DNA on Agarose Gels

Papilloma DNA was loaded on 1.2% (W/V) vertical agarose gels and electrophoresis carried out at 70 V for approximately 8 hours. The appearance of papilloma DNA on such gels is demonstrated in fig. 5. Because of the proximity of form 1 and form 11 DNA on CsCl-ethidium bromide gradients, separation of the bands resulted in a mixture of both forms. Furthermore, the possibility of nicks arising in form 1 DNA, as a result of the handling process, may have occurred. This would account for the presence of both forms of DNA in a single track on the agarose gel (fig. 5a).

Form 1 and form 11 DNA isolated from plantar virions of an individual isolate were pooled and alcohol precipitated and resuspended in a small volume of .01M Tris-HCl, .001M EDTA pH 7.9 and loaded onto an agarose gel (fig. 5d). Electrophoresis of this material resulted in the appearance of an atypical DNA band which migrated faster than from 11 DNA. This band may represent a lower molecular weight class of form 11 DNA and

1. particles/ml
fig. 5. Appearance of undigested papilloma DNA on 1.2% agarose gels. Electrophoresis is from top to bottom. The arrows indicate the position of the bands. The gel was loaded with a) .5 ug of form I BPV DNA. b) .6 ug of form II BPV DNA. c) with .5 ug of plantar wart DNA isolated from a Hirt extract of plantar wart tissue. d) with a mixture of form I and form II DNA which had been alcohol precipitated and resuspended in a small volume of .01M Tris-HCl, .001M EDTA pH 7.9. Note the presence of the atypical band indicated by the arrow. Gels were stained in .5 ug/ml ethidium bromide and electrophoreograms visualized under U.V. light.
this DNA species probably appears in such low quantities that it is impos-
sible to detect the superhelical and linear forms, as detection of these
bands is beyond the sensitivity of this particular gel system.

F. Electron Microscopic Examination of HPV (Plantar) DNA and Contour
Length Measurement

Form II DNA was extracted from the virions, previously
isolated from the plantar wart tissue of the single plantar patient (same
patient bearing extensive plantar warts described in Materials and Methods
B1). The DNA was spread according to the Kleinschmidt technique
(Kleinschmidt et al 1967) and examined in the electron microscope. Contour
length measurements were made using form II DNA and SV40 DNA as an internal
standard. An electron micrograph of plantar viral DNA spread along with
SV40 is shown in fig. 6. The papilloma form II molecules can easily be
distinguished from the smaller SV40 molecules.

84 papilloma DNA molecules and 58 SV40 molecules were
measured using a coordinometer attached to a digital computer (see Material
and Methods). The error of measurement in this procedure was less than 1%.
The average contour length for 84 papilloma DNA molecules was 33.20 ± 1.96 cm.
(a standard deviation of 6% of the mean) while the average contour length of
SV40 was 22.05 ± 1.32 cm. (a standard deviation of 6% of the mean). When
converted to um, this is 2.22 ± .13 um. and 1.47 ± .09 um. respectively. A
histogram of the length distribution of HPV DNA plotted against the frac-
tional length of SV40 DNA is given in fig. 7. As can be seen the SV40
molecules are quite homogeneous in length while the papilloma DNA molecules
show a greater variation in length distribution. Inset within fig. 7 are
two papilloma DNA molecules which appeared in this spread and are good
fig. 6. Electronmicrograph of a HPV plantar DNA spread. Grids were Pt-Pd shadowed. SV40 was used as an internal standard.

A. HPV DNA. B. SV40 DNA. Final magnification is 48,000 x. Bar represents 500 nm.
fig. 7. Histogram of the length distributions of plantar DNA extracted from virions of a single wart isolate and SV40 DNA. The number of molecules were plotted against the fractional length of SV40.

Inset: Two papilloma DNA molecules which are examples of the size heterogeneity exhibited by this plantar DNA isolate. The DNA molecules are shown at a magnification of 50,000 x. Measurements made at a final magnification of 148,200 x showed that A was 30.09 cm. and B was 34.9 cm. Bar represents 500 nm.
examples of the size heterogeneity found in this preparation. Molecule A measured 30.09 cm. (after the electron micrograph had been magnified and image traced onto paper) while molecule B was 34.90 cm. using the same procedure, and this represents a 13% difference in size. This heterogeneity in size distribution can probably account for the atypical DNA band, which appeared when form I, and form II DNA molecules (isolated from the same patient whose wart DNA was used to do contour length measurements) were mixed and run on an agarose gel (see fig. 5d). However, a distinct population of papilloma DNA molecules representative of this atypical band were not found when contour length measurements were performed.

The average molecular weight of this papilloma DNA preparation could be calculated knowing the M.W. of SV40 to be $3.5 \times 10^6$ (Dr. John Hasse, personal communication) and was found to be $5.25 \times 10^6$ daltons. This is in agreement with other reported average M.W. determinations for papilloma DNA. Using SV40 as their internal standard Yoshiike and Defendi (1977) found the M.W. of papilloma DNA isolated from pooled common warts to be $5.3 \times 10^6$. Using $\phi$ x 174 RF DNA as their standard, Orth et al (1977a) have found the M.W. of papilloma DNA, isolated from a pool of plantar warts and from the common warts of one individual to be $5.26 \times 10^6$ and $5.23 \times 10^5$ respectively. Gissmann and Zur Hausen (1976) reported a M.W. of $4.9 \times 10^6$ for papilloma virus DNA isolated from a pool of plantar warts, using lambda DNA as a standard. It should be noted here that these previous measurements of plantar viral DNA were made using pooled material while the measurements performed here were from an individual plantar patient.
G. Restriction Endonuclease Digestion of DNA Isolated from Papilloma Virions

1. Restriction Enzyme Analysis of BPV (Multiple) DNA

BPV DNA (.5 ug) was digested with restriction endonucleases under the conditions described in the Materials and Methods. Cleavage products were run on 1.2% agarose gels for 8 hours. The results of such cleavage are shown in fig. 8. Hind III cleaved SV40 DNA (8h) was used for calibration of the gels. As can be seen in track b EcoR1 cleaved at one site only to give linear DNA fragments. Bam and Hind III, (tracks c and e respectively) also cleaved BPV DNA to linear fragments. Hind II digestion resulted in the appearance of 5 fragments: Hind II A, Hind II B and Hind II C and 2 fragments indicated by the arrows. These 2 fragments seem to be resistant to cleavage at multiple sites and may represent a different class of BPV DNA molecules. Increasing the enzyme concentration 2 fold did not result in the disappearance of these two bands (fig. 9b). Hpa II digestion resulted in at least 7 bands, only 5 of which are detected in fig. 8f and an additional 2 bands (F and G) which appear in fig. 9c. A mixture of Hind II and Hind III enzymes (8g) gave the same pattern as Hind II digestion alone.

1. The photographs of some of the gels which appear in this thesis have been cut for labelling purposes and in the process of developing these prints, some of the more weakly visible bands have disappeared. These weakly detectable bands may be seen more distinctly in the electrophoreograms which appear in Appendix B and have the same fig. no. as those which appear in the results section.
The M.W. of all these fragments were determined from the calibration of the gels with Hind III digested SV40 DNA which is seen both in fig. 8h and 9a. Fig. 8h has only 5 of the 6 reported bands for SV40 digested with Hind III while fig. 9c has this missing band (indicated by the arrow). The M.W. of these Hind III fragments are given in the Materials and Methods. By plotting the logarithm of the M.W. of the Hind III fragments versus distance migrated a straight line was obtained from which the M.W. of the papilloma DNA fragments could be estimated by interpolation.

