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Identification of Papillomaviruses in Butchers' Warts

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We have studied the papillomaviruses found in the hand warts of 60 butchers, most of them from 2 distant slaughterhouses. Warts differing in morphology and location were studied separately. The viruses were identified by molecular hybridization, restriction enzyme analysis and immunofluorescence. Four known human papillomaviruses (HPV-1, HPV-2, HPV-3, HPV-4) were detected and one hitherto unknown papillomavirus was identified in 9 butchers. The DNA of the latter virus did not anneal with any of the RNAs complementary to either HPV-1 to HPV-5 or bovine papillomavirus type 1 (BPV-1) DNAs, and showed a *Hind* II+III restriction enzyme cleavage pattern distinct from those of known HPVs and BPVs. This virus showed distinct antigenic properties, as shown by immunofluorescence, using HPV-1, -2, -3, -5, and BVP-1 antisera. It may represent a new type of human papillomavirus (HPV-7) or a yet unidentified animal papillomavirus. In addition, 6 butchers were found to be infected with a papillomavirus, distinct from the known skin HPVs and from BPV-1, which could not be characterized by restriction enzyme analysis. Eleven butchers were found to be infected by 2 viruses.

A characteristic histological pattern was found to be associated with the different papillomaviruses.

It is now well established that the incidence of warts is significantly higher in butchers and meat-cutters than in the general population [1–4]. Although some authors have raised the question of the possible transmission of animal papillomaviruses to man [1,2,5–8], no identification of the viruses found in butchers' warts had yet been reported. The problem is of special interest, since at least 5 types of cutaneous human papillomaviruses (HPV) [9–12], and 4 types of bovine papillomaviruses (BPV) [12–16] have recently been identified. Furthermore, 2 of these viruses, HPV-5 and BPV-4, have been found associated with naturally occurring carcinomas [11,16–18], and 2 other viruses, BPV-1 and BPV-2, have been found associated with natural cases of tumors of the connective tissue occurring in an alien host, the horse [19].

The aim of our work was to determine the types of viruses in butchers' warts, and to look for a possible correlation between the types of viruses and the histological patterns of the lesions.

MATERIALS AND METHODS

Subjects

Our studies have been performed in 60 butchers whose work was either to slaughter cattle, sheep and pigs, or to cut meat and viscera. Most of the butchers were from 2 slaughterhouses located in different cities. All the warts were located on the hands: in 21 butchers, they were dorsal and palmar; in 8, they were dorsal, palmar and around the nails; and in 14 they were dorsal only. The clinical characteristics of the warts and the epidemiological data will be reported elsewhere.* The warts differed considerably in morphology, size, and growth pattern (endophytic or exophytic). When clinically different types of warts were present, each type was usually studied separately. The specimens used for the biochemical characterization of the viral DNA were obtained from 1 to 40 lesions, either by removing the warts by curretage or by scraping their surfaces. The specimens were kept in Eagle medium before freezing at −70°C. The samples for immunofluorescence (IF) and histological studies were obtained by punch biopsies. The samples for IF studies were either frozen immediately, or placed in Eagle medium before processing.

Preparation of Viral DNA

The extraction of viral DNA was performed according to a modified Hirt's method [10,20,21]. The DNA was precipitated with ethanol and redissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7.9 (200 μl). The concentration of circular viral DNA molecules was evaluated by electron microscopy [22], by comparison with a sample of the replicative form of bacteriophage Ø × 174 DNA (Ø × 174 RF DNA) of known concentration. The formamide technique described by Davis, Simon, and Davidson [23] was used for mounting undiluted or diluted samples (5 μl). The grids were observed at a magnification of 8000. Supercoiled (form I) and relaxed (form II) circular DNA molecules were scored by crossing 5 squares per grid. One molecule per 5 squares corresponded to 0.06 μg/200 μl. With this method, the concentration of viral DNA is underestimated, since linear viral DNA molecules and fragments present in the Hirt' supernatants are not taken into account.

