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A study of HPV 1, 2 and 4 antibody prevalence in patients presenting for treatment with cutaneous warts to general practitioners in N. Ireland

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SUMMARY

Three hundred and seventy-six patients attending their general practitioner with cutaneous warts at five health centres in Northern Ireland were screened for human papilloma virus (HPV) types 1 and 2 IgM antibody using an indirect immunofluorescence test. Eighty-eight (23·4%) patients were positive for HPV type 1 IgM and 156 (41·5%) for HPV type 2 IgM. HPV 1 IgM antibody was significantly more likely to be associated with plantar warts than warts elsewhere ($P < 0{\cdot}0001$). HPV 2 IgM was present in 45 (34·1%) patients with plantar warts and 99 (45·6%) patients with warts at other sites ($P = 0{\cdot}1$). Evidence of multiple infection by HPV types 1 and 2 was demonstrated by the finding of HPV 1 and 2 IgM antibodies in the sera of 16 (4·3%). HPV 4 was found in only 1 out of 30 biopsies and HPV 4 IgM was undetectable in 50 randomly chosen sera.

INTRODUCTION

Disclosure of the plurality of human papilloma virus (HPV) types associated with cutaneous warts (Orth, Brietburd & Favre, 1977; Gissmann, Pfister & Zur Hassen, 1977) raised the problem of clinical morphology to the distinct types of HPV. At present more than 40 different types of HPV are recognized with several subtypes for many of them (Pfister, 1986). Certain HPV types are specifically associated with cutaneous warts namely HPV 1, 2, 3, 4, 7, 10, 26–29 (Abłońska & Orth, 1983). The problem of specific or preferential association of distinct HPVs with morphological types of cutaneous warts is controversial, since only a limited number of cases have been studied in biopsy series to date and no widely accepted clinical criteria are available for differentiation between wart types (Rook & Nagington, 1979; Laurent *et al.* 1982).

Most antibody prevalence surveys described in the literature have used a highly selected or poorly defined sample, along with non specific often pooled antigen as a means of antibody detection (Almedia, Goffe & Grown, 1966; Pyrhonen & Penttinen, 1972; Pass & Maizel, 1973; Matthews & Shoridaria, 1973). Various

tests have been used to detect antibody. These tests vary in sensitivity and in the type of antibody detected and this has made comparison between studies difficult.

Kienzler, Lemoine & Orth (1983) using a highly purified suspension of HPV 1 particles used immunodiffusion and microcomplement fixation methods to detect HPV type 1 antibody in 162 patients with cutaneous warts. Their study sample was poorly defined and the authors suggested that future surveys should employ type-specific antigen to detect type-specific antibody. Immunofluorescence using the indirect method is known to be a very sensitive if laborious method for detecting IgG or IgM antibody. It has been used with non-specific antigen in previous studies of antibody prevalence to papilloma virus (Matthews & Shirodaria, 1973). IgM has been shown to be the predominant antibody associated with papilloma virus infection (Almedia, Goffe & Brown, 1966; Ogilvie, 1970) IgG antibody to human papilloma virus is usually found in conjunction with IgM and rarely in isolation. The prolongation of IgM response has been observed well beyond the usual 2-month period post infection (Shirodaria & Matthews, 1975).

The prevalence of antibody to papilloma virus has not been studied in the United Kingdom and no studies involving HPV 2 and 4 have been described. Our main aims were to survey a population of patients with non-regressing warts attending for treatment, and to determine the prevalence of IgM antibody to HPV types 1, 2 and 4 in these patients by indirect immunofluorescence employing type-specific antigen. The antibodies to types 1, 2 and 4 were selected in this study because HPV types 1, 2 and 4 are most commonly associated with cutaneous warts (Jabłońka & Orth, 1983).

MATERIALS AND METHODS

Subjects. The study population consisted of patients attending five wart clinics in the area of Belfast and its environs. Practitioners in the area agreed to refer all new patients presenting with warts to these clinics for a period of 6 months. Patients were interviewed using a questionnaire. The latter was assisted with the aid of photographs as inquiry was made into previous history of cutaneous warts. A total of 826 patients with cutaneous warts were interviewed. The duration of infection ranged from less than 1 month to 2 years.

