Int. J. Cancer: **21**, 161-165 (1978)

SEROEPIDEMIOLOGICAL STUDIES OF HUMAN PAPILLOMA VIRUS (HPV-1) INFECTIONS

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At least two groups of human papilloma viruses can be distinguished serologically: on the one hand HPV 1-3, which are closely related but differ in the restriction enzyme pattern of their DNA, and on the other hand HPV-4. The age distributions of patients with warts induced by HPV 1-3 or by HPV-4, respectively, differ markedly. HPV 1-3 predominates between 5 and 15 years of age, whereas HPV-4 could be isolated more often between the ages of 20-25 years. The large number of HPV-1-3-induced warts in children is paralleled by a high percentage of HPV-1 antibody-positive sera in the same age group (about 50%). With increasing age the percentage of HPV-1 antibody-positive sera declines gradually. This pattern of seroreactivity was compared to that of patients with various papillomas and with several malignant tumors. There is no evidence to suggest a link between HPV 1-3 and *condylomata acuminata*, laryngeal papillomas or any of the malignant tumors tested.

Human papilloma viruses are the agents responsible for a variety of warts (reviewed by zur Hausen, 1977). Much attention has been paid to such proliferations to analyze the role of immune surveillance in tumor development, since spontaneous regression of multiple papillomas has frequently been observed simultaneously (Rowson and Mahy, 1967). The relative importance of humoral antibodies and cell-mediated immunity in wart regression is not yet clear. IgM antibodies against structural antigens of human wart viruses were demonstrated by immune electron microscopy in 38% of a series of wart patients (Goffe *et al.*, 1966) and by immunofluorescence in all patients with regressing warts (Matthews and Shirodaria, 1973; Shirodaria and Matthews, 1975). A correlation was shown to exist between wart regression and the presence of complement-fixing antibodies of the IgG class, which appeared in 12% of a set of wart patients (Pyrhönen and Penttinen, 1972; Pyrhönen and Johannson, 1975).

Recently, the heterogeneity of human wart viruses was established by analysis of viral DNA with restriction enzymes and by comparing the protein patterns of individual isolates after SDS-gel electrophoresis (Gissmann *et al.*, 1977). Virus isolates designated HPV-1 and HPV-4, both frequently found in common warts *(verrucae vulgares)*, lacked significant cross-reactivity in complement-fixation assays. In view of these results, a re-evaluation of the serological data seems to be important.

This manuscript describes the development of a solid-phase radioimmunoassay for the detection of HPV-1-specific antibodies. The antibody distribution in a non-selected population is compared to the frequency of HPV-1-induced warts. In addition, the presence of specific antibodies in patients with warts and with various malignant tumors is investigated.

MATERIAL AND METHODS

Material

Verrucae vulgares and plantar wart biopsies were kindly provided by Drs. G. Schmid, W. Meinhof and W. Steiner, Erlangen; T. Nasemann, Frankfurt; B. R. Balda, Munich; G. Weber, Nuremberg; and Ph. Baumeister, Fürth. Wart virus antigen was prepared and purified from these biopsies as described previously (Gissmann and zur Hausen, 1976). Sera from healthy individuals and from patients with infectious diseases other than warts analyzed in a routine diagnostic laboratory, were used as a control population. Sera from wart patients and from tumor patients were taken from the serum collection of this Institute.

Preparation of antisera

Antisera against HPV 1 and HPV 4 were obtained after immunization of two rabbits, respectively, with three injections each of 50 μg viral protein (two injections IM with complete Freund's adjuvant and one injection IV) (Gissmann *et al.*, 1977). The viral antigens were prepared from individual warts, characterized by their protein pattern after SDS-gel electrophoresis, and pooled according to the result of this protein analysis.

Complement fixation

This test was performed as described previously (Gissmann *et al.*, 1977), using 2 units of complement and an antigen solution with 5.4 μg protein per ml.

Iodination of antibodies

The globulin fraction of the rabbit anti-HPV-1 serum was enriched by three precipitations with 16% Na_2SO_4. Antibodies were labelled with ^{125}I isotope of sodium iodine by the chloramine T method (Greenwood *et al.*, 1963). To remove free iodine the sera were dialyzed against phosphate-buffered saline (PBS) for 15 h at 4° C. The specific activity of the globulin fraction was determined to be 2×10^8 cpm/10 μg protein. The preparations were diluted to 5×10^6 cpm/ml in PBS, containing 10% of an anti-HPV-1-negative human serum, and stored at −20° C until use.