In preparations containing more than 2.5 μg of DNA, form I DNA molecules were separated from form II and form III (linear) and from cellular DNA by sedimentation in sucrose gradients in the presence of ethidium bromide (less than 10 μg) [18,24], or by CsCl-ethidium bromide equilibrium centrifugation (more than 10 μg) [10]. After dialysis against 10 mM Tris-HCl, 1 mM EDTA, pH 7.9, the DNA concentrations were determined by electron microscopy, and the DNA preparations were kept at −20°C.

cRNA-DNA Hybridization

Complementary RNAs were obtained by *in vitro* transcription of form I DNA molecules, as previously described [10,21]. The viral DNAs were: HPV-1 DNA (subtype a) [12,18] obtained from a single deep plantar wart; HPV-2 DNA (a mixture of subtypes b and c)† obtained from the pooled common warts of a single patient; HPV-3 DNA (subtype a)† [18] isolated from pooled scrapings of flat wart-like lesions in a patient with EV [17,18]; HPV-4 DNA [9,18] obtained from the pooled palmar warts of a butcher; HPV-5 DNA (subtype a)† [18]

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Abbreviations:
B: bovine
cRNA: complementary RNA
EV: epidermodysplasia verruciformis
H: human
IF: immunofluorescence
MH: molecular hybridization
PV: papillomavirus

* Jablonska S, Croissant O, Obalek S, Favre M, Rzesa G, Orth G: Morphology and epidemiology of butchers' warts in relation to the type of papillomavirus, in preparation.

† Favre M, Jablonska S, Croissant O, Obalek S, Orth G: On the genetic heterogeneity of skin HPVs as analysed by restriction endonucleases, in preparation.

obtained from the pooled scrapings of pityriasis versicolor-like lesions of a patient with EV [17,18]; and BPV-1 DNA [13,14] isolated from skin fibropapillomas of cattle. The cRNA-DNA filter hybridizations under paraffin [25] were performed using amounts of form I and II viral DNA varying from less than 5 ng to 50 ng per filter, under the conditions previously described [10,21].

Restriction Enzyme Analysis

The digestion of viral DNAs with a mixture of *Hind* II and *Hind* III endonucleases (Boehringer, Mannheim) and the separation of the cleavage products by vertical slab gel electrophoresis in agarose (1.2%) were performed as previously described [18]. The molecular weights of the fragments were evaluated from their electrophoretic mobilities, by comparison with λ *Hind* III DNA fragments [26] and *Hae* III Ø × 174 RF DNA fragments [27].

Immunofluorescence

Direct and indirect immunofluorescence (IF) studies were performed as previously described [17,28]. Fluorescein-labeled anti-HPV-1 guinea pig IgG (G 121, G 122) and anti-HPV-3a (G 281) or HPV-5a (G 251) guinea pig sera were the same used in our previous studies [10,17,18]. Fluorescein-labeled anti-HPV-2 IgG were prepared from a guinea pig serum (G 314) obtained against full particles of a mixture of subtypes b and c.† This serum had a specificity similar to that of the HPV-2a antiserum (G 206) used previously [10,11,17,28].

Histological Studies

Sections prepared from specimens fixed in Bouin's solution or in 10% formalin and embedded in paraffin, or cryostat sections adjacent to sections studied by IF, were stained with hematoxylin and eosin.

RESULTS

Identification of Viruses by DNA Analysis

The viral DNA was extracted from 68 samples obtained from 58 butchers and the yields varied considerably. The procedures used for the characterization of the viral DNAs are summarized in Table I.

Molecular hybridization studies were performed in 49 preparations from 41 butchers, using RNAs complementary to HPV-1 to 5 and BPV-1 DNAs (Table II). Significant annealing (values of at least 5% of the control values) was observed in 31 preparations, thus permitting the identification of HPV-1, HPV-2, HPV-3, and HPV-4 in 2, 19, 6 and 4 preparations, respectively. In 18 preparations, the annealing was not significant, although for 10 of them molecular hybridization experiments were performed in our standard conditions (about 50 ng of viral DNA per filter). This indicates the existence of at least one papillomavirus not related to the presently known skin HPVs and BPV-1, which will be referred to as papillomavirus × (PVX). The extent of annealing was variable, especially for HPV-3 isolates (Table II). This may be due to the infection of some butchers with 2 or more viruses (this was indeed observed in 5 butchers) and/or to the presence of viruses only partially related to the prototypes.

Twenty-four DNA preparations (from 22 butchers) were further fractionated by gradient centrifugation (Table I) and characterized by *Hind* II+III endonuclease cleavage patterns (Table 3, Fig 1).

Sixteen DNA preparations had previously been characterized by MH. Two HPV-1 preparations yielded patterns characteristic of subtypes a (Fig 1*a*), or b [9,12]; three out of 4 HPV-2 preparations gave a pattern corresponding to subtype b† (Fig 1 c). The HPV-3 preparations yielded 2 distinct cleavage patterns, quite different from those of the 4 HPV-3 subtypes previously characterized,† which has led to the identification of 2 new subtypes, referred to as HPV-3e and HPV-3f, respectively (Fig 1*d,e*). One DNA preparation, which annealed with both

TABLE I. *Procedures used for the characterization of papillomavirus DNA from butchers' warts*

Form I+II DNA yields[a] (Hirt extract)	Number of preparations	Fractionation by gradient	Molecular hybridization	*Hind* II+III cleavage
<0.06 μg	20		10	
0.06 to 2.5 μg	18		17	
2.5 to 37.5 μg	30	24	22	24
Total	68	24	49	24

[a] As determined by electron microscopy [22].