The main clinical varieties of cutaneous warts were recognized, namely plantar and non-plantar. The latter were sub-divided according to clinical appearance into common, filiform (papillomatous), periungual and plane. The classifications of intermediate wart, endophytic wart and myrmeciae were not used because of difficulty with clinical differentiation (Laurent *et al.* 1982).

Sera. Out of 826 patients interviewed, 751 (91%) patients donated blood. The sera from these patients were stored in small aliquots at −20 °C until tested.

Wart biopsies. Plantar and common warts were excised under local anaesthetic, snap-frozen in liquid nitrogen and stored at −70 °C. Common wart biopsies were taken from the hands. In the study, 14 plantar and 16 common wart biopsies were included, two patients with plantar warts provided two biopsies. All lesions of less than 1 year duration were included because it has been previously reported that warts that have been present for less than 1 year tended to have more virus

ntigen than older warts (Shirodaria & Matthews, 19775). The biopsies were ectioned at 5 μm on a Bright cryostat. Sections were allowed to dry at room emperature for 20 min, fixed in acetone at room temperature for 10 min and tored at −70 °C.

Extraction of DNA from biopsies and DNA blot hydridization

Nine unfixed frozen sections from each biopsy were covered with 50 μl lysis buffer 10 mM Tris HCl pH 7·5, 10 mM EDTA; 0·1 M NaCl 0·5 SDS) and incubated for h at 37 °C in a moist chamber. The lysates were pooled into one Eppendorf tube nd digested with proteinase K (500 μg/ml) at 37 °C for 30 min. Following RNase A treatment (50 μg/ml, 5 min, room temperature) the DNA was extracted with phenol–chloroform (1:1). Residual phenol was extracted twice with ether. The DNA was precipitated with ethanol, digested with BamHI and subjected to gel electrophoresis. Southern blots were prepared on nylon membranes (Gene Screen R) according to the recommendation of the manufacturer.

Reference DNAs for HPV 1 (the identity) of the DNA, which was cloned from a plantar wart, was demonstrated by physical mapping), HPV 1 (Fuchs & Pfister, 1984) and HPV 4 (Heilman, Law & Israel, 1980) were labelled by nick translation using radioactive phosphorous to a specific activity of about 10^8 cpm/μg DNA. The filters were preincubated with 2 × SSC, 0·1 % Denhardt solution, and 100 μg/ml yeast RNA for 3 h at 65 °C. Hydridization with probe DNA (50000 cpm/cm filter) occurred in the presence of 50 % formamide 5 × SCC, 80 mM Na-phosphate buffer (pH 6·2), 100 μg/ml yeast RNA, 0·02 % Denhardt solution and 0·1 = SDS for 2 days at 37 °C. Filters were washed as described (Pfister, Gassenmaier & Nurnberger, 1983).

Detection of wart virus antigens by immunofluorescence

After the HPV type associated with each biopsy was identified by hybridization, the acetone-fixed sections from these biopsies were stained by an indirect immunofluorescence technique using polyclonal rabbit antiserum to purified wart virus (Shirodaria & Matthews, 1975) and fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit globulin (Behringer Diagnostics). The staining procedure and the specificity of the serum was determined as described previously (Shirodaria & Matthews, 1975). The rabbit serum was found to stain HPV 1 and HPV 2 antigens but not HPV 4 antigens. This was not surprising because purified wart virus used to immunize rabbits was prepared from the pooled biopsies of plantar and common warts.

Detection of wart virus specific antibodies by immunofluorescence

For detection of antibodies to wart virus, the source of antigen was the wart biopsies. This limits the number of sera which can be analysed for type-specific antibodies. In our study most patients with cutaneous warts were from a younger population and not keen on surgical excision. This method of treatment could not ethically be offered as the treatment of choice when a less traumatic method such as liquid nitrogen and ointment are available.