Received: November 4, 1977.

Radioimmunoassay

The wells of flexible polyvinyl microtiter plates (Linbro) were filled with anti-HPV-1 rabbit antiserum at a dilution of 1:640 in PBS. The antibodies were allowed to adsorb to the plastic by incubation for 50 h at 4° C. Thereafter the fluid was removed and the plates were washed three times with 0.1 M phosphate buffer, pH 7.8. One hundred μl of HPV-1 antigen were added and incubated overnight at 4° C. For antigen determination, labelled HPV-1 rabbit antiserum was filled into each well after the antigen had been removed and the plate washed. A 90-min incubation at 37° C sufficed for the reaction. After washing, the individual wells were cut from the plate and counted in a gamma counter. In order to determine the HPV-1 antibody content of human sera, the radioimmunoassay was used as a blocking test. Sera of patients were added to plates and coated with antigen by incubation with 150 μg per well. The sera were allowed to react for 90 min at 37° C. Afterwards, labelled rabbit antiserum was added as described for antigen determination. A serum dilution was regarded as positive when two parallel test results were lower than the mean value of 20 unblocked controls minus the 1.5-fold standard deviation of usually 15%.

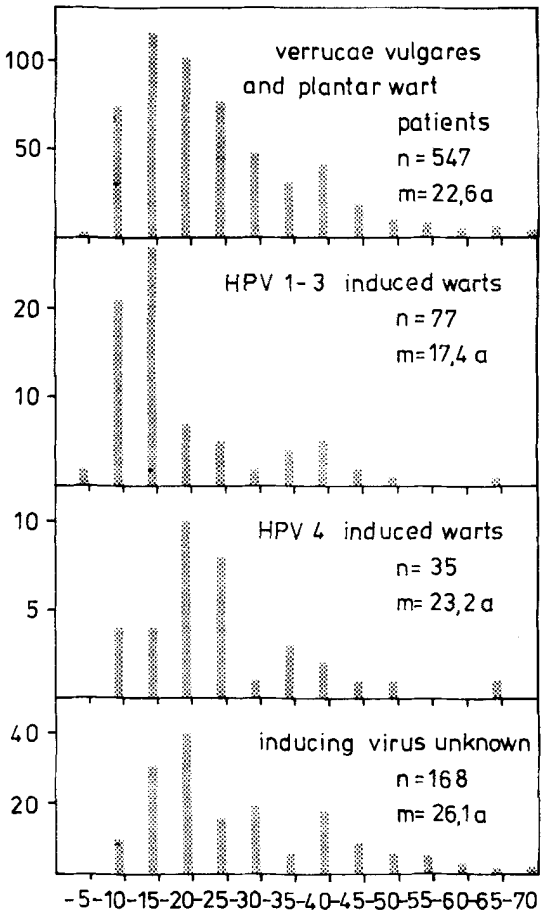

FIGURE 1 — Overall age distribution of patients with *verrucae vulgares* and plantar warts at the time of surgical removal (first upper panel) and age distribution of patients with warts, induced by HPV 1-3, HPV-4 or uncharacterized papilloma viruses.

TABLE I

DIFFERENT PAPILLOMA VIRUS ISOLATES FROM WARTS OF PLANTA PEDIS AND VERRUCAE VULGARES

	Warts of planta pedis		Verrucae vulgares	
	Number	%	Number	%
HPV-1	50	36	27	16
HPV-4	18	13	21	14
Non 1-4 HPV	6	4	17	11
No virus isolated	66	47	89	58
Total	140		154	

RESULTS

Age distribution of wart patients

It is well established that warts most frequently occur in the 5-20 year age-group (Bosse and Christophers, 1964; Cubie, 1972). The upper panel of Figure 1 shows the age distribution of patients with *verrucae vulgares* and plantar warts at the time of surgical removal. In order to determine whether different human papilloma viruses prevail in different age-groups, virus isolates from biopsies of 300 patients were characterized by their reactivity with rabbit anti-HPV-1 and anti-HPV-4 antisera in a complement-fixation assay. Ninety of these warts contained HPV-1-3, which could not be further differentiated serologically. Forty-two virus preparations were shown to be HPV-4. In 23 cases, the electron microscope revealed papilloma virus particles, which reacted neither with anti-HPV-1 nor with anti-HPV-4 antiserum. However, the particle yield was too low to permit a positive characterization. In 161 cases, no viruses were detected with our techniques. HPV-1 and HPV-4 were frequent both in plantar warts and in *verrucae vulgares* although HPV 1 was more often found in plantar warts ((Table I).