TABLE II. *Identification of papillomaviruses by cRNA-DNA hybridization*[a]

Radioactivity bound to filters (% of control)	Number of butchers found infected with						
	HPV-1	HPV-2	HPV-3	HPV-4	HPV-5	BPV-1	PVX
>50	1	11	1	2			
25 to 50	1	5	1	1			
5 to 25		3	4	1			
<5							10
Total	2	19	6	4	0	0	10

[a] The amount of viral DNA available for 8 preparations showing no annealing with any of the probes was smaller than 5ng per filter, and the results were considered as unconclusive.

TABLE III. *Analysis of papillomavirus DNAs by restriction enzymes cleavage*

No. of butchers	Virus type as determined by	
	Molecular hybridization	*Hind* II+III cleavage patterns
2	1	1a; 1b
4	2	2b; 2b + Xa; 2b + 3f; 2x[a] + Xa
4	3	3e; 3f; 3f; 3f + Xa
1	2 + 3	2c + 3g
1	4	4
4	X	Xa; Xa; Xa; Xa
6	ND[b]	1a + 1b; 3f; 3g + 4[c]; 4; Xa; Xa[c]

[a] An incomplete cleavage pattern did not allow identification of the subtype.
[b] ND, not determined.
[c] Two DNA preparations from different locations were studied.

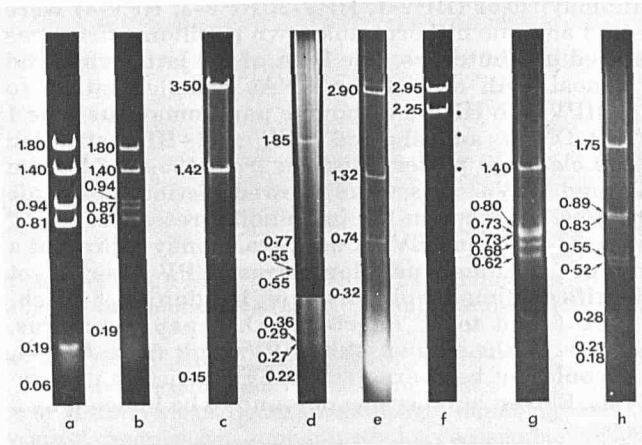

FIG 1. Cleavage patterns of papillomavirus DNAs obtained after treatment with *Hind* II+III endonucleases. The viral DNAs were obtained from single (*a*, HPV-1a) or pooled (*b*, HPV-1a+b) deep palmar warts; pooled dorso-palmar common warts (*c*, HPV-2b); pooled dorso-palmar flat warts (*d*, HPV-3e; *e*, HPV-3f; *f*, HPV-3g); pooled endophytic palmar warts (*g*, HPV-4); pooled dorso-palmar common warts (*h*, PVXa). Numbers on the side of the gels indicate the fragments' size expressed in kilodaltons. The sum of the molecular weights of the fragments generated, 5.20 (*a*), 5.07 (*c*), 4.86 (*d*), 5.28 (*e*), 5.20 (*f*), 4.96 (*g*), 5.21 (*h*), nearly equals the molecular weight of one papillomavirus genome. The presence of HPV-1a and b in (*b*) is shown by the existence of both fragments 0.94 and 0.87 [9]. Stars (*f*) indicate HPV-2c pattern with fragments with molecular weights of 2.2, 1.75 and 1.20×10^6.

HPV-2 and HPV-3 probes, yielded, in addition to the pattern specific for HPV-2c,† a pattern distinct from those of HPV-3a to HPV-3f: it may thus correspond to a new subtype referred to as HPV-3g (Fig 1*f*). One HPV-4 preparation yielded the pattern specific of this type (Fig 1*g*) [18]. Four PVX preparations showed an identical cleavage pattern (Fig 1*h*), which had not been observed previously and will be referred to as PVXa. It should be stressed that in 4 cases a second virus could be identified by restriction enzyme analysis, PVXa in 3 of them, and, in one case, a small amount of HPV-3f.