Partial cleavage products of Hind III digested SV40 (M.W. - 1.5 x 10^6, 2.2 x 10^6 and 3.08 x 10^6) indicated by the position of the arrows in fig. 8h were also used in calibration of the gel. The results of the restriction enzyme digestions are summarized in Table 5.
fig. 8. Agarose slab gel electrophoresis of cleavage products of BPV DNA by restriction endonuclease a) undigested DNA b) EcoRI digested c) Bam d) HindIII e) HindIII f) HpaII g) HindIII + HindIII h) SV40 DNA digested with HindIII.
fig. 9. Agarose slab gel electrophoresis of cleavage products of BPV by Hind III, Hind II, Hpa II. Effect of increasing enzyme concentration: a) Hind III digested BPV DNA: the enzyme concentration was double of that in 8e b) Hind II digested BPV DNA: enzyme concentration was increased two fold c) Hpa II digested BPV DNA with appearance of 2 additional lower M.W. fragments not seen in fig. 8 d) SV40 DNA cleaved with Hind III.
<table>
<thead>
<tr>
<th>Column</th>
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**TABLE 5:** Molecular weights of BPV DNA fragments after cleavage with restriction endonucleases.

As can be seen in Table 5 the sum of the M.W. of the Hpa II fragments is 5.06 x 10^6 which falls in the range of the reported molecular weights for papilloma DNA (see pg. 60). Digestion of BPV DNA with the various restriction nucleases was repeated and the results were reproducible.

<table>
<thead>
<tr>
<th>Cleavage Products</th>
<th>Mol. Wt. x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>ND^1</td>
</tr>
<tr>
<td>Bam</td>
<td>ND</td>
</tr>
<tr>
<td>Hind II</td>
<td>ND (&lt;3.08 x 10^6)</td>
</tr>
<tr>
<td></td>
<td>A 0.67 x 10^6</td>
</tr>
<tr>
<td></td>
<td>C 0.53 x 10^6</td>
</tr>
<tr>
<td>Hind III</td>
<td>ND</td>
</tr>
<tr>
<td>Hpa II</td>
<td>2.6 x 10^6</td>
</tr>
<tr>
<td></td>
<td>B 1.05 x 10^6</td>
</tr>
<tr>
<td></td>
<td>C 0.43 x 10^6</td>
</tr>
<tr>
<td></td>
<td>D 0.38 x 10^6</td>
</tr>
<tr>
<td></td>
<td>E 0.25 x 10^6</td>
</tr>
<tr>
<td></td>
<td>F 0.2 x 10^6</td>
</tr>
<tr>
<td></td>
<td>F' 0.15 x 10^6</td>
</tr>
<tr>
<td></td>
<td>5.06</td>
</tr>
</tbody>
</table>

1. ND - M.W. could not be estimated from the gel as there were no high M.W. markers in the range of 5 x 10^6.
2. Restriction Enzyme Digestion of Common Viral DNA Isolated from HPV (Leukemic)

.5 ug of HPV DNA (form II) was digested with various restriction endonucleases and the products run on 1.2% agarose gels. The results of digestion can be seen in fig. 10. EcoR I, digestion (track b) resulted in DNA fragments which co-migrated with forms 1, 11 and 111 of undigested DNA (a). It would thus appear that this DNA is not sensitive to digestion with EcoR I. In order to see if the enzyme preparation was active .5 ug of Lambda (λ) DNA (Miles Co.) was digested with EcoR I under the same conditions and the cleavage products were run on the same gel (10g). As can be seen λ DNA was cleaved into 5 visible fragments (one of the bands is a doublet). The reported M.W. values of λ DNA cleaved by EcoR I are 13.7 x 10^6, 4.49 x 10^6, 3.54 x 10^6, 3.54 x 10^6, 3 x 10^6 and 2.3 x 10^6 (Albert et al 1973). Thus the EcoR I enzyme preparation is active and HPV DNA isolated from the virions of the leukemic are resistant to digestion with this enzyme. Digestion with Bam endonuclease (10C) resulted in 2 fragments while Hind 11 digestion resulted in 3 fragments labelled A, B and C and 3 higher M.W. fragments. Hpa 11 digestion resulted in multi-cleavage (at least 8 fragments were detected). Fragments A - G would appear to be the predominant products of digestion while the fragment indicated by the dotted arrow would appear to be the product of either partial digestion or an atypical DNA fragment resulting from cleavage of a heterologous population of DNA molecules (derived from a different type of papilloma virus). This digestion was repeated and identical results were obtained even after increasing the enzyme concentration.

SY40 DNA cleaved by Hind 111 (10f) was used for calibration of the gels along with some of the cleavage products of EcoR I digested λ DNA.
fig. 10. Agarose slab gel electrophoresis of cleavage products of HPV DNA (Leukemic) by restriction endonucleases. a) undigested DNA b) EcoR I digested c) Bam d) Hind III (arrows indicate atypical fragments) e) Hpa II digestion, the dotted arrow represents an atypical fragment f) Hind III digested SV40 DNA g) λ DNA digested with EcoRI h) superhelical DNA (HPV Leukemic).
HPV DNA (Leukemic) appeared to be resistant to cleavage with Hind III and these results appear in fig. 11b. The gel system employed here was 2% agarose. This gel system was used in order to detect any lower M.W. Hind III fragments which might run off 1.2% gels. However, Hind III cleaved DNA co-migrated with undigested DNA (11a). The Hind III enzyme preparation was the same one used to cleave SV40 DNA (10f) and thus was active.

A summary of the M.W. of the cleavage products after enzyme digestion is given in Table 6.
TABLE 6: Molecular weights of HPV DNA (Leukemic) fragments after cleavage with restriction endonucleases.

Increasing the Hind III enzyme concentration (2-fold) did not result in the disappearance of the higher M.W. fragments (indicated by the arrows in track d (fig. 10). Thus it would appear that there is a population of molecules which are resistant to cleavage (don't generate A, B and C fragments). These could represent the cleavage products of a heterologous population of papilloma DNA molecules (see Hpa II digestion pg. 66).
fig. 11. Agarose slab gel electrophoresis (2% agarose) of undigested HPV DNA (Leukemic) and Hind III digested DNA
a) undigested DNA  b) Hind III digested
3. Restriction Enzyme Digestion of Common Viral DNA Isolated from HPV (Renal Transplant)

The DNA isolated from the common wart virions of the immunosuppressed patient were also subjected to restriction enzyme analysis. The DNA was digested with Bam and EcoR₁, and these results are shown in fig. 12. Digestion with Hind II, Hind III, and Hpa II did not allow for visualization of the resulting fragments on agarose gels as the quantity of DNA in the initial reaction mixtures (< 0.3 µg) was too low for visualization of DNA cleaved into multiple pieces. However, the common viral DNA of the immunosuppressed patient was shown to be sensitive to EcoR₁ (12b) and was cleaved to unit length (as the DNA migrated to the position of form III DNA). Bam cleavage (11c) showed the DNA to be resistant as the DNA remained in the position of form II molecules (the bottom band in track 11c is superhelical DNA as it was shown to co-migrate with form 1 DNA in a separate gel). As low quantities of this DNA prevented visualization of form III (linear molecules) papilloma DNA isolated from a Hirt extract was used as a marker (track 8).

4. Restriction Enzyme Analysis of Plantar Wart Virus DNA Isolated from a Single Patient

Form I and Form II DNA isolated from the virions of the plantar wart patient were combined and to this was added 2 x volume ethanol (NaCl concentration was adjusted to 0.1M). The mixture was stored at -20°C overnight and then centrifuged to pellet the DNA. The DNA was resuspended in a small volume of 0.01M Tris-HCl, 0.001M EDTA buffer pH 7.9 and restriction enzyme analysis carried out and products run on 1.2% agarose gels. The cleavage patterns obtained for the various restriction enzymes are shown in
fig. 12. Agarose gel electrophoresis of cleavage products of EcoR1 and Bam digested common wart viral DNA (renal transplant)
a) uncleaved DNA (.2 ug) b) EcoR1 digested (.3 ug) c) Bam digested (.3 ug) d) papilloma viral DNA isolated by Hirt extraction.
fig. 13. Agarose slab gel electrophoresis of the cleavage products of plantar DNA isolated from purified virions. a) undigested DNA the arrow indicates the position of an atypical DNA band b) EcoRI digested c) Bam d) Hind III e) Hind III f) Hpa II g) partial cleavage products of SV40 DNA digested with Hind III.
fig. 13. EcoR₁ digestion (13b) resulted in DNA bands which migrated to the equivalent positions of undigested DNA (13a), however, the atypical band (same one examined previously pg. 55) immediately below form 11 DNA has disappeared. So it seems that we have both resistant (majority) and sensitive DNA molecules in the papilloma DNA pool isolated from a single patient.

