THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 65:324–330, 1975

Vol. 65, No. 3
Printed in U.S.A.

THE VIRUS OF EPIDERMODYSPLASIA VERRUCIFORMIS: ELECTRON MICROSCOPIC AND FLUORESCENT ANTIBODY STUDIES

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Five cases of epidermodysplasia verruciformis were studied for viral particles and antigens. In all benign lesions tested, viral particles and antigens were observed by electron microscopy of ultrathin sections and/or tissue extracts and by fluorescent antibody staining with an antiserum against human wart virus. Both viral particles and antigens were observed in the cells of the stratum granulosum and the stratum corneum and not in those of deeper layers. Viral particles and antigens were observed in nuclei. Viral particles resembled morphologically the virus of common human warts. In two, one on the forehead and the other on the inner aspect of the upper thigh, of six lesions showing the histology of early malignancy, viral particles were observed by electron microscopy of ultrathin sections and/or tissue extracts. Four advanced malignant lesions, two primary ulcerated squamous cell carcinomas and two recurrent carcinomas, were similarly studied. In none of them, were viral particles or antigens detected. These results suggest that (1) the virus of epidermodysplasia verruciformis is related with that of common human warts both morphologically and antigenically, (2) at least some of the virus-induced lesions of epidermodysplasia verruciformis become malignant, and (3) when the lesions are completely replaced with malignant cells, neither viral particles nor antigens are recognizable in them.

Reports on the successful auto-inoculation and hetero-inoculation of epidermodysplasia verruciformis (e.v.) [1–4] and on the electron microscopic observation of papova viral particles in its skin lesions [5–15] have suggested that e.v. is an extensive eruption of verrucae in genetically predisposed patients. One of the most remarkable characteristics of patients with e.v. is, however, the frequent development of malignant lesions at relatively young ages, a phenomenon not observed in patients with common warts [16]. It appears, therefore, that the identity of the virus of e.v. with that of common warts is still debatable. In malignant lesions of e.v., moreover, virus particles are rarely if ever observed [6,10,11,13–15,17–20]. Hence, as emphasized by Ruiter [21], there still remains the question whether the virus-induced lesions of e.v. actually become malignant. If they do, the role of virus in the malignant transformation must be studied.

We have already reported briefly the electron microscopic studies of 4 cases of e.v. [10,15]. Recently we found an additional case of e.v. and performed similar electron microscopic studies. In addition, with the specimens of this and previous patients and with an antiserum against the virus of common warts, we carried out fluorescent antibody studies. In this paper, the results of these electron microscopic and fluorescent antibody studies will be reported and their significances discussed in relation to the above-described problems.

Manuscript received January 16, 1975; in revised form April 4, 1975; accepted for publication April 8, 1975.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan.

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MATERIALS AND METHODS

Patients Examined

Five patients of e.v. were studied. Case 1: Y. Y., 38-year-old female, had typical skin lesions on the whole body (Fig. 1) and a skin lesion with the histology of early malignancy on the left forehead. Case 2: Y. K., 21-year-old female, had no grossly recognizable malignant lesions. Case 3: K. K., 40-year-old male, had a lesion showing the histology of early malignancy on the inner aspect of the right upper thigh (Fig. 2). Besides the skin lesions of e.v., this patient had a rhabdomyosarcoma on the right chest wall which was reported previously [22,23]. Case 4: M. Y., 39-year-old male, had an ulcerated invasive squamous cell carcinoma on the right forehead (Fig. 3). A carcinoma recurred at the site of operation 11 months after the primary excision. Case 5: E.I., 64-year-old male, had an ulcerated invasive squamous cell carcinoma on the left forehead. A carcinoma recurred at the site of operation 10 months after the primary excision. Cases 2 to 5 have been briefly reported previously [10,15].

None of these patients was related to another. Case 2 was the only patient who was born to consanguineous parents (cousins).

Specimens

Specimens were obtained at the time of biopsy or operation, and were cut into three pieces: one was used for histologic sections, one for ultrathin sections, and one was preserved at −70°C.

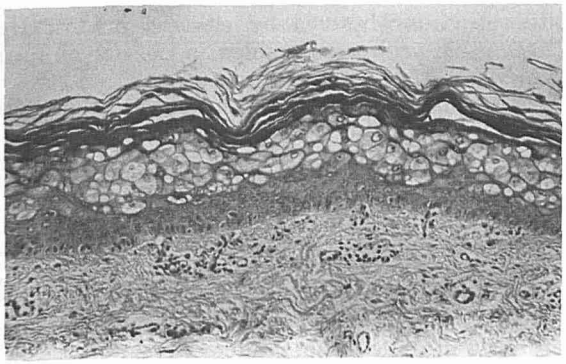

FIG. 1. Histology of a typical benign lesion of epidermodysplasia verruciformis (case 1, hand) (× 110).