Eight preparations from 6 butchers had not been previously studied by MH. In one case, the restriction enzyme analysis indicated a mixture of HPV-1 subtypes a and b (Fig 1*b*). In another case the preparation from dorsal lesions contained HPV-3g, while that from palmar lesions contained HPV-4. In still another case, HPV-4 was identified, while three other preparations from 2 butchers contained PVXa.

Identification of Viruses by Immunofluorescence

Immunofluorescence studies were performed in 56 specimens obtained from 52 butchers, using guinea pig HPV-1, HPV-2, HPV-3 and HPV-5 antisera (Table IV). In 16 out of 21 butchers found to be infected with HPV-1, HPV-2, or HPV-3 (2 of them being infected with 2 viruses), the results of molecular hybridization and/or restriction enzyme analysis were confirmed by immunofluorescence. Negative results were obtained in specimens from butchers infected with HPV-4, or PVX (including 5 specimens from workers infected with PVXa). This result provides further evidence that PVX viruses represent a distinct group. In the butchers whose virus types had not been determined by other methods, IF studies revealed infection with HPV-1 in one case and with HPV-2 in 8 cases.

Frequency of Infection with Different Papillomaviruses

The data obtained from 60 butchers, using three methods of viral identification, are summarized in Table V and VI. Table V shows that at least 5 different types of papillomaviruses were found in butchers' warts. The most frequent virus was HPV-2. It should be noticed that about 25% of the butchers were found to be infected with a virus (or viruses) not previously identified, and that in 15% of the cases the virus was shown to be a distinct type by restriction enzyme analysis. In 11 cases, that is in 18.3% of the butchers, the results were unconclusive. Table VI shows that a significant number of butchers (11 out of 60, or 18.3%) were found to be infected with at least 2 viruses.

TABLE IV. *Identification of viruses from butchers' warts by immunofluorescence tests*[a]

Virus type (DNA analysis)	Number of cases				
	With antigens				Yielding negative results
	HPV-1	HPV-2	HPV-3	HPV-5	
HPV-1	1				
HPV-2		11			4
HPV-3			5		2
HPV-4					6
PVXa					5
PVX					3
Unconclusive assays					5
Not done	1	8			5
Total	2	19	5	0	30

[a] No HPV-4 antiserum was available for this study.

TABLE V. *Frequency of papillomavirus types in butchers' warts*

Virus type[a]	Butchers infected	
	Number	Percentage[b]
HPV-1	4	6.7
HPV-2	27	45.0
HPV-3	9	15.0
HPV-4	6	10.0
HPV-5	0	
PVXa	9	15.0
PVX	5	8.3
Unconclusive assays	11	18.3

[a] As determined by molecular hybridization, and/or restriction enzyme analysis, and/or IF.

[b] Total number of butchers studied:60.

TABLE VI. *Summarized pattern of papillomavirus infection in butchers*

Virus types	No. of butchers
HPV-1	3
HPV-1, HPV-2	1
HPV-2	18
HPV-2, HPV-3	2
HPV-2, HPV-4	2
HPV-2, PVXa	2
HPV-2, PVX	2
HPV-3	4
HPV-3, HPV-4	2
HPV-3, PVXa	1
HPV-4	3
PVXa	6
PVX	3
Unconclusive assays	11

TABLE VII. *Histological characteristics of warts as related to the papillomavirus type*

Virus type as determined by DNA analysis and/or immunofluorescence	Number of lesions with histological features					
	Characteristic of infection with					Unconclusive
	HPV-1	HPV-2	HPV-3	HPV-4	PVXa	
HPV-1	2					
HPV-2		25			4	1
HPV-3			7			2
HPV-4				7		
PVXa					10	
PVX					4	
Not determined		2	1	1	3	3
Total	2	27	8	8	21	6

Histological Features of Butchers' Hand Warts, as Related to the Type of Papillomaviruses

Seventy-two specimens from 50 butchers were studied and the histological findings were related to the type of virus identified in the same lesion by IF (21 specimens) and/or viruses found in lesions of similar morphology and/or location of the same subject (Table VII). In all cases characteristic histological features could be determined.

HPV-1-induced warts (Fig 2): Two palmar warts showed all the features described for myrmecia [29]: (1) pronounced papillomatosis; (2) eosinophilic cytoplasmic keratohyalin inclusions, first detectable in the lower malpighian layers, becoming numerous, irregularly shaped, and very large in the upper layers; (3) a progressively increasing size of the cells and clearing of the cytoplasm observed from the lower to the upper layers; (4) enlarged nuclei showing marginated chromatin and light basophilic inclusions in the upper granular layers; (5) extensive hyperkeratosis with large, highly basophilic nuclei.