Treatment with Bam (fig. 13c) resulted in 2 fragments while Hind 11 digestion resulted in 3 fragments A, B and C and 4 higher molecular weight fragments (partial cleavage products as determined from subsequent experiments). Hind 111 digestion resulted in fragments which co-migrated with uncleaved DNA but the "atypical" band has disappeared. Hpa 11 digestion resulted in 5 low M.W. fragments indicated by the arrows in track f. These bands may be seen more clearly in fig. 13 which appears in the appendix and also were more evident when restriction enzyme analysis was carried out on papilloma DNA extracted by the Hirt procedure from this same patient.

The partial cleavage products (13g) of SV40 Hind 111 digested DNA were used for calibration purposes. A summary of the M.W. of the cleavage products of restriction digestion appear in Table 7.
TABLE 7: Molecular weights of plantar HPV DNA fragments after cleavage with restriction endonucleases.

Assuming the M.W. of this plantar DNA preparation to be $5.25 \times 10^6$ (as determined by contour length measurements see pg. 60) then one would expect the Bam A fragment to have a M.W. of $4.06 \times 10^6$. The cleavage of the plantar viral DNA was repeated in separate experiments, but using viral DNA isolated from a Hirt extraction of plantar tissue of the same patient (see pg. 78).
H. Examination of Papilloma DNA Isolated from Wart Tissue by the Hirt Procedure

1. Isolation of Papilloma-Sized DNA in Condylomata Acuminata

Attempts to isolate papilloma virus from condylomata tissue excised from a patient with genital warts proved negative (see pg. 47). This same tissue (.43 g) and tissue excised from 2 other patients with genital warts (approximately .3 g in each case) were examined for the presence of papilloma DNA molecules using a modification of the Hirt procedure (Hirt 1967). The DNA was extracted as described in the Materials and Methods (pg. 40) and DNA appearing at a density of 1.54 g/ml on CsCl-ethidium bromide gradients was collected, ethidium bromide removed by isopropanol extraction, dialyzed and then loaded onto 1.2% agarose gels. These DNA preparations will be provisionally called Condy 1 (tissue from this patient was used in the attempted extraction of virus), Condy 2 and Condy 3.

When 2.5 ug of Condy 1 DNA was loaded onto 1.2% agarose gels (see fig. 14e) and electrophoresis carried out it was possible to detect papilloma-sized DNA molecules which co-migrated with formal DNA isolated from a) BPV b) HPV common c) plantar tissue by the Hirt procedure d) common wart tissue.

When 3.8 ug of Condy 2 DNA was loaded on a 1.2% agarose gel (fig. 15a), it was possible to detect papilloma-sized DNA molecules as well as a larger class of DNA molecules. The Condy 3 DNA preparation also yielded both types of DNA.

The background smear in these preparations probably represent DNA molecules (probably human in origin) found in the Hirt supernatant which appear at the same density as papilloma DNA on CsCl gradients. They occurred in all Hirt preparation of condylomata tissue. Delap et al (1976)
   a) BPV DNA  b) HPV DNA (common)  c) HPV DNA (plantar)  d) DNA isolated from Hirt extraction of common wart tissue  e) Condy 1 DNA preparation isolated from genital warts, the arrow indicates position of papilloma-sized DNA.

fig. 15. Agarose gel electrophoresis of DNA extracted from genital warts.
   a) Condy 2 DNA preparation isolated from genital warts.
      A. position of a large M.W. DNA  B. papilloma-sized DNA
   b) BPV DNA isolated from virions  c) plantar DNA isolated from Hirt extraction of plantar tissue.
have reported the presence of poly-disperse circular DNA molecule in their condylomata DNA preparations which obscured their detection of papilloma-sized DNA molecules. However, this group has used an alternate procedure, one involving alkali denaturation, for their extraction procedure.

2. Extraction of Papilloma DNA from Plantar and Common Wart Tissue

The Hirt procedure proved to be an invaluable method for extracting papilloma DNA from tissue where low quantity prevented extraction of virus by the procedure described previously (pg. 35). Extraction of papilloma DNA could be achieved from small quantities of papilloma tissue. As little as 0.1 g was sufficient in certain instances for DNA extraction, however, on the average at least 0.3 g was required. Several papillomatamas tissue were subjected to the Hirt extraction process. The following specimens were examined and these were designated Common (PANT), Common (ESK), Common (ARS), Plantar (MIL) and Plantar (NOR).

a) Isolation and Characterization of Plantar Wart Virus DNA (NOR)

Approximately 1 gram of papilloma tissue (excised from the same plantar patient described previously) was extracted by the Hirt procedure. The DNA isolated was subjected to restriction enzyme analysis. The results appear in fig. 16 and fig. 17. The digestion pattern compares favourably to that obtained when restriction enzyme analysis was performed on DNA isolated directly from purified virions. The DNA was shown to be resistant to cleavage with EcoRI (f/6g) and was seen to co-migrate with forms II and III of undigested DNA (i). Under the same conditions λ DNA was shown to be sensitive (f). HindII digestion (e) resulted in 3 lower M.W. fragments A, B and C and 3 bands which represent partial cleavage.
products, as these bands diminish in intensity with increased enzyme concentration (17d). The DNA appears resistant to Hind III (17e). SV40 DNA however was cleaved into the expected number of fragments using the same enzyme preparation (16b) and 17h). Hpa II digestion resulted in the appearance of 5 low M.W. fragments previously reported on pg. 75. Bam cleavage resulted in two fragments (17c).

A summary of the results appear in Table 8.

<table>
<thead>
<tr>
<th>Cleavage Products</th>
<th>Mol. Wt. x 10^6</th>
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<tbody>
<tr>
<td>EcoR₁ (resistant)</td>
<td></td>
</tr>
<tr>
<td>Bam A</td>
<td>ND</td>
</tr>
<tr>
<td>Bam B</td>
<td>1.1</td>
</tr>
<tr>
<td>Hind III A</td>
<td>2.3</td>
</tr>
<tr>
<td>Hind III B</td>
<td>1.75</td>
</tr>
<tr>
<td>Hind III C</td>
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</tr>
<tr>
<td></td>
<td>5.25</td>
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<td>Hpa II (resistant)</td>
<td></td>
</tr>
<tr>
<td>Hpa II A</td>
<td>.85</td>
</tr>
<tr>
<td>Hpa II B</td>
<td>.75</td>
</tr>
<tr>
<td>Hpa II C</td>
<td>.57</td>
</tr>
<tr>
<td>Hpa II D</td>
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</tr>
<tr>
<td>Hpa II E</td>
<td>.40</td>
</tr>
<tr>
<td></td>
<td>3.06</td>
</tr>
</tbody>
</table>

TABLE 8: Molecular weights of the cleavage products of HPV DNA (NOR) after restriction endonuclease digestion.
fig. 16. Restriction enzyme analysis of plantar papilloma DNA (NOR) isolated from a single patient by the Hirt procedure.

a) BPV DNA  b) Hind III digested SV40 DNA  c) Hpa II digested HWV DNA (NOR)  d) Hind III digested HWV DNA (NOR)

e) λ DNA digested with EcoRI  f) EcoRI digested HWV DNA (NOR) (0.4 ug)  g) undigested HWV DNA (NOR) (0.2 ug).
fig. 17. 1. Additional analysis of plantar papilloma DNA isolate NOR.

2. Examination of undigested HPV DNA PANT.

Tracks a - f show the digestion patterns of papilloma DNA isolate NOR after restriction endonuclease cleavage.

a) undigested DNA b) EcoR1 c) Bam d) Hind II digestion.