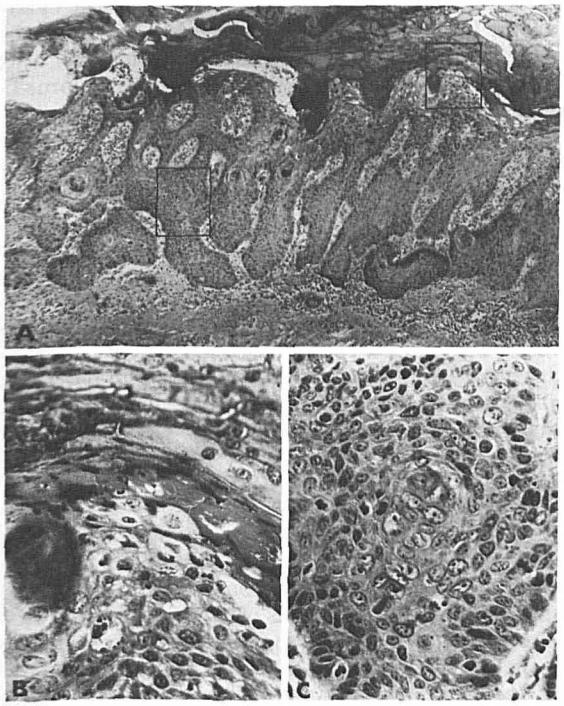

FIG. 2. Histology of a lesion showing the early stage of malignancy (case 3, inner thigh). *A:* (× 42). *B:* Large vacuolated cells in superficial layers (× 215). *C:* Multiple mitotic figures in deeper layers (× 215).

Electron Microscopy

The ultrathin sections were prepared following fixation in 6% glutaraldehyde, postfixed in 1% OsO_4, dehydrated, embedded in Epon 812, sectioned, and stained with uranyl acetate and lead citrate. The tissue extracts for electron microscopy were prepared as follows: frozen tissues were thawed, ground in a chilled mortar, suspended in distilled water, centrifuged at 1,000 rpm for 10 min and at 3,150 × *g* for 15 min, the supernatant was centrifuged at 66,492 × *g* for 120 min or at 134,220 × *g* for 60 min, and the pellet was suspended in a drop of distilled water, mounted on grids, and negatively stained with 1% potassium phosphotungstate. The electron microscopic observation of tissue extracts and the photographic enlargement of electron micrographs were done as described by Klug and Finch [24].

Antiserum to Wart Virus

The upper-half layers were removed from 40 human warts (verrucae vulgares), ground in a chilled mortar, suspended in phosphate-buffered saline, centrifuged at 1,000 rpm for 10 min and twice at 3,150 × *g* for 15 min, and the supernatant was centrifuged at 134,220 × *g* for 60 min. The pellet thus obtained was suspended in 0.5 ml of phosphate-buffered saline, overlaid on a 29–61% linear sucrose density gradient, and centrifuged at 68,480 × *g* for 90 min. The main band containing whole virions was collected, suspended in phosphate-buffered saline, centrifuged at 134,220 × *g* for 60 min, and the virus obtained was further purified by repeating the same sucrose density gradient centrifugation. The final viral pellet was suspended in 0.2 ml of phosphate-buffered saline, and the purity and the high concentration of virions were confirmed by electron microscopy of the negatively stained preparations (Fig. 4*A*). The purified virus thus obtained was suspended in 0.6 ml of phosphate-buffered saline, containing penicillin and streptomycin, and preserved at −70°C. Before injection, 0.2 ml of virus suspension was mixed with 0.2 ml of Freund's complete adjuvant, and injected intramuscularly into a guinea pig. After 3 injections at 1- and 2-week intervals, the serum was taken and absorbed with the homogenate of normal human skin. This serum was designated as the "purified-virus-antiserum."

A partially purified virus suspension was prepared with 30 warts by the fractional centrifugation of tissue extract at 3,150 × *g* and 134,220 × *g*, and was similarly

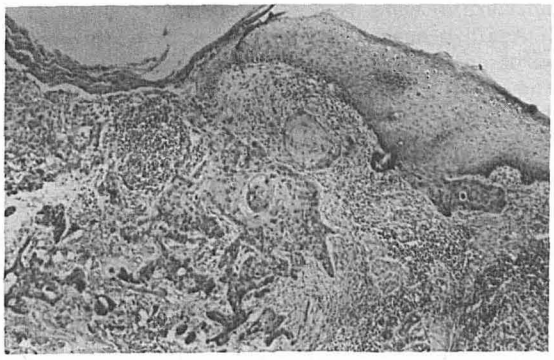

FIG. 3. Histology of a lesion showing ulcerated squamous cell carcinoma to the left and early malignant downward growth to the right (case 4, forehead) (× 42).