The age distribution of patients with warts induced either by HPV 1-3, by HPV-4, or by unidentified viruses, differed markedly (Fig. 1). HPV 1-3 was predominantly isolated from patients between 5 and 15 years of age. The percentage of HPV-1-3-induced warts drops drastically after the age of 15. HPV-4-induced warts were found more often at the age of 20-25 years. Both HPV 1-3 and HPV-4 were only rarely isolated from patients between 25 and 30 years. All profiles show a small second peak at the age of 40 as does the overall distribution in the upper panel.

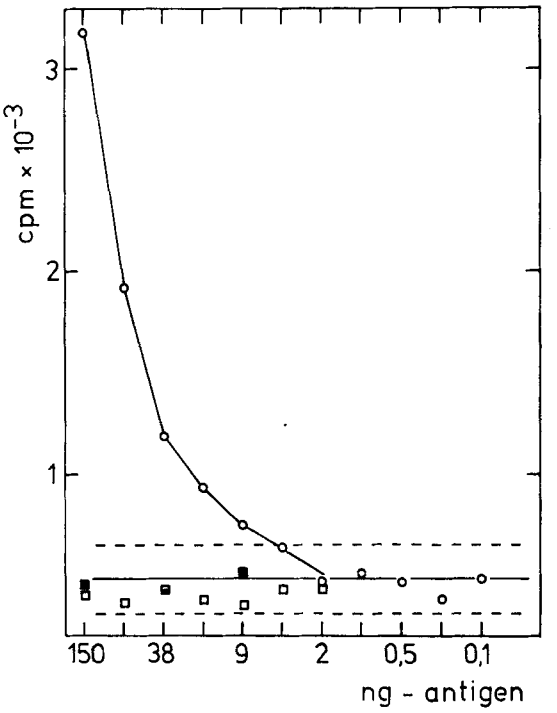

FIGURE 2 — Direct radioimmunoassay of a serial dilution of purified HPV 1 (○——○). The protein concentration was estimated from the optical density at 260 nm and 280 nm (Kalckar, 1947). Purified HPV-4 (□——□) and bovine papilloma virus (■——■) were used as controls.

Antibody response against HPV-1

The prevalence of HPV-1-induced warts in children should correlate with a significant percentage of HPV-1 antibody-positive persons in the same age-group. This assumption was confirmed by testing different age groups for humoral antibodies, using a solid-phase radioimmunoassay.

Also in this test, no cross-reactivity was observed between HPV-1 and HPV-4 (Fig. 2, 3). As an additional control, bovine papilloma virus antigens also failed to cross-react with HPV-1 (Fig. 2). The test detected approximately 10 ng of protein from purified HPV-1 particles. HPV-1 antibodies in human sera were detected by their ability to block the binding of radioactively labelled rabbit antiserum. Figure 3 gives an example of serum titration by means of this blocking assay.

The percentage of anti-HPV 1-3-positive sera in a non-selected population reached a maximum of about 50% between 11 and 20 years (Fig. 4). Thereafter it gradually declined and only about 25% of the sera were positive between 40 and 60 years. Usually, dilution steps 1/10 and 1/100 were tested. Sixty per cent of the positive sera had a titer of 1/10. Sera which were positive at both dilutions were titrated. Twenty-one sera had a titer of 1/100 and five proved to be positive up to 1/400. Sera from patients with warts gave similar values for the age-groups tested until the age of 30 (Table II). At higher ages, a significantly ($p<0.05$) higher percentage of sera from wart patients was positive when compared to the non-selected group of the same age. In three cases we obtained sera from children whose warts were characterized earlier as being induced by HPV-1. Two patients had a titer of 1/100 against HPV-1. The third child whose wart had persisted for several months was negative.