Enzyme concentration was 2 x greater than that in fig. 16d.

The partial cleavage products have almost entirely disappeared. e) Hind III f) Hpa II. h) SV40 DNA digested with Hind III.

Tract i shows the migration pattern of undigested HPV DNA PANT which migrated with form II DNA of HPV DNA isolated from the renal transplant (tract g) and undigested HWV NOR.
The results obtained here are almost identical to those which appear in Table 7. Once again however, the sum of the M.W. of the Hpa I digestion products are well below the reported M.W. of papilloma DNA. This would suggest the presence of very low M.W. fragments whose detection is beyond the sensitivity of this gel system.

b) Isolation and Characterization of Common Wart Virus DNA (PANT)

.1 gram of tissue was extracted according to the procedure in the Materials and Methods. The yield of DNA from this particular isolate was 10.5 ug/ml. The DNA .5 ug was loaded onto agarose gels for purpose of examination. This can be seen in fig. 16i. As can be seen 2 bands appear in the position where form 11 DNA normally migrates. Thus it appears that there are at least 2 different size classes of papilloma DNA within the same isolate. This same DNA was then spread by the Kleinschmidt technique for electron microscopic examination. An electronmicrograph of this DNA preparation can be seen in fig. 18. The DNA molecules shown appear at a magnification of 50,000 x. At a magnification of 149,760 x, A measured (using a map measurer) approximately 35 cm, while B measured 30 cm. Thus these 2 DNA molecules are representative of the size variation which may occur within this single isolate (but may not be necessarily representative of two size classes which appear in fig. 16i).

The DNA was then subject to analysis with restriction endonucleases. The results can be seen in fig. 19. Prior to enzyme digestion this DNA was concentrated by centrifugation. EcoR I digestion (track d) resulted in the appearance of two adjacent fragments (A and B) which migrated with form 11 (linear DNA) and 2 lower M.W. fragments (C and D) as well as a resistant fraction which remained in the position where form 11
fig. 18. Variation in size of papilloma DNA (common) extracted from a single patient. Bar represents 500 nm. When magnified at 149,760 x, A measured approximately 35 cm. while B measured 30 cm.
fig. 19. Agarose gel electrophoresis of cleavage products of HPV DNA (ESK), HPV DNA (PANT) and HPV DNA (ARS) by restriction endonucleases. a - c HPV DNA (ESK), d - f HPV (PANT) h - j HPV DNA (ARS).

a) undigested HPV DNA (ESK) b) EcoRI digested HPV DNA (ESK)
c) Bam d) EcoRI digested HPV DNA (PANT) e) Bam f) Hpa II
g) BPV DNA digested with Hpa II h) EcoRI digested HPV DNA (ARS) i) Bam j) undigested HPV DNA (ARS).
DNA migrates. The DNA appeared resistant to digestion with Bam endonuclease (track e). Hpa II digestion resulted in appearance of at least 6 fragments, designated A-F. Fragment A was seen to co-migrate with linear DNA. A summary of the M.W. of these fragments is given in Table 9. The fragments generated by the Hpa II digestion of BPV DNA were used for calibration purposes. The M.W. of these fragments were previously determined using SV40 DNA (pg. 65).

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<thead>
<tr>
<th>Cleavage Products</th>
<th>Mol. Wt. x 10^6</th>
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<tbody>
<tr>
<td>EcoRI</td>
<td>A</td>
</tr>
<tr>
<td>B</td>
<td>ND (linear)</td>
</tr>
<tr>
<td>C</td>
<td>2.9 x 10^6</td>
</tr>
<tr>
<td>D</td>
<td>2.7 x 10^6</td>
</tr>
<tr>
<td>Bam (resistant)</td>
<td></td>
</tr>
<tr>
<td>Hpa II</td>
<td>A</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>2.9 x 10^6</td>
</tr>
<tr>
<td>D</td>
<td>2.2 x 10^6</td>
</tr>
<tr>
<td>E</td>
<td>1.15 x 10^6</td>
</tr>
<tr>
<td>F</td>
<td>1.05 x 10^6</td>
</tr>
</tbody>
</table>

TABLE 9: Molecular weights of HPV DNA (PANT) fragments produced by cleavage with EcoRI, Bam I and Hpa II endonucleases.

As can be seen in Table 9 the sum of the molecular weights of the fragments generated by Hpa II and EcoRI digestions would be well above the reported M.W. of HPV DNA (which falls in the range of 4.8 to
5.3 \times 10^5). This would suggest the presence of atypical fragments in the DNA pool after enzyme digestion. These atypical fragments would be generated from the cleavage of a second population of HPV DNA present in the papilloma DNA isolated from the wart tissue of this particular patient.

c) Isolation and Characterization of Common Wart Virus DNA (ESK)

Approximately 1 gram of tissue was used in this particular extraction. DNA appearing at a density of 1.54 g/ml was collected and after removal of the ethidium bromide with isopropanol and dialysis the concentration was found to be approximately 10 \mu g/ml. The DNA was then analyzed by restriction endonuclease cleavage. This can be seen in fig. 19. Undigested papilloma DNA (a) was seen to separate into form 11 and form 1. The DNA appeared to be resistant to EcoR1 (b) but was sensitive to Bam yielding 2 fragments, however, there also appeared to be a fraction of the DNA which was cleaved to linear DNA (track c). The DNA was concentrated by alcohol precipitation and was subjected to digestion with Hind I, Hind III and Hpa II enzymes. These results appear in fig. 20. Hind I digestion resulted in the appearance of 6 bands: 3 lower M.W. fragments A, B and C and 3 fragments of higher M.W. which may be the partial cleavage products of Hind I digestion or a population of resistant molecules. The Hind I digestion was carried out with 8 units of enzyme and therefore the latter explanation may seem more reasonable. Unfortunately limited quantities of this DNA preparation did not allow repetition of this particular digestion. Hind III digestion resulted in DNA molecules which were resistant to cleavage and thus co-migrated with form 1 and form 11 DNA (track a) (the intensity of the DNA bands in track a are lower because this sample came from the DNA pool which had not been alcohol precipitated).
fig. 20. Agarose gel electrophoresis of cleavage products of HPV DNA (ESK) by restriction endonucleases. a) undigested DNA b) Hind II digested c) Hind III digested. The arrow indicates the position of an atypical fragment. d) Hpa II digested e) BPV DNA digested with Hpa II.
In the Hind III digestion however, a band of low intensity (indicated by the arrow) appeared and this would suggest the presence of a sensitive fraction. Hpa II digestion resulted in multi-cleavage products. At least 6 fragments can be seen below fragment A. The DNA fragments indicated by the arrows are probably partial cleavage products as their intensity is quite low. The amount of ethidium bromide bound by DNA is proportional to the M.W. of the DNA and as these fragments are of higher M.W. than fragment A, they should have bound more ethidium bromide and appear at greater intensity. A summary of the molecular weights of the fragments obtained after digestion with the various enzymes is given in Table 10. The Hpa II digestion products of BPV DNA were used for calibration purposes.
### Cleavage Products

<table>
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<th>Restriction Enzyme</th>
<th>Cleavage Product</th>
<th>Mol. Wt. x 10^6</th>
</tr>
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<tbody>
<tr>
<td>EcoR₁ (resistant)</td>
<td>A</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1.2</td>
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<td>Bam</td>
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<td></td>
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<td>1.7</td>
</tr>
<tr>
<td></td>
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<td>Hind II</td>
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</tr>
<tr>
<td></td>
<td>*</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* Partial cleavage products indicated by the arrows in fig. 20 track d.