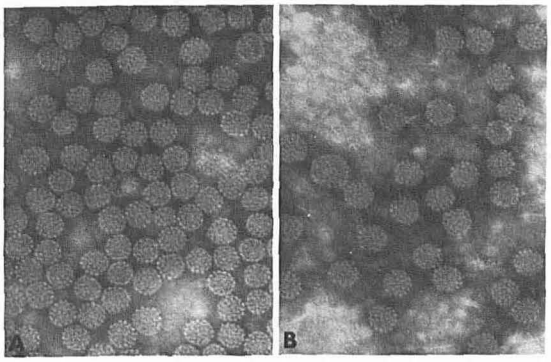

FIG. 4. Negatively stained human wart virus (× 53,000). *A:* Purified virus used for immunization of guinea pigs. *B:* Partially purified virus used as the antigen for the precipitation reaction.

injected into 2 guinea pigs. The serum obtained from the guinea pigs of this group was designated as the "crude-virus-antiserum."

Fluorescent Antibody Staining

Frozen sections were fixed in acetone and stained by the indirect method, using the guinea-pig antiserum to wart virus and fluorescein-labeled rabbit antiserum to guinea-pig gamma globulin.

RESULTS

Specificity of Antiserum Used for Fluorescent Antibody Staining

The specificity of antiserum against wart virus was tested by precipitation reaction in 0.6% agarose gel plates using a method similar to that of Almeida and Goffe [25], and by fluorescent antibody staining of frozen sections of common human warts (verrucae vulgares). The partially purified preparation of human wart virus was used as the antigen for the precipitation reaction. As shown in Figure 4*B*, this preparation had a large number of wart viral particles and some tissue debris. An extract of normal human epidermis was prepared by the same method and used as control antigen. The guinea-pig antiserum against the purified wart virus (purified-virus-antiserum) and that against the partially purified wart virus (crude-virus-antiserum) were used as antisera.

Against normal epidermal extract, the crude-virus-antiserum produced a precipitation line in the middle or a little closer to the antiserum well, but the purified-virus-antiserum, the undiluted one, produced no precipitation line (Fig. 5*A*). Against the partially purified wart virus preparation, the purified-virus-antiserum produced a precipitation line closer to the virus well (Fig. 5*B*). These results suggested that the purified-virus-antiserum contained an antibody only against human wart virus.

Fluorescent antibody reactions with the purified-virus-antiserum stained nuclear sites of cells in the stratum granulosum and the stratum corneum of verrucae vulgares (Fig. 6), which were the sites of virus observed by electron microscopy [26,27]. No cells were stained in normal human skin.

The purified-virus-antiserum was used to search for viral antigens in lesions of e.v.

Search for Viral Particles and Antigens in Benign Lesions

Regardless of the site of lesions, viral particles, which appeared to belong to the papova group, were observed in ultrathin sections of 16 out of 18 benign lesions tested (Table). Viral particles were observed in the stratum granulosum and the stratum corneum (Fig. 7*A*). They were rarely found in the upper layer of the stratum spinosum, and were not found in deeper layers. The particles were observed in the nucleus and rarely in the cytoplasm. In the stratum corneum, they were mostly observed in the nucleus of parakeratotic cells, and occasionally in the breaking nuclei of desquamating cells. The particles frequently showed crystalline arrangements; most frequently hexagonal and rarely square or pentagonal. Virus-containing cells were not evenly distributed in lesions but appeared in groups of cells.

In tissue extracts, viral particles, 50 to 60 nm in diameter, were observed in all 18 benign lesions tested (Fig. 7*B*, Tab.). Even in 2 benign lesions where viral particles were not observed by electron microscopy of ultrathin sections, a few viral particles were observed in tissue extracts. The particles showed a discernible surface structure, in which capsomeres appeared to be arranged in the order of pentamer–hexamer–hexamer–pentamer of the right-handed form (Fig. 7*B*).