Patients with genital warts *(condylomata acuminata)* have an average age of 26 years (zur Hausen *et al.*, 1975): 46% of their sera (N = 37) contained antibodies against HPV-1, which agrees very well with the data obtained from the non-selected population. Patients with juvenile laryngeal papillomas did not differ significantly from the control population: only 23% of the 22 patients, with a mean age of 8.8 years, had antibodies against HPV-1.

If HPV-1 or a serologically cross-reacting virus plays any role in the induction of specific malignant tumors one would expect a higher percentage of positive sera among such patients. A first analysis revealed that a relatively high number of sera from patients with genital cancer contained antibodies against HPV-1. In contrast, by using sera from patients with other carcinomas, lymphomas, sarcomas and melanomas (Table III), no significant difference to the control populations was observed.

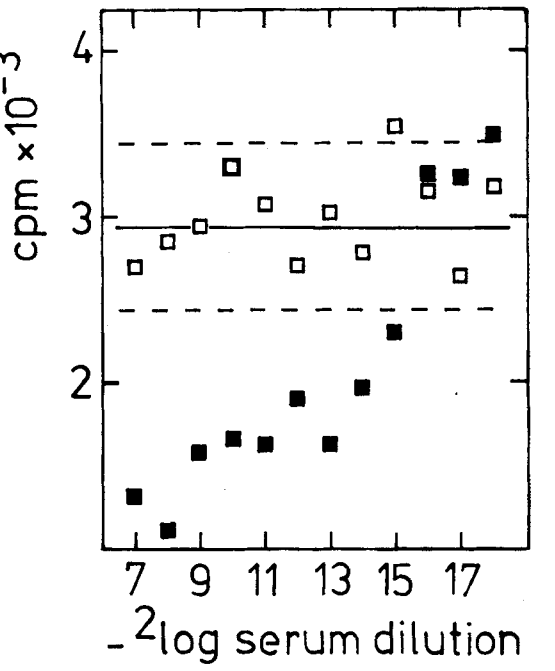

FIGURE 3 — Titration of an anti-HPV-1 rabbit antiserum (■) by means of a blocking assay. An anti-HPV-4 rabbit antiserum was used as a control (□). The horizontal straight line represents the mean value of the unblocked label binding. The dotted lines indicate the 1.5-fold standard deviation.

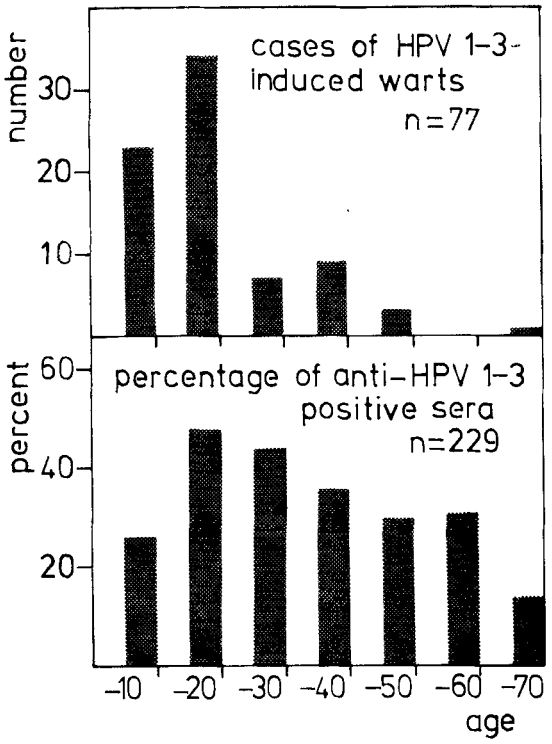

FIGURE 4 — Comparison of the age distribution of patients with HPV-1-3-induced warts to the age distribution of people with anti HPV-1-3-positive sera in a non-selected population.