**TABLE 10:** Molecular weights of HPV DNA (ESK) fragments after cleavage with restriction endonucleases.

1. The M.W. of the atypical fragment in the Hind III digested indicated by the arrow could not be determined as there were no large M.W. markers on the gel.
d) Isolation and Characterization of Common Wart Virus DNA (ARS)

0.3 grams of wart tissue was used in this extraction process. The papilloma DNA extracted from this tissue was then examined by restriction endonuclease digestion. The results appear in fig. 19 and 21. The electrophoretic migration pattern of undigested HPV DNA (ARS) appears in track j of fig. 19. As can be seen there are 2 bands which appear in the position where form 11 DNA migrates and once again we see the size heterogeneity which was displayed by HWV DNA (PANT) (in fig. 17j). The results of EcoR1 cleavage are shown in fig. 19h. As can be seen a fragment (designated A) of unit genome size has been formed as well as two other lower M.W. fragments (B and C) both of which appear at low intensity on this gel. HWV DNA (ARS) was sensitive to Bam cleavage. A fragment which migrated with linear DNA, (a fragment cleaved to unit length), and 3 lower M.W. fragments B, C and D were formed. This is shown in track i of fig. 19. The DNA was subsequently analyzed with restriction endonucleases Hpa I1 and Hind III and these results can be seen in fig. 21. Hpa I1 digestion (track b) resulted in the formation of 7 major fragments as well as a partial cleavage product which is indicated by an arrow. This DNA preparation was shown to be resistant to Hind III digestion. The Hpa I1 digestion products of BPV DNA were used for calibration of the gels (19 g and 21 e). A summary of the M.W. of the cleavage products of HPV DNA (ARS) after enzyme digestion are shown in Table 11.
Fig. 21. Agarose gel electrophoresis of the cleavage products of HPV DNA (ARS) and HPV DNA (MIL) by restriction endonucleases.

a) undigested HPV DNA (ARS) b) HPV DNA (ARS) digested with Hpa II

c) HPV DNA (ARS) digested with Hind III d) undigested HPV DNA (MIL)

e) BPV DNA digested with Hpa II

f) EcoRI digested HPV DNA (MIL).
TABLE 11: Molecular weights of HPV DNA (ARS) fragments after cleavage with restriction endonucleases.

<table>
<thead>
<tr>
<th>Cleavage Products</th>
<th>Mol. Wt. x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoR₁</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>ND (linear)</td>
</tr>
<tr>
<td>B</td>
<td>2.8 x 10^6</td>
</tr>
<tr>
<td>C</td>
<td>2.6 x 10^6</td>
</tr>
<tr>
<td>Bam</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>ND (linear)</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>1.25</td>
</tr>
<tr>
<td>D</td>
<td>1.10</td>
</tr>
<tr>
<td>Hind III (resistant)</td>
<td></td>
</tr>
<tr>
<td>Hpa II</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.8</td>
</tr>
<tr>
<td>B</td>
<td>1.4</td>
</tr>
<tr>
<td>C</td>
<td>1.3</td>
</tr>
<tr>
<td>D</td>
<td>0.95</td>
</tr>
<tr>
<td>E</td>
<td>0.92</td>
</tr>
<tr>
<td>F</td>
<td>0.87</td>
</tr>
<tr>
<td>G</td>
<td>0.75</td>
</tr>
<tr>
<td>*</td>
<td>1.75</td>
</tr>
</tbody>
</table>

* a partial cleavage product

e) Isolation and Characterization of Plantar Wart Virus DNA (MIL)

The quantity of DNA isolated from this plantar isolate was quite small and therefore analysis by restriction enzymes was not possible other than with EcoR₁ endonuclease. The results are shown in fig. 21 in
tracks d and f. HWV DNA (MIL) was cleaved to genome length with \( \text{EcoR1} \) (track f) and thus was seen to co-migrate with form III DNA of the uncleaved preparation (track d).
DISCUSSION AND CONCLUSION

The genetic heterogeneity of papilloma viruses has only recently been demonstrated. It has previously been thought that the different types of papilloma skin tumours were caused by a single papilloma virus (Rowson and Mahy 1967). However, Orth et al (1977a) were able to demonstrate differences between papilloma virus isolated from skin common warts and plantar warts, using cRNA-DNA hybridization studies and restriction enzyme analysis. Our own studies confirm these reports but also demonstrate the existence of virus heterogeneity within the common and plantar wart virus groups themselves. Due to the lack of an in vitro culture system our examination of papilloma viruses had to rely on extraction of papilloma viruses directly from wart tissue. The difficulty in obtaining enough wart tissue from a single isolate (approximately 5 grams) proved to be an experimental drawback and as a result our supply was quite limited. However, we were able to harvest enough tissue from two compromised individuals, one being a leukemic farmer and the other a renal transplant patient undergoing immunosuppressive therapy, and from a patient with extensive plantar warts.

Extraction of papilloma virus from the lesions of each of these patients (approximately the same rough wet weight of tissue was used in each case) yielded varying concentrations of papilloma virus as can be seen in Table 2. The concentration of the common wart virus isolated from the leukemic's tissue was three fold higher \((6.5 \times 10^{11} \text{ particles/ml})\) than that of the renal transplant \((2 \times 10^{11} \text{ particles/ml})\), yet these viruses were isolated from morphologically and anatomically
similar lesions. The plantar tissue of the patient with extensive plantar warts yielded particularly low amounts of virus, considering that plantar lesions have been reported to be high virus yielding (Orth et al 1978d).

The viruses isolated from these individual isolates were morphologically identical to one another and to BPV, isolated from a cow with multiple warts. However, using the techniques of immune electron microscopy and restriction enzyme analysis the viruses extracted from each individual human wart isolate were shown to be antigenically unrelated and biochemically distinct from one another and BPV.

In serological studies, using the technique of IEM, the virions of the individual plantar isolate were shown not to be aggregated or coated with antisera raised against either HPV Common (LEUK.) or HPV Common (R.T.). This confirms the results which Orth et al (1977a) obtained, using immunofluorescence and immunodiffusion techniques, when they characterized papilloma viruses isolated from common and plantar warts.

Despite the fact that veterinarians and butchers have a high incidence of warts (Rowson and Mahy 1967) and that plantar wart virions have been shown to share antigenic determinants with Shope papilloma virus (Orth et al 1978b), we were not able to find any antigenic relationship between any of the HPV's and BPV. We were particularly interested in the reactions between the HPV isolated from the leukemic and BPV, as the leukemic patient was a farmer whose cattle had a high incidence of bovine fibropapillomatosis (cow warts). As seen in fig. 3 the HPV (LEUK.) was not reactive with antisera raised against BPV (unfortunately isolates from the leukemic's cattle were not
available). Thus it would appear that the wart virus infection of the leukemic was caused by a human strain of papilloma virus and confirms the host specificity reported by Le Bouvier et al (1966). However, the demonstration of distinct types of BPV by Lancaster and Olson (1978) might still favor an antigenic relationship between the virions isolated from the leukemic patient and a second distinct BPV isolate, however, this may be pure conjecture.

HPV (LEUK.) and HPV (R.T.) were also shown to be antigenically unrelated and thus suggests heterogeneity within the group of common wart viruses. The results obtained in IEM were confirmed when restriction enzyme analysis was carried out.

The restriction enzyme analysis of BPV DNA with EcoRI, BamHI, HindIII and HpaII, resulted in 1, 1, 5, 1 and 7 fragments respectively. However, digestion with HindIII endonuclease generated fragments, the total M.W. of which, would exceed the molecular weight of the entire genome. Increasing the HindIII enzyme concentration did not result in any change in the digestion pattern, which suggests the presence of partially resistant DNA molecules which originate from a second population of BPV DNA molecules and thus supports the existence of viral heterogeneity within the bovine papilloma viruses.