HPV-2-induced warts (Fig 3): The main features are: (1) papillomatosis and acanthosis; (2) granulosis, especially in rete ridges, associated with more or less pronounced clearing of the cytoplasm, and characterized by numerous keratohyalin granules of variable size, shape and stainability; (3) hyperkeratosis with parakeratosis, sometimes arranged characteristically in columns.

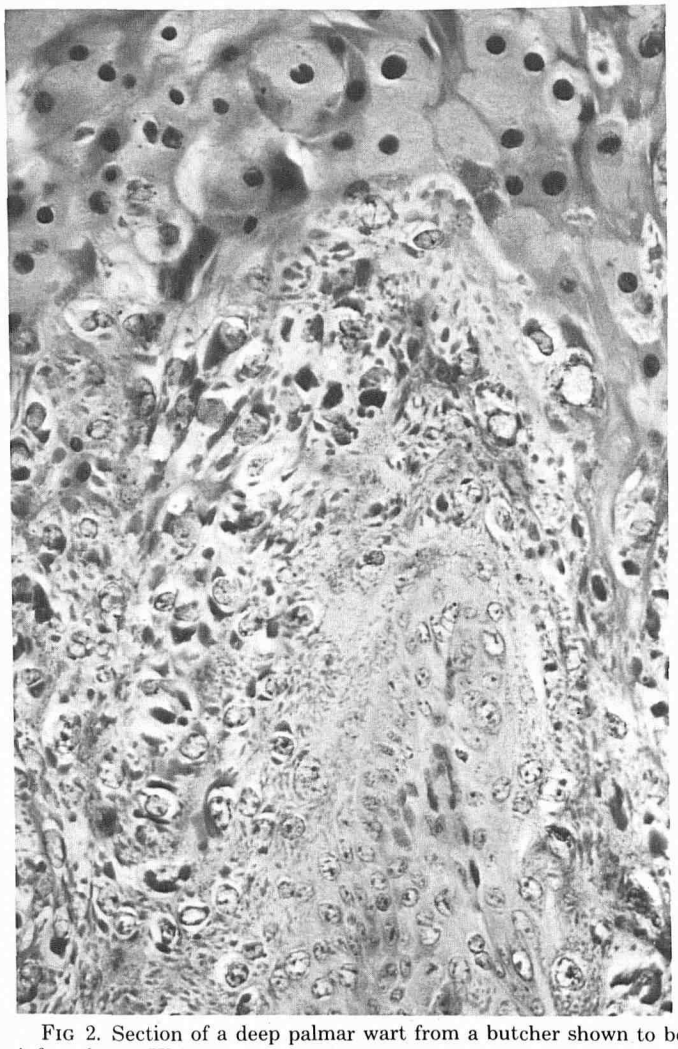

FIG 2. Section of a deep palmar wart from a butcher shown to be infected with HPV-1a (× 260).

HPV-3-induced warts (Fig 4): The characteristic features are: (1) some papillomatosis, though less pronounced than in HPV-2-induced warts; (2) slight to prominent perinuclear vacuolization of the cells in the upper malpighian layer, especially in granular cells where keratohyalin granules are small and pushed away to the periphery; (3) heavy staining of superficial granular cells where keratohyalin granules may not be discernable; (4) hyperkeratosis, often with more or less prominent parakeratosis.

HPV-4-induced warts (Fig 5): The characteristic features are: (1) pronounced papillomatosis; (2) large cells in squamous and granular layers with small, crescent-shaped, off-centered nuclei and no detectable keratohyalin granules; these cells are often arranged in clusters and surrounded, in the granular layer, by heavily stained cells with irregularly distributed, very small, keratohyalin granules; (3) pronounced hyperkeratosis with parakeratosis; the nuclei of parakeratotic cells are large, basophilic and overlay areas containing clusters of clear cells.

PVXa-induced warts (Fig 6): The characteristics are: (1) papillomatosis and acanthosis, (2) large clear cells, isolated or in clusters, with centered nuclei, sometimes binucleated, with no keratohyalin granules; these cells are surrounded by heavily stained cells, often containing small to medium-sized keratohyalin granules; (3) pronounced hyperkeratosis, with parakeratosis often arranged in columns.

As seen in Table VII, there is a close correlation between the virological findings and the histological features. The unconclusive histological findings in 3 out of the 58 specimens in which the virus type has been identified, were due to the absence of clear cells, that is to the absence of viral cytopathogenic effects. The only discrepancy was the demonstration of a histological picture characteristic for PVXa in 4 specimens from butchers shown to be infected with HPV-2. In these cases, the virus had been identified by the IF study of another lesion, and/or by molecular hybridization experiments which gave less than 50% the annealing value observed in controls. Such results are compatible with infection by an additional virus not detected by these techniques. The specimens from butchers infected with PVX had features characteristic of PVXa infection. Finally 7 out of 10 specimens from butchers with undetermined papillomavirus type had histological pictures suggestive of infection with HPV-2, HPV-3, HPV-4 or PVXa.