TABLE II

PERCENTAGE OF ANTI-HPV-1-POSITIVE SERA FROM WART PATIENTS (*VERRUCAE VULGARES* AND PLANTAR WARTS) OF DIFFERENT AGE-GROUPS

	Age-groups					
	0-10	11-20	21-30	31-40	41-50	51-70
No. of patients	14	40	30	11	9	6
No. of positive sera	4	18	15	5	5	5
Percentage of positive sera	28	45	50	45	55	83

DISCUSSION

Previous data on humoral antibody response to human papilloma virus (Almeida and Goffe, 1965; Pyrhönen and Penttinen, 1972; Pass and Maizel, 1973; Matthews and Shirodaria, 1973) were commonly obtained by using pools of purified virus as antigens. Recent results clearly indicate that under these conditions antigenetically distinct human papilloma viruses (HPV-1, HPV-4 and possibly others) are present in such mixtures (Gissmann *et al.*, 1977).

This manuscript reports on the evaluation of type-specific antibodies directed against HPV 1 antigens. The antibody response is correlated to the age incidence of HPV-1 warts. Since the antigens were prepared exclusively from clinical material for the radioimmunoassay, the available concentrations were rather limited and prevented a large-scale titration of every individual serum. Thus, screening was only performed at two serum dilutions.

The data show that, corresponding to the prevalence of HPV-1 warts during childhood and adolescence, a parallel increase in the percentage of antibody-positive sera is noted. This percentage then declines gradually with increasing age, levelling off after approximately 40 years of age. The age distribution curve of HPV-1 warts drops in the age-group 20-30, revealing a small second peak at higher ages. In spite of this striking parallel between a high percentage of antibody-positive sera and the drop in the number of HPV-1 warts, these data do not permit any conclusions as to the protective role of antibodies. One individual serum from an HPV-1 wart patient was found to be devoid of detectable HPV-1 seroreactivity. This fact and the usually low titers of seroreactive patients frequently escaping their demonstration by conventional complement fixation tests (Pyrhönen and Pentinnen, 1972) seem to point to a very limited antigenic stimulation of HPV-1 wart carriers.

No seroepidemiological evidence has been obtained linking HPV-1 to *condylomata acuminata*, laryngeal papillomas or certain malignant human tumors. Although sera from patients with genital cancer reacted at a significantly higher percentage with HPV-1 antigens, a substantial percentage of such sera (47%) lacked any measurable response.

The low seroreactivity of patients with Hodgkin's

TABLE III

Sera from patients with	Number tested	Percentage of anti-HPV-1-positive sera	Mean age	Age range
Genital cancer (cervical and penile)	47	53	44.7	30-70
Carcinoma of the larynx	66	39	57.9	33-80
Carcinoma of the nasopharynx	13	38	46.4	15-70
Other carcinomas	10	40	22.8	15-45
Morbus Hodgkin	19	26	18.5	3-29
Acute lymphatic leukemia	8	12	10.1	1-16
Sarcoma	9	33	23.4	3-74
Melanoma	16	31	— [1]	— [1]

[1] Data not known.

disease is of some interest. It contrasts markedly with the significantly elevated antibody titers of these patients against persisting herpes virus antigens (Levine *et al.*, 1970; Hesse *et al.*, 1973; Johansson *et al.*, 1975).

The establishment of type-specific serological tests against various human papilloma viruses may provide an additional parameter to assess their role in oncogenesis.

ACKNOWLEDGEMENTS

We gratefully acknowledge helpful suggestions by Drs. R. Thomsen and W. Gerlich, Göttingen, concerning the radioimmunoassay. The skilful technical assistance of Miss Irene Bauer and Miss Ingeborg Hettich is also greatly appreciated. These studies were supported by the Deutsche Forschungsgemeinschaft (Ha 449/12).

ÉTUDES SÉROÉPIDÉMIOLOGIQUES DES INFECTIONS A VIRUS DU PAPILLOME HUMAIN (HPV-1)

On peut distinguer sérologiquement deux groupes au moins de virus du papillome humain: les HPV 1-3, qui sont étroitement apparentés, mais qui diffèrent dans les enzymes de restriction de leur ADN, et le HPV-4. Les distributions par âge des malades présentant des verrues induites par les HPV 1-3 ou par le HPV-4, respectivement, diffèrent nettement. Les HPV 1-3 prédominent entre cinq et quinze ans, alors que le HPV-4 a pu être isolé plus souvent entre 20 et 25 ans. Le grand nombre de verrues induites par les HPV 1-3 chez les enfants s'accompagne, dans le même groupe d'âge, d'un pourcentage élevé de sérums contenant l'anticorps contre le HPV-1 (environ 50%). A mesure que l'âge augmente, le pourcentage de sérums positifs diminue progressivement. Cette courbe de séroréactivité a été comparée à celle de malades présentant divers papillomes et certaines tumeurs malignes. Rien ne prouve qu'il y ait un lien entre les HPV 1-3 et les condylomes acuminés, les papillomes du larynx ou l'une quelconque des tumeurs malignes soumises à expérience.