The digestion patterns for the HPV DNA's isolated from the individual human sources differed from BPV DNA as expected, but were also distinct from one another which correlates with the IEM studies. A comparison of the digestion patterns of HPV DNA (LEUK.) and BPV DNA revealed marked differences in the number and molecular weights of the fragments, thus providing further evidence for the host specificity...
which had been observed using IEM. Examination of the digestion patterns of HPV DNA (LEUK.), HPV DNA (R.T.) and HPV DNA (plantar) revealed differences between these individual isolates. The HPV DNA (LEUK.) could be distinguished from HPV DNA (R.T.) on the basis of their sensitivity towards the restriction endonucleases EcoR\textsubscript{I} and Bam 1. The HPV-DNA (LEUK.) was shown to be resistant to EcoR\textsubscript{I} but sensitive to Bam, yielding 2 fragments, while the HPV DNA (R.T.) was shown to be sensitive to EcoR\textsubscript{I}, yielding 1 fragment of unit genome length, but was resistant to Bam cleavage. Low yields of HPV DNA (R.T.) prevented further comparisons with other restriction endonucleases. The resistance of HPV DNA to restriction endonucleases has also previously been observed by Orth et al (1978a). They have reported the resistance of HPV DNA, isolated from a patient bearing numerous hand common warts, to cleavage with Bam and EcoR\textsubscript{I} restriction endonucleases. Using 3 preparations of HPV DNA isolated from this patient they were able to demonstrate DNA molecules which were either resistant to Bam 1 and sensitive to EcoR\textsubscript{I}, or sensitive to Bam and resistant to EcoR\textsubscript{I}.

The HPV DNA (plantar) could be distinguished from HPV DNA (R.T.) on the basis of the Bam reaction, as HPV DNA (plantar) was sensitive to cleavage with this enzyme, yielding 2 fragments. The digestion patterns of HPV DNA (plantar) and HPV DNA (LEUK.) appeared to be similar after digestion with EcoR\textsubscript{I}, Bam, Hind 11 and Hind 111 but differed in their reaction with Hpa 11 as can be seen from comparing the results which appear in Table 6 and Table 7. Agarose gel electrophoresis of undigested HPV DNA (plantar) (form 1 and form 11 combined) revealed the presence of an atypical papilloma DNA band which
migrated in the vicinity of form II DNA (fig. 13). The variation in the length distribution of this HPV plantar DNA isolate (fig. 7) may account for this band, but no distinct population of DNA molecules representative of this size could be found. EcoR1 digestion of the HPV DNA (plantar) resulted in the disappearance of this atypical DNA band, however, the majority of DNA molecules were shown to be resistant. Thus there appears to be heterogeneity within plantar papilloma DNA isolated from a single individual. Favre et al (1977) have also reported heterogeneity within HPV plantar DNA but used pooled material in doing so. They showed that the Hpa I site was absent in 40 to 50% of papilloma DNA molecules examined and they claim that these resistant molecules originated from HPV DNA variants. Furthermore Gissmann and Zur Hausen (1976) have reported the existence of an additional Bam site in one of their plantar DNA isolates, the majority of which contain only a single Bam site.

The plantar papilloma DNA isolate which we examined was shown to have 2 Bam cleavage sites however the M.W. of our Bam B fragment was 1.19 x 10^6 while the Bam B fragment found in one of Gissmann's and Zur Hausen's isolates was .45 x 10^6. Thus it would appear that our plantar HPV DNA isolate is different from those previously characterized. Hpa I digestion of our plantar DNA isolate resulted in the appearance of 5 low molecular weight fragments whose total M.W. was 2.94 x 10^6 which is much less than the average molecular weight (5.25 x 10^6) determined when contour length measurements were performed.

---

1. Can be seen more clearly in the fig. 13 which appears in the appendix.
(section F). This digestion was repeated several times and the digestion pattern obtained was always reproducible and therefore we can only assume that very small M.W. fragments are generated which cannot be detected in our gel system or that fragments of very similar M.W. are formed which cannot be individually resolved.

Favre et al (1977b) have reported the Hpa 11 cleavage products of plantar papilloma DNA (pooled) to be fragments of M.W. $2.3 \times 10^6$, $1.88 \times 10^6$, $0.40 \times 10^6$ and $0.15 \times 10^6$ while Lancaster and Meinke (1976) report the presence of fragments of M.W. $2.29 \times 10^6$, $1.78 \times 10^6$, $0.47 \times 10^6$ and $0.36 \times 10^6$ after Hpa 11 digestion of papilloma DNA isolated from pooled material. Thus it would further appear that our plantar isolate is different from those previously characterized.

Attempts to isolate papilloma virus from condylomata acuminata proved unsuccessful (A3). This is not unusual in that papilloma virus has been shown to occur irregularly in these types of lesions (Dunn and Oglivie 1968; Oriel 1970b). The absence of papilloma virus from these lesions might be explained if the process of replication is examined. It appears that the production of papilloma virus depends on an activation step which occurs during keratinization. However, in condylomata acuminata there appears to be little if any keratinization process. When virus particles have been found in these lesions they have been shown to be antigenically different from papilloma virus found in other types of skin warts (Almeida et al 1969) and DNA isolated from condylomata acuminata was shown not to hybridize with cRNA that was derived from other skin HPV DNA's. Thus it would appear that condylomata acuminata is caused by a distinct type of papilloma virus.
Despite the fact that we were not able to isolate HPV from these lesions we were still able to selectively extract papilloma-sized DNA from 3 cases of condylomata acuminata, using a modification of the Hirt procedure (Hirt 1967; Orth et al 1977a). When the DNA, isolated from these condylomata specimens, was loaded onto 1.2% agarose gels it was possible to detect papilloma-sized DNA molecules which migrated with form II papilloma DNA isolated from skin warts (fig. 14). Orth et al (1978c) have been able to detect papilloma-sized DNA in their preparations using the same procedure, but they have detected superhelical forms in their preparations. The results which we obtained also show the presence of a much larger class of DNA (fig. 15a) which may be papilloma DNA dimers or the mitochondrial-sized DNA which Delap et al (1976) have observed in their DNA preparations of condylomata acuminata, using a reversible alkali denaturation procedure. However, this same group were not able to demonstrate the presence of a distinct papova-like circular DNA, as the presence of small polydisperse circular DNA in their preparations obscured such detection. In all our condylomata DNA preparations there exists (as can be seen in fig. 14 and fig. 15) an intense "band" of DNA which spans the entire length of the gel. The appearance of these intense smears in all our condylomata preparations and in some of our common wart preparations may or may not correlate with the appearance of polydisperse circular DNA which Delap et al (1976) have reported exist in condylomata DNA preparations as two different experimental procedures have been used. In summary it appears that even though papilloma virus cannot be isolated from these lesions there still exists papilloma-sized DNA, which may originate from a distinct type of papilloma virus or may be an autonomously
replicating form.

When low quantities of plantar and common wart tissue precluded virus purification we were able to selectively extract papilloma virus DNA in certain instances. Restriction enzyme analysis of these DNA preparations revealed both size and genetic heterogeneity.

The results (fig. 16 and fig. 17) of the restriction enzyme analysis of HPV DNA (NOR), selectively isolated from the patient with extensive plantar warts compared favorably to those obtained when papilloma DNA was extracted from purified plantar virions (fig. 13) however, it was not possible to detect the "atypical" band which appeared in fig. 13a.

Examination of undigested papilloma DNA (PAWT) (fig. 16i) and papilloma DNA (AR5) revealed the presence of size heterogeneity. Two DNA bands were seen in the vicinity where form 11 DNA migrates. The faster migrating band (lower M.W.) may represent a specific class of deletion molecules which have arisen in the virion population during replication in vivo. Defective DNA molecules have been shown to occur frequently both in SV40, polyoma and BK viruses, which are all smaller papovaviruses. These deletion mutants have been shown to arise during serial high multiplicity passage of virus in vitro (Yoshiike 1968; Tai et al 1972; Fried et al 1974; Padgett and Walker 1976). Yoshiike and Defendi (1977) have previously examined HWV DNA (pooled) for the presence of such deletion molecules. Using length distribution and heteroduplex studies they have shown that deletion mutants occur occasionally in the virus population but that the majority of virion DNA was homogeneous in length. Delap et al (1977) have also examined papilloma DNA for size heterogeneity. Using reassociation kinetic studies and contour length measurements they showed papilloma
DNA isolated from a pool of common warts to be a homogeneous unique population, yet restriction enzyme analysis of their DNA revealed the presence of atypical fragments. Such atypical fragments might arise from the digestion of a second class of papilloma DNA, such as those which were shown to occur in HPV DNA isolated from the patient with extensive plantar warts, HPV DNA (PANT) and HPV DNA (ARS). Although Delap's group report the presence of atypical fragments after enzyme digestion, gel electrophoresis of their undigested papilloma DNA molecules did not reveal the presence of any specific class of atypical molecules. However, this group carried out their electrophoresis at 150 V for 4 hours which might limit the resolution of such atypical DNA molecules.