DISCUSSION

The present study has revealed the presence of different types of papillomavirus in butchers' warts. Of the 5 HPVs known to induce skin warts [9–12], 4 (HPV-1, HPV-2, HPV-3, HPV-4) were found in the butchers' warts. HPV-2, previously shown to be preferentially associated with common warts [10,30] was the most frequent finding (45% of butchers), whereas the potentially oncogenic HPV-5, usually associated with epidermodysplasia verruciformis [11,17,18] was not detected. The present study reveals a greater heterogeneity of papillomavi-

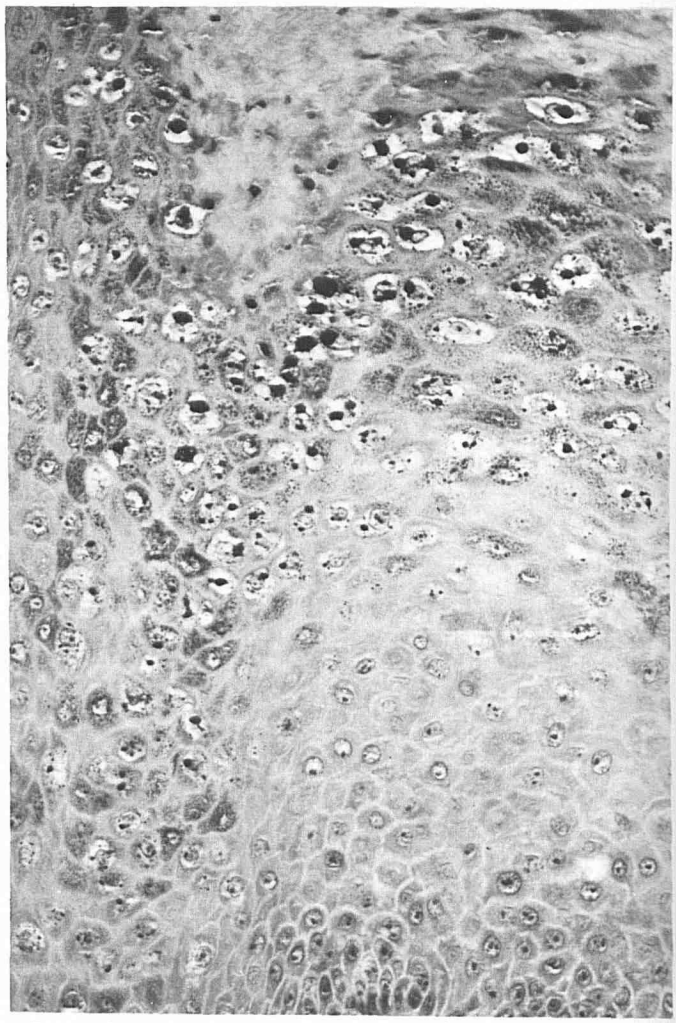

FIG 3. Section of a dorsal common wart from a butcher found to be infected with HPV-2b (× 260).

ruses infectious for man than previously expected. HPV-1, HPV-2 and HPV-4, identified by restriction enzyme analysis of the viral DNAs, corresponded to previously characterized molecular species† [9–12,18,31]. However, most HPV-3 isolates obtained from butchers' warts were found to show only a rather low annealing value with the probe specific for the prototypical HPV-3 DNA [18]. Restriction enzyme analysis led to the characterization of 3 distinct molecular species, which are provisionally regarded as new subtypes of HPV-3 (HPV-3e,f,g). HPV-3f has since been found in flat wart-like lesions of 2 patients with epidermodysplasia verruciformis (including the patient JG previously studied) [11,17], while HPV-3e, the most closely related to the prototypical HPV-3a (as shown by MH experiments), and HPV-3g have not yet been found in other patients.