REFERENCES

ALMEIDA, J. D., and GOFFE, A. P., Antibody to wart virus in human sera demonstrated by electron microscopy and precipitin tests. *Lancet*, **2**, 1205-1207 (1965).

BOSSE, K., and CHRISTOPHERS, B., Beitrag zur Epidemiologie der Warzen. *Hautarzt*, **15**, 80-86 (1964).

CUBIE, H. A., Serological studies in a student population prone to infection with human papilloma virus. *J. Hyg. Camb.*, **70**, 677-690 (1972).

GISSMANN, L., and ZUR HAUSEN, H., Human papilloma virus DNA: physical mapping and genetic heterogeneity. *Proc. nat. Acad. Sci. (Wash.)*, **73**, 1310-1313 (1976).

GISSMANN, L., PFISTER, H., and ZUR HAUSEN, H., Human papilloma viruses (HPV): characterization of four different isolates. *Virology*, **76**, 569-580 (1977).

GOFFE, A. P., ALMEIDA, J. D., and BROWN, F., Further information on the antibody response to wart virus. *Lancet*, *ii*, 607-608 (1966).

GREENWOOD, F. C., HUNTER, W. M., and GLOVER, J. S., The preparation of ^{131}J-labelled human growth hormone of high specific radioactivity. *Biochem. J.*, **89**, 114-123 (1963).

HESSE, J., ANDERSEN, E., LEVINE, P. H., EBBESEN, P., HALBORG, P., and REISHER, J. I., Antibodies of Epstein-Barr virus and cellular immunity in Hodgkin's disease and chronic lymphatic leukemia. *Int. J. Cancer*, **11**, 237-243 (1973).

JOHANSSON, B., KILLANDER, D., HOLM, G., MELLSTEDT, H., HENLE, G., HENLE, W., KLEIN, G., and SÖDERBERG, J., Epstein-Barr virus (EBV)-associated antibody patterns in relation to the deficiency of cell-mediated immunity in patients with Hodgkin's disease. *Onc. Herpes viruses*, **11**, 237-247 (1975).

KALCKAR, H. M., Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. *J. biol. Chem.*, **167**, 401 (1947).

LEVINE, P. H., ABLASHI, D. V., BERARD, C. W., CARBONE, P. P., WAGGONER, D. E., and MALEM, L., Elevated antibody titers to Epstein-Barr virus in Hodgkin's disease. *Cancer*, **27**, 416-421 (1970).

MATTHEWS, R. S., and SHIRODARIA, P. V., Study of regressing warts by immunofluorescence. *Lancet*, **1**, 689-691 (1973).

PASS, F., and MAIZEL, J. V., Wart-associated antigens. II. Human immunity to viral structural proteins. *J. Invest. Dermat.*, **60**, 307-311 (1973).

PYRHÖNEN, S., and PENTTINEN, K., Wart-virus antibodies and the prognosis of wart disease. *Lancet*, **2**, 1330-1332 (1972).

PYRHÖNEN, S., and JOHANSSON, E., Regression of warts. An immunological study. *Lancet*, **1**, 592-595 (1975).

ROWSON, K. E. K., and MAHY, B. W. J., Human papova (wart) virus. *Bact. Rev.*, **31**, 110-131 (1967).

SHIRODARIA, P. V., and MATTHEWS, R. S., An immunofluorescence study of warts. *Clin. exp. Immunol.*, **21**, 329-338, (1975).

ZUR HAUSEN, H., Human papilloma viruses and their possible role in squamous cell carcinomas. *Curr. Top. Microbiol. Immun.*, **78**, 1-30 (1977).

ZUR HAUSEN, H., GISSMANN, L., STEINER, W., DIPPOLD, W., and DREGER, I., Human papilloma viruses and cancer. *Bibl. Haemat.*, **43**, 569-571 (1975).