Restriction enzyme digestion of these atypical DNA molecules would result in the production of atypical fragments. When HPV DNA (PANT) was digested with EcoR₁ (fig. 19d) four fragments were formed and the sum of the M.W. of these fragments would be much greater than the molecular weight of papilloma DNA. Two of the fragments produced co-migrated with linear (form III) DNA but two bands of low intensity (C and D) were also formed and these had M.W. of 2.9 x 10⁶ and 2.7 x 10⁶ respectively. These fragments would appear to be atypical digestion products. Using pooled common wart material Delap et al (1977) have shown the presence of atypical fragments of M.W. 3.5 x 10⁶ and 2.9 x 10⁶ after EcoR₁ digestion in 3 out of 5 pooled wart preparations. Using pooled plantar wart material Favre et al (1975) were able to show the production of two weak bands corresponding to M.W.'s of 2.3 x 10⁶ and 2.6 x 10⁶, after EcoR₁ digestion. In contrast we have been able to show the existence of such atypical fragments in DNA isolated from wart tissue of a single isolate. It therefore appears that
more than one common HPV exists and that in a single papilloma infection more than one type of virus may be present.

HPV DNA (NOR) also yielded atypical fragments after Hpa II digestion as the total of the M.W. of the fragments generated vastly exceeds the M.W. of HPV DNA. Fragments C, E and F are probably the predominant digestion products of Hpa II digested HPV DNA (NOR), as the sum of the molecular weights of these fragments is $5.1 \times 10^6$ which is quite close to the reported M.W. of papilloma DNA. Fragments A, B and D would appear to be atypical cleavage products (as judged by their intensity).

The production of atypical fragments after enzyme digestion was also seen with HPV DNA (ARS) preparation (fig. 19). This is not surprising considering that two size classes of DNA were detected in the undigested material. EcoR I digestion resulted in the appearance of a fragment of genome unit length as well as 2 atypical fragments of approximately the same M.W. as those observed after EcoR I digestion of HPV DNA (PANT). Bam digestion of this isolate also resulted in the appearance of atypical bands (C and D) with M.W.'s of $1.25 \times 10^6$ and $1.1 \times 10^6$ respectively as well as fragments of genome length. Hpa II digestion resulted in the production of 7 fragments, whose total M.W. is twice that of HPV DNA, which might infer that two populations of HPV DNA exist in this single isolate which differ entirely in their Hpa II recognition sites. Enzymatic digestion with EcoR I, Bam and Hpa II have clearly established the presence of more than one type of HPV DNA in the papilloma DNA (ARS) pool which may originate from distinct types of HPV.

Comparison of the HPV DNA (PANT) with HPV DNA (ARS) (Table 9 and Table 11) after digestion with the various restriction
endonucleases reveals certain similarities. Both produced atypical fragments of the same M.W. after EcoR₁ digestion and also shared some of the same Hpa II digestion products. However, they differ in their sensitivity to Bam endonuclease and therefore cannot be identical. As both these preparations are sensitive to EcoR₁ they can be distinguished from HPV DNA (LEUK.) which was shown to be resistant to this enzyme. HPV DNA (ARS) and HPV DNA (PANT) can also be distinguished from plantar HPV DNA (NOR) as the latter is resistant to digestion with EcoR₁ and yields fragments of lower M.W. after Hpa II digestion.

As HPV DNA (R.T.) was shown to be resistant to Bam and sensitive to EcoR₁ it displays similarities to HPV DNA (PANT) however, no atypical fragment were produced in the EcoR₁ digestion. Limited restriction analysis of HPV (R.T.) prevented further comparisons.

Agarose gel electrophoresis of undigested HPV DNA (ESK) did not reveal the presence of any atypical DNA molecules. HPV DNA (ESK) could be distinguished from HPV DNA (ARS) and HPV DNA (PANT) on the basis of the EcoR₁ reaction, as the former is resistant to digestion with this enzyme while the latter two are sensitive. Restriction enzyme analysis with Hind III showed the majority of molecules to be resistant but also demonstrated the presence of a sensitive fraction as an atypical band was detected (indicated by the arrow in fig. 20c). The restriction enzyme digestion pattern of HPV DNA (ESK) bears certain similarities to that of HPV DNA (LEUK.) (see Table 6 and Table 10) however, they differ in their Hpa II digestion pattern. It would appear on the basis of the Hind III and Hpa II reactions that HPV DNA (ESK) also exhibits genetic heterogeneity (fig. 20).
Restriction enzyme analysis of HPV DNA (MIL) with EcoR₁ (fig. 21f) showed it to be different from the only other plantar DNA isolate in our study (HPV DNA NOR) as HPV DNA (MIL) was sensitive to EcoR₁ cleavage while HPV DNA (NOR) was resistant. Low yields of HPV DNA (MIL) prevented further characterization with other restriction endonucleases.

In summary, it would appear that there is a wide spectrum of human papilloma viruses. The plantar wart virus was shown to be antigenically and biochemically different from the group of common wart viruses. However, our study cannot exclude the association of papilloma viruses of the common type with plantar lesions as the number of plantar specimens which we examined were limited. It would appear that variations can occur in the composition of DNA within both the plantar and common wart viruses themselves. This variation may be a result of alterations in the integration excision process which may occur when warts are transmitted in vitro, as this has been reported to occur in other members of the papova group of viruses such as polyoma, SV40 and BK (Yoshiike 1968; Freid et al 1974). Furthermore there may exist more than one type of HPV in a single human wart infection (at a specific anatomical site). The development of an in vitro culture system would certainly aid in the investigation of these agents, however, this still remains to be done.
CLAIMS TO ORIGINAL KNOWLEDGE

1. Agarose gel electrophoresis of DNA extracted from genital warts revealed the presence of papilloma-sized DNA which co-migrated with form 11 DNA purified from virions.

2. Agarose gel electrophoresis of virion DNA isolated from Hirt extracts of common wart tissue from single donors revealed the presence of size heterogeneity.

3. Restriction enzyme analysis of HPV DNA isolated from single sources (non-pooled) revealed the presence of atypical DNA fragments after digestion and thus demonstrates the existence of viral heterogeneity within individual wart isolates.

4. Restriction enzyme digestion of BPV DNA with EcoRI, Bam, Hind II, Hind III and Hpa II resulted in the production of 1, 1, 5, 1 and 7 fragments respectively.
APPENDIX A

A. Glassware

All glassware (centrifuge tubes) was washed with detergent, thoroughly rinsed in tap water, then in distilled water, and then autoclaved at 15 lbs pressure for 20 minutes.