Because of limitation on the availability of antigen, we decided to take a 50 % random sample which would be representative of those patients who donated a

blood sample for antibody analysis. Therefore 376 sera out of 751 sera were analysed for HPV 1 and HPV 2 IgM antibody. The sera were tested at a dilution of 1 in 10 on acetone fixed cryostat sections from HPV 1 or HPV 2 biopsies using the indirect immunofluorescence technique. The FITC-conjugated goat anti-human IgM (Behringer Diagnostics) was used at an optimal dilution of 1 in 40. The details of the staining procedure have been described previously (Shirodaria & Matthews, 1975). As only one biopsy was found to be associated with HPV 4, this further limited the number of sera which could be analysed. Therefore a random sample of 50 sera was chosen for the analysis of HPV 4 IgM antibody. The specificity of HPV 1 IgM or HPV 2 IgM staining was established by the fact that the pattern of positive staining was similar to that obtained with rabbit anti-wart virus serum and the specific staining was abolished after the absorption of positive sera with purified wart virus.

Statistics

The statistical analysis of the data was carried out using the χ^2 test. The conventional level of significance ($P < 0{\cdot}05$) was used throughout.

RESULTS

Typing of HPV types by hybridization

When DNA from the 14 plantar wart biopsies was electrophoresed and hybridized with reference DMAs, 9 (64·3%) were shown to contain HPV 1, 3 (21·4%) HPV 2 and none contained HPV 4.

When viral DNA from 16 common wart biopsies was analysed, 5 (31·25%) contained HPV 2, 3 (18·75%) HPV 1 and 1 (6·25% HPV 4 (examples are given in Figure 1).

HPV type IgM antibodies

Eight-eight (23·4%) of 376 sera tested contained HPV type 1 IgM antibody and *156 (41·5%) HPV type 2 IgM antibody. Seventy-two (19·1%) patients sera* contained type 1 IgM alone, 140 (37·2%) type 2 IgM alone, 16 (4·3%) contained types 1 and 2 IgM antibodies and 148 (39·4%) contained no detectable IgM antibody.

Table 1 shows the relationship between the type of lesion and whether the patients' sera contained HPV 1 IgM antibody. Fifty-four (40·9% patients with plantar warts, 2 (18·2%) with periungual warts, 16 (11·3%) with common warts. 1 (7·1%) with a filiform wart and none with plane warts exclusively were shown to be HPV 1 IgM antibody positive. For the purposes of statistical analysis the patients were grouped into those with plantar warts, those with non-plantar warts and those with mixed lesions. Fifty-four (40·9%) patients with plantar warts compared to 23 (10·6%) with non-plantar warts were shown to have HPV 1 IgM in their sera ($P < 0{\cdot}0001$).

Table 2 shows the relationship between HPV type 2 IgM antibody and the type of lesion. Forty-five (34·1%) patients with plantar warts, 70 (44·3%) with common warts, 9 (64·3%) with filiform warts, 5 (45·4%) patients with periungual warts and 1 (16·7%) patient with a plane wart were shown to have HPV 2 IgM in

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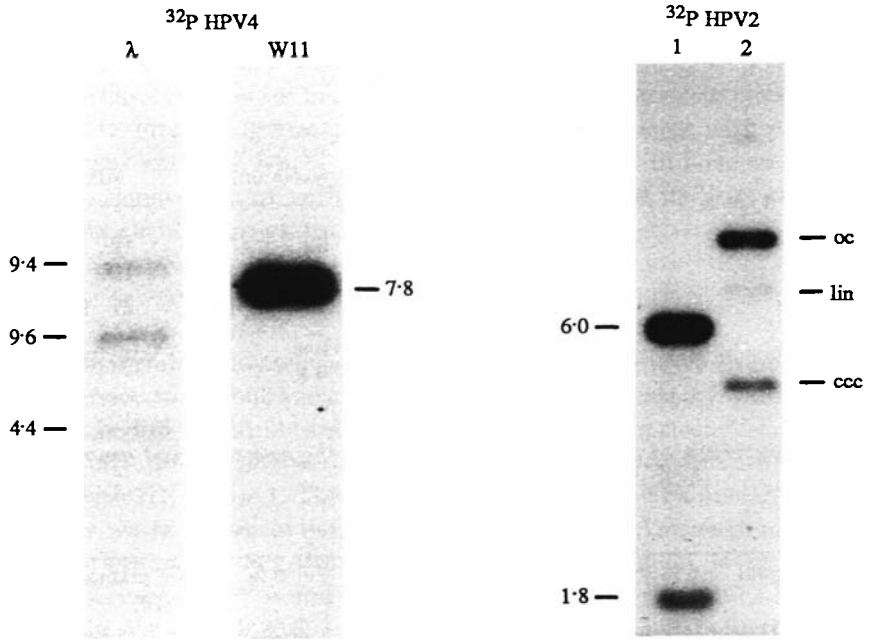