In addition to the known HPVs, a hitherto unidentified papillomavirus, referred to as PVXa, has been demonstrated: its DNA did not anneal with either HPVs or BPV-1 probes, and restriction enzyme analysis revealed it to be of a distinct type. This virus has been found in 15% of the butchers and its incidence is most probably underestimated since restriction enzyme analysis of viral DNAs was performed in only about one-third of the cases. PVXa may be of human or animal origin. The lack of annealing of the viral DNA with BPV-1 probe, as well as the cleavage pattern obtained with *Hin*d II+III endonucleases, clearly different from those reported for BPV-1 to 4 DNAs [14–16], suggest a nonbovine origin. However, it is conceivable that other, still unrecognized, BPVs may exist, for instance the virus responsible for the teat rice grain lesions [32]. Furthermore, the possible role of a papillomavirus of ovine [33] or porcine origin cannot be excluded. On the other hand, PVXa has all characteristics of a new HPV type: (a) lack of DNA sequence homology with known skin HPVs; (b) *Hin*d II+III, as well as *Eco* RI, *Bam* HI and *Hae* III cleavage patterns clearly different from those of skin HPVs† [9–12,18,31] or HPV-6, recently characterized in genital warts [34]; (c) distinct antigenic properties. PVXa may thus represent a 7th HPV type (HPV-7)[18].

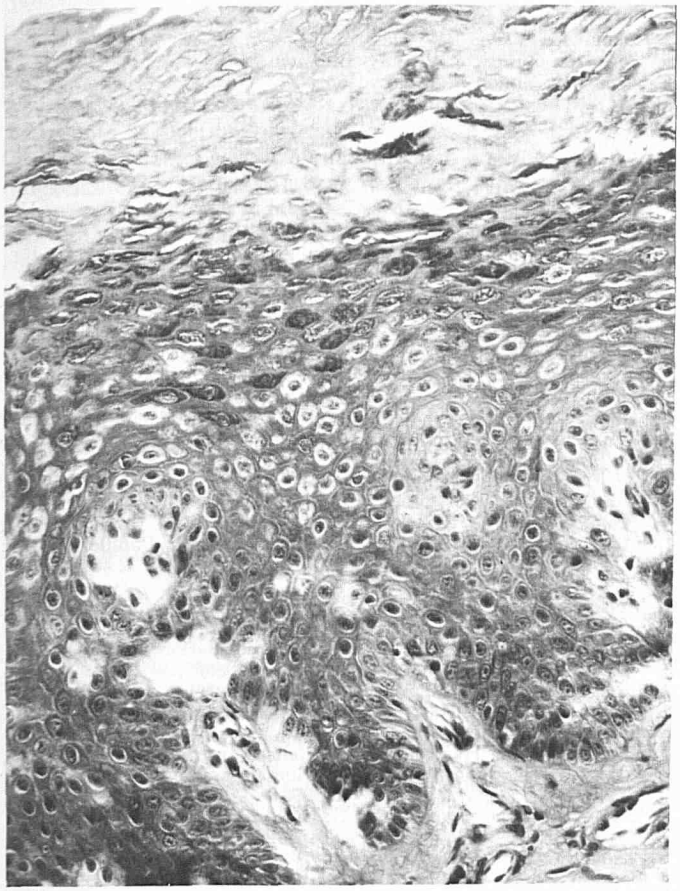

FIG 4. Section of a flat wart located on the dorsal side of the finger of a butcher shown to be infected with HPV-3g (× 260).