B. Solutions and Buffers for Virus Extraction

1. Preparation of Phosphate Solutions
   a) Sodium dihydrogen orthophosphate<sup>1</sup>, 0.02M
      \[ \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} \] 3.12 g
      Distilled water 1000 ml
   b) Sodium phosphate dibasic<sup>2</sup>, 0.02M
      \[ \text{Na}_2\text{HPO}_4 \] 2.84 g
      Distilled water 1000 ml

2. Preparation of Phosphate Buffer
   a) 1M NaCl, 0.05M phosphate buffer pH 8
      .02M NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O 10.6 ml
      .02M Na<sub>2</sub>HPO<sub>4</sub> 189.4 ml
      NaCl 46.76 g
      Distilled water 600 ml

1. BDH Laboratories
2. Fischer Scientific Co.
b) .05M NaCl, .01M EDTA, .05M phosphate buffer pH 7.4

\[
\begin{align*}
&\text{.02M } \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} & 19 \text{ ml} \\
&\text{.02M } \text{Na}_2\text{HPO}_4 & 81 \text{ ml} \\
&\text{.05M NaCl} & 40 \text{ ml} \\
&\text{1M EDTA} & 40 \text{ ml} \\
&\text{Distilled water} & 220 \text{ ml}
\end{align*}
\]

*All buffers were adjusted to indicated pH with either 2N NaOH or 2N HCl

3. .5M NaCl\(^3\) solution

\[
\begin{align*}
&\text{NaCl} & 14.62 \\
&\text{Distilled water} & 500 \text{ ml}
\end{align*}
\]

4. .1M EDTA\(^4\) (dissodium salt) ethylene diamine tetraacetic

\[
\begin{align*}
&\text{EDTA} & 7.3 \text{ g} \\
&\text{Distilled water} & 250 \text{ ml}
\end{align*}
\]

All solutions and buffers were sterilized by autoclaving for 15 min. at 15 lb pressure (121°C) and then stored at 4°C.

5. Trypsin\(^5\) solution .1%

\[
\begin{align*}
&\text{Trypsin} & .1 \text{ g} \\
&1\text{M NaCl} .05\text{M phosphate buffer} & 100 \text{ ml}
\end{align*}
\]

\[\text{NOT autoclaved}\]

3. Fischer Scientific Co.
4. Sigma Chemical Co.
5. Difco (1:250)
6. Phosphate Buffered Saline (PBS) pH 7.3
Dulbecco's A tablets

- NaCl: 8.00 g/l
- KCl: .20 g/l
- Disodium hydrogen phosphate: 1.15 g/l
- Potassium dihydrogen phosphate: .20 g/l

Dissolve one tablet in 100 ml distilled water autoclave at 10 lbs (115°C) for 10 min.

C. Reagents for E.M. Examination of Virus

1. Phosphotungstic Acid 2% pH 7.2
   - Phosphotungstic acid: 1.0 g
   - Distilled water: 50 ml

   Filtered and adjusted to pH 7.2 with 2NaOH and stored at 4°C

D. Reagents used in immune electron microscopy

1. Freund's Complete Adjuvant
   - Anlacel A: 1.5 ml
   - Bayol F: 8.5 ml
   - Mycobacterium butyricum (killed and dried): 5 mg

2. Physiological Saline: .85% saline
Estimation of Virus Concentration by Latex Counting

The virus concentration of the saline lavages was estimated by latex counting using the method of Watson (1962a), employing Dow latex of particle size .091 μ.

E. Buffers and Reagents Used in Extraction of Papilloma DNA

1. Tris (hydroxymethyl) aminomethane (Tris) Buffer
   a) Tris stock solutions \(0.2M\)
      - Tris aminomethane: 24.2 g
      - Distilled water: 1000 ml
   b) \(0.05M\) Tris - HCl pH 7.8
      To make a 200 ml solution:
      - Tris Stock: 50 ml
      - \(2N\) HCl: 32.5 ml
      - Distilled water: 117.5 ml

2. Ethidium Bromide (500 µg/ml)
   - Ethidium Bromide: 0.05 g
   - Tris Buffer: 100 ml

3. 5% Solution of N-lauroyl - Sarcosine (Sarkosyl) (Sodium Salt)
   - Sarkosyl: 5 g
   - \(0.01M\) EDTA: 100 ml

4. Isopropanol (Saturated with 2M CsCl)

9. Sigma Chemical Co.
10. Sigma Chemical Co.
11. Fischer Scientific Co.
5. DNA Dialysis Buffer - .01M Tris-HCl - .001M EDTA pH 7.9

- Tris stock 50 ml
- .2N HCl 26.8 ml
- .1M EDTA 10 ml
- Distilled water 913.2 ml

F. Buffers for Selective DNA Extraction (Hirt)

1. .01M Tris, .01M NaCl, .01M EDTA - .5% SDS

- 50 µg per ml Proteinase K

To make 50 mls

- .05M Tris pH 7.8 10 ml
- .05M NaCl 10 ml
- .1M EDTA 5 ml
- SDS (dodecyl sodium sulfate) .25 g
- Proteinase K .002 g
- Distilled water 25 ml

G. Solutions for DNA Spreading

1. .25M Ammonium Acetate

- NH₄COOH 19.27 g
- Doubly distilled water 1000 ml

12. Sigma Chemical Co.
13. Boehringer Mannheim
2. .5M Ammonium Acetate pH 8
   \(\text{NH}_4\text{COOH}\) 38.54 g
   Doubly distilled water 100 ml

3. Cytochrome c Solution .1%
   Cytochrome c\textsuperscript{15} 1 mg
   Doubly distilled water 1 ml

H. Restriction Enzyme Digestion

1. Restriction Endonuclease Digestion Buffers
   All buffers were made 10 x more concentrated and were diluted in the reaction mixtures.
   All buffers were at pH 7.5 and were stored at \(-20^\circ\text{C}\)

a) EcoR\textsubscript{I}: .1M Tris-HCl pH 7.5, .5M NaCl, .01M MgCl\textsubscript{2}
   To make 25 ml
   \begin{align*}
   \text{Tris} & \quad 3.03 \\
   \text{MgCl\textsubscript{2}} & \quad .5 \\
   \text{NaCl} & \quad .73 \\
   \text{Distilled water} & \quad \text{to make 25 ml}
   \end{align*}

b) Bam: .1M Tris-HCl MgCl\textsubscript{2}
   \begin{align*}
   \text{Tris} & \quad 3 \text{ g} \\
   \text{MgCl\textsubscript{2}} & \quad .5 \text{ g} \\
   \text{Distilled water} & \quad \text{to make 25 ml}
   \end{align*}

15. Sigma Chemical Co.
c) Hind II: .1M Tris-HCl pH 7.5, .1M MgCl₂, .5M NaCl, .15M dithiothreitol

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>Tris</td>
<td>.3 g</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>.5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>.73 g</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>.54 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to make 25 mls</td>
</tr>
</tbody>
</table>

d) Hind III: .06M Tris-HCl pH 7.5, .5M MgCl₂, .5M NaCl

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>.18 g</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>.3 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>.73</td>
</tr>
<tr>
<td>Bovine Serum Albumin¹⁶</td>
<td>50 ug</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to make 25 mls</td>
</tr>
</tbody>
</table>

e) Hpa II: .1M Tris-HCl pH 7.5, .1M MgCl₂, .15M Dithiothreitol

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>.3 g</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>.5 g</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>.037 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to make 25 mls</td>
</tr>
</tbody>
</table>

¹⁶ ICN Pharmaceuticals
2. Composition of Polyacrylamide Plug (15%)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyacrylamide A*</td>
<td>13.5 ml</td>
</tr>
<tr>
<td>Buffer E</td>
<td>15 ml</td>
</tr>
<tr>
<td>Temed</td>
<td>25 ul</td>
</tr>
<tr>
<td>Ammonium Persulfate</td>
<td>1.5 ml of</td>
</tr>
<tr>
<td></td>
<td>150 mg/10 ml distilled water</td>
</tr>
<tr>
<td>*Polyacrylamide</td>
<td>22.2 g</td>
</tr>
<tr>
<td>Bis</td>
<td>.6 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>made up to 100 ml</td>
</tr>
</tbody>
</table>

*Polyacrylamide

17 & 18. Bio Rad Laboratories
19. Sigma Chemical Co.
fig. 12
BIBLIOGRAPHY


FAVRE, M., G. Orth, O. Croissant and M. Yaniv. 1977b. Human papilloma virus DNA: physical mapping of the cleavage sites of Bacillus amyloli-quefaciens (Bam I) and Haemophilus parainfluenza (Hpa I) endonuclease and evidence for partial heterogeneity. J. Virol 21: 1210-1214