Fig. 1. Southern blot hybridization of DNA extracted from wart tissue and digested with BamHI. Phage Lambda DNA (λ) digested with HindIII was run in parallel as a molecular weight marker. Blot W11 was hybridized with ^{32}P-labelled HPV 4 DNA revealing a 7·8 kb viral band. Lanes 1 and 2 were hybridized with ^{32}P-labelled HPV 2 DNA and show HPV 2 DNAs with two BamHI cleavage sites (leading to 6·0 and 1·8 kb fragments) and no BamHI cleavage site, respectively. Bands in lane 2 represent covalently closed circles (ccc), open circles (oc) and linear form (lin) of HPV DNA unaffected by BamHI cleavage. The sizes of DNA fragments beside each lane are given in kp.

Table 1. *Comparison of the incidence of human papilloma virus type 1 IgM antibody in sera of three groups of patients with cutaneous warts*

	Type of lesion					
	Plantar warts only		Non-plantar warts only		Mixed	
HPV type 2						
Yes	54	40·9*	23	10·6*	11	4017
No	78	59·1	194	89·4	16	59·3
Total	132	100	217	100	27	100

* Significance of difference.
$\chi^2 = 46{\cdot}93$, D.F. = 2, $P < 0{\cdot}0001$.

their sera. Thus 45 (34·1%) patients with plantar warts only and 99 (45·6%) patients with non-plantar warts were associated with HPV 2 IgM antibody. The difference in these percentages is not significant.

Sixteen patients' sera contained both HPV 1 and 2 IgM antibody. Six of these patients had non-plantar warts, 6 had plantar warts and 4 had mixed lesions. Nine

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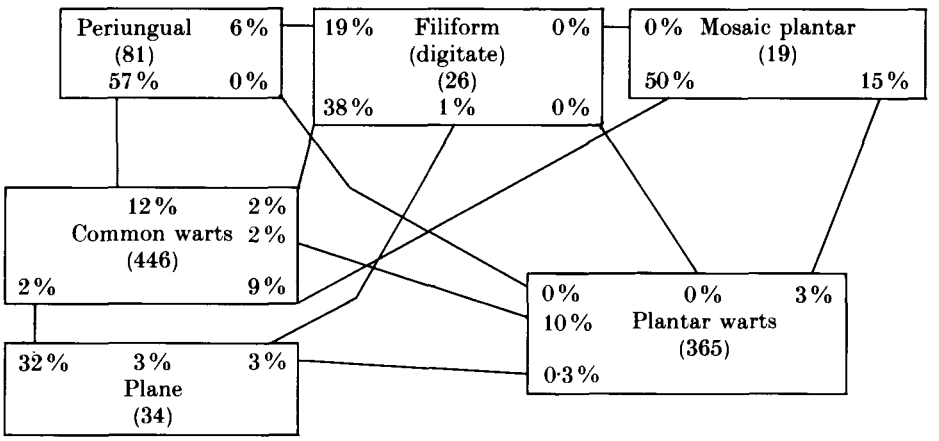
Table 2. *Comparison of the incidence of human papilloma virus type 2 IgM antibody in sera of three groups of patients with cutaneous warts*

	Type of lesion					
	Plantar warts only		Non-plantar warts only		Mixed	
HPV type 2						
Yes	45	34·1*	99	45·6*	12	44·4
No	87	65·9	188	54·4	15	55·6
Total	132	100	217	100	27	100

* Significance of difference.
$\chi^2 = 4{\cdot}6$, D.F. = 2, $P = 0{\cdot}1$.