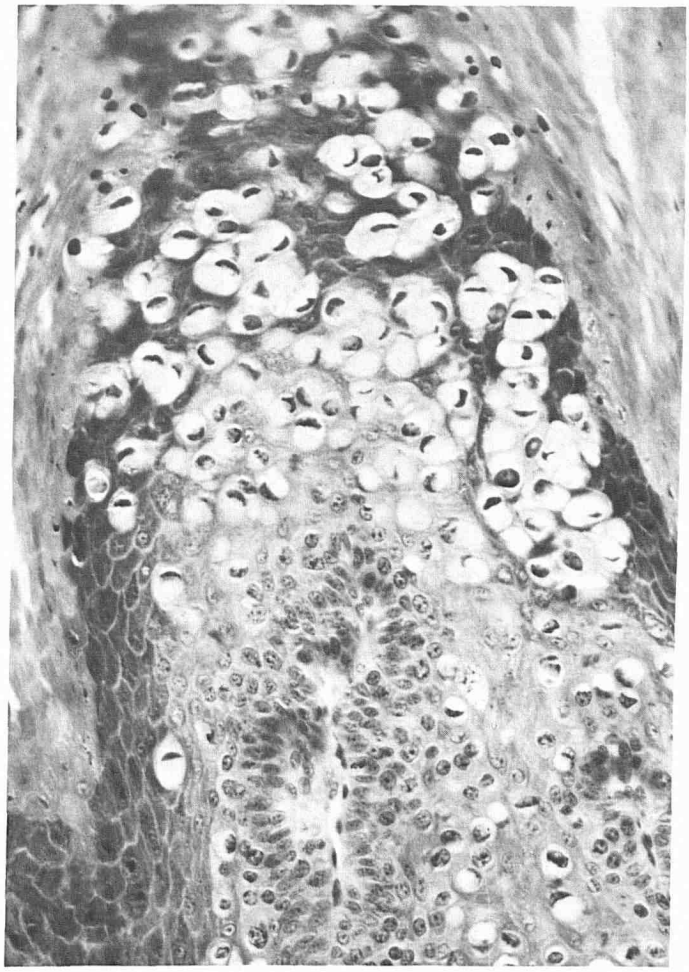

FIG 5. Section of an endophytic palmar wart of a butcher found to be infected with HPV-4 (× 260).

Our studies have also disclosed that different types of papillomaviruses produce distinct histological changes in the skin. The differences in the histological pictures of butchers' warts consist especially in various characters of the clear cells, that is the so-called vacuolated cells [35], which have been shown to represent sites of virus replication by *in situ* molecular hybridization experiments [18]. The patterns found to be associated with HPV-1, HPV-2 and HPV-3 have been previously observed in myrmecia, common warts, and flat warts, respectively [29,36,37]. This is in agreement with our previous studies which have shown a preferential association of HPV-1 with deep plantar warts, of HPV-2 with common warts, and of HPV-3 with flat warts [30], a similar correlation having also been noticed in butchers' warts.* In addition, 2 distinct histological pictures have been identified. A very characteristic pattern has been found to be associated with HPV-4. Gissmann, Pfister, and zur Hausen [9] had reported the presence of HPV-4 in "common warts," but no histological data were presented. Another characteristic histological pattern has been found in warts shown to be induced by the newly identified papillomavirus PVXa. This pattern has been observed in 21 of the 72 specimens

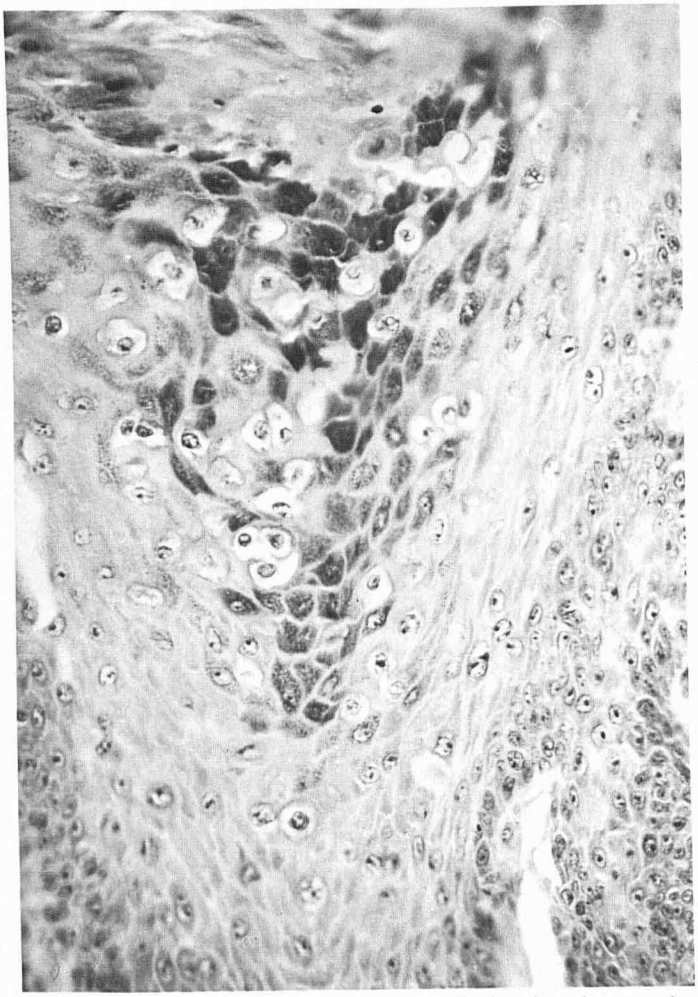

FIG 6. Section of a dorsal common wart of a butcher shown to be infected with PVXa (× 260).

studied, suggesting a high incidence of infection of butchers by this virus.

In conclusion, the multiple types of virus found in the hand warts of butchers illustrate the remarkable plurality of the human papillomaviruses recently disclosed [9–12,18,30,34]. The data reported in this paper further indicate that this plurality is reflected in the diversity of the histological features of the warts, thus providing a helpful mean to distinguish warts associated with different types of papillomaviruses.

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