Table 3. *Occurrence of different types of wart in the same patient amongst the 826 studied*



patients gave a past history of cutaneous wart infection ranging from less than 6 months to greater than 5 years previously and seven gave no history of previous infection. Three patients with singular lesions gave no past history of infection.

The relationship between age group and HPV 2 IgM antibody revealed that 30 (39%) of patients in the under 11 age group, 43 (38·7%) in the 11–15 age group, 39 (36·1%) in the 16–30 age group and 44 (55%) of the 31+ age group were identified as having HPV type 2 IgM antibody in their serum ($P = 0{\cdot}049$). The relationship between age group and HPV 1 IgM antibody revealed that 22 (28·5%) of patients in the under 11 age group, 31 (28·9%) of the 11–15 age group, 22 (20·4%) of the 16–30 age group and 13 (16·2%) of the 31+ age group were identified as having HPV type 1 IgM antibody in their serum. The difference in these percentages is not significant. Forty-three (46%) patients presenting with plantar warts who had HPV 1 and/or 2 antibodies in their blood compared to 18 (46%) who were negative for antibody, self-medicated with ointment prior to attendance at the clinic. The difference in these percentages is not significant. A

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similar distribution for patients presenting with non-plantar warts was not significant.

None of the 50 randomly selected sera contained HPV 4 IgM antibody.

The occurrence of different clinical types of warts in the same patient is shown in Table 3. The interesting findings were that 10% of patients with plantar warts had co-existent common warts, none had periungual or filiform and 0·3% had plane warts. Some 57% of patients with periungual and 38% of patients with filiform warts also had co-existent common warts.

DISCUSSION

In order to determine the association of HPV type with a wart lesion, a biopsy or excision is necessary, but surgical treatment is unnecessary and unpopular. Therefore it is difficult to obtain a random sample of biopsies.

In our study, nine (64·3%) biopsies from patients with plantar warts were associated with HPV type 1. This confirmed the preferential association of HPV 1 with plantar warts shown in earlier French, Polish and American studies (Orth, Faure & Brietburd, 1980; Jabłońska *et al.* 1981; Jenson *et al.* 1982). The association between HPV 4 and plantar warts shown by Heilman, Law & Israel (1980) and Jenson *et al.* (1982) could not be confirmed and adds further weight to the hypothesis that plantar warts in France, Poland and Northern Ireland may be associated with a different distribution of virus types than in Germany and the U.S. Conclusions, however, must be guarded due to the high degree of selectivity associated with all these series of biopsies. Our inability to detect HPV 4 IgM antibodies in our random sample of 50 patients would, however, support our biopsy findings.

Five (31·25%), three (18·75%) and one (6·25%) of the common wart biopsies were associated with HPV type 2, 1 and 4 respectively. We were unable to confirm the highly preferential association between HPV 2 and common warts described by Jabłońska *et al.* (1981) and Kienzler, Lemoine & Orth (1983). The weak association of HPV 4 with common warts as described by Laurent *et al.* (1982) was confirmed. We were also able to confirm the finding of Gissmann, Pfister & Zur Hausen (1977) that common and plantar warts can both be caused by HPV types 1 and 2.

The epidemiological association between HPV type and clinical variety of wart is important because of differences in clinical course, contagiousness and response to treatment (Laurent *et al.* 1982). It also provides important information to add to our understanding of the epidemiology of this infection.

From our biopsy series there was a suggestion that HPV type 1 virus was more strongly associated with plantar than non-plantar warts. We were able to confirm this in that 54 (40·9%) patients with plantar warts alone compared to 19 (11·5%) of those with periungual, filiform or common warts exclusively were shown to contain HPV 1 antibody in their sera. The association of HPV type 1 antibody with plantar warts when compared with non-plantar warts was highly significant ($P < 0·0001$). This confirmed previous findings in French patients (Kienzler, Lemoine & Orth, 1983).

The frequency of HPV type 2 antibodies has not previously been determined in

a population of patients with cutaneous warts. The highest frequencies were found in 70 (44·3%) patients with common warts alone, 5 (45·5%) with only periungual warts, 9 (60%) patients with periungual and common warts, and 9 (64·3%) patients with filiform (papillomatous) warts alone. We would postulate that the strong association between filiform warts and antibody may in part be related to the excellent blood supply of the former. This confirms previous biopsy surveys associating common warts and their clinical variants (periungual and filiform) with HPV 2 (Jabłońska *et al.* 1981; Kienzler, Lemoine & Orth, 1983). The high percentage of patients with plantar warts exclusively who had HPV 2 antibodies in their sera was surprising (45; 34·1%). Unlike the studies cited above we could not show a preferential association between HPV type 2 IgM antibody and non-plantar warts ($P < 0{\cdot}1$), when compared to patients with plantar warts. In our survey a larger proportion of plantar warts would appear to be related to HPV type 2 compared with biopsy surveys described elsewhere. This could be due either to differences in the epidemiology of papilloma infection in the areas studied, factors inherent in biopsy selection, or inclusion of a large number of patients with endophytic plantar warts in our sample. These lesions are known to be associated with HPV 2 (Jabłońska *et al.* 1981).

The associations between various clinical types of wart occurring in the 826 patients in our survey, is in some way explained by these findings. Some 57% of patients with periungual and 38% with filiform also had common warts in keeping with their common HPV type 2 aetiology. Patients with plantar warts were unlikely to present with co-existing non-plantar warts (Table 3). The preferential association of plantar warts with HPV 1 would explain these findings.

Some 71·4% of patients who had plantar warts previously compared to 53·4% of patients with a previous history of non-plantar warts, had types 1 and/or 2 antibody ($P < 0{\cdot}004$) in their sera, and this finding may be related to the higher viral and hence antigenic content of the former (Shirodaria & Matthews, 1975).

Sixteen patients (4·2%) possessed HPV 1 and 2 IgM antibody supporting infection with both HPV types 1 and 2. Whether these patients were infected with two different strains of virus concurrently or at different points in time with a resulting persistent antibody response is unclear. Evidence of multiple infection by several HPV types has previously been described by Jabłońska & Orth (1983) who took multiple wart biopsies from butchers. The natural immune response to warts is type specific and this may help to explain why in some patients the majority of lesions will naturally resolve leaving only a few warts. The latter may be related to a different type of virus. Three patients who presented with singular lesions and gave no previous history of infection were shown to possess types 1 and 2 antibody. Either these patients had concurrent lesions of which they were unaware or didn't notice a previous infection. Furthermore from our results it is difficult to ascertain whether there are cross reacting antibodies against a common polypeptide shared by HPV 1 and 2. Further studies using techniques such as immunoblotting will reveal whether such cross reactions exist.

This association between a negative history of infection and being antibody positive has been described by Cubie (1972) in University students. De Peuter, De Ciereg & Minelle (1977) in their survey of meat workers showed the unreliability of clinical history in that a large proportion of workers denied they had warts and were subsequently found to be infected on examination.

Older patients were more likely to have HPV type 2 IgM antibody in their sera = 0·049). This might be explained by the increased but not significant sociation of HPV 2 with non-plantar warts and the association of the latter with ler age groups. No such association was found between HPV 1 antibody and age = 0·15). The French results for prevalence of HPV 1 antibody distributed by e agree with the findings shown in this study. This and other similarities to dings in the French population would lead us to hypothesize that the idemiological characteristics of HPV 1 infection are similar in the two pulations (Kienzler, Lemoine & Orth, 1983).

Patients who had used self medication prior to presenting for treatment were no ore likely to possess antibodies in their sera than patients who did not. This plied to both patients presenting with plantar warts exclusively ($P = 0·99$) and atients presenting with non-plantar warts ($P = 0·81$). These results do not pport the theory that ointment provokes an immune response (McConaghy, 976).

We could postulate from the results of our biopsy series and antibody survey at HPV type 4 was a rare cause of cutaneous warts. This contrasts with the ndings of Gissman, Pfister & Zur Hausen (1977) who showed that HPV 4 was esponsible for approximately 13% of warts analysed in their biopsy series. These esults may reflect a true epidemiological difference between the association of IPV 4 and cutaneous warts in patients in Northern Ireland and West Germany r may reflect selectivity in the biopsy series in the study in Germany.

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