Eight benign lesions from 4 cases (1, 2, 4, and 5) were studied by fluorescent antibody staining. In all, antigens stainable by the antiserum against human wart virus were demonstrated (Tab.). The antigens were found in cells of the stratum granulosum and the stratum corneum and rarely in those of the upper layer of the stratum spinosum (Fig. 8). In deeper layers, no antigens were observed. The antigens appeared to exist in the nucleus (Fig. 8*C*). The antigen-positive cells did not appear diffusely

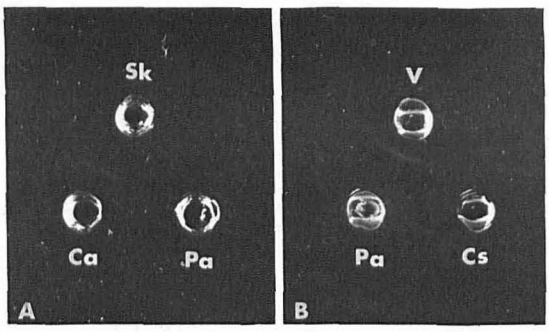

FIG. 5. Precipitation reaction in agarose gel. *A:* Against normal skin extract. *B:* Against wart virus. *Ca*, guinea-pig antiserum against crude wart virus; *Pa*, guinea-pig antiserum against purified wart virus; *V*, partially purified wart virus; *Cs*, serum of nonimmunized guinea pig.

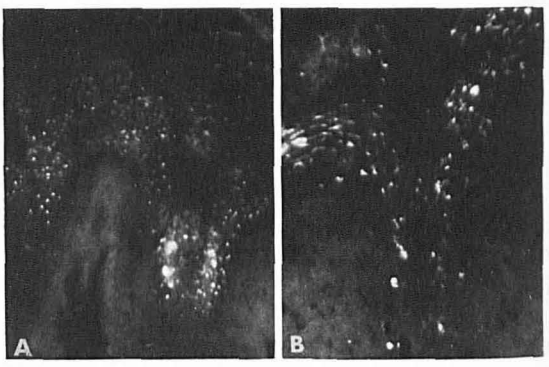

FIG. 6. Fluorescent photomicrographs of verruca vulgaris stained with the antiserum against purified wart virus. *A:* (× 42). *B:* (× 110).

TABLE. *Virus in lesions of epidermodysplasia verruciformis*

Case (Age, sex)	Site of lesion	Histology	Viral particles by electron microscopy[a]		Viral antigens by fluorescent antibody staining
			Ultrathin section	Tissue extract	
1	Hand	Benign	+	+	+
(38, ♀)	Back	Benign	+	+	NT[e]
	Forehead	Early malignancy[b]	+	+	−
2	Forehead	Benign	+	+	+
(21, ♀)	Neck	Benign	+	+	+
3	Hand	Benign	+	+	NT
(40, ♂)	Foot	Benign	+	+	NT
	Neck	Benign	+	+	NT
	Thigh	Early malignancy[b]	−	+	NT
4	Forehead	Benign	+	+	+
(39, ♂)	Forehead	Benign	+	+	+
	Leg	Benign	+	+	+
	Knee	Benign	+	+	NT
	Breast	Benign	+	+	NT
	Back	Benign	−	+	NT
	Forearm	Early malignancy[b]	−	−	NT
	Forehead	Early malignancy[b]	−	−	−
	Forehead	Advanced malignancy[c]	−	−	−
	Forehead	Advanced malignancy[d]	−	−	−
5	Forehead	Benign	+	+	+
(64, ♂)	Hand	Benign	+	+	+
	Hand	Benign	+	+	NT
	Thigh	Benign	+	+	NT
	Breast	Benign	−	+	NT
	Forehead	Early malignancy[b]	−	−	NT
	Forehead	Early malignancy[b]	−	−	NT
	Forehead	Advanced malignancy[c]	−	−	−
	Forehead	Advanced malignancy[d]	−	−	−
Total (No. positive/ no. examined) (%)		Benign	16/18 (89%)	18/18 (100%)	8/8 (100%)
		Early malignancy	1/6 (17%)	2/6 (33%)	0/2 (0%)
		Advanced malignancy	0/4 (0%)	0/4 (0%)	0/4 (0%)

[a] In all cases the viral particles resembled those of papova virus.
[b] Intraepidermal epithelioma.
[c] Ulcerated, invasive squamous cell carcinoma.
[d] Squamous cell carcinoma recurring after removal of primary carcinoma.
[e] NT: not tested.

in a lesion, but frequently showed a tendency to form groups.

Search for Viral Particles and Antigens in Lesions of Early Malignancy

Six lesions showing the histology of early malignancy were studied electron microscopically. In ultrathin sections, viral particles were observed in only one lesion from the forehead of case 1. In this lesion, the particles were observed in the nucleus of cells of the stratum granulosum and the stratum corneum which were superficial to those which showed downward malignant proliferation (Figs. 9*A*,*B*). The virus-containing cells and the number of viral particles in each cell were much fewer than in benign lesions; crystals were not formed. Viral particles of the same morphology as those in benign lesions were observed also in the tissue extract of this lesion (Fig. 9*C*). By fluorescent antibody staining, however, viral antigens were not observed in this lesion (Tab.). In a lesion from the inner aspect of the upper thigh of case 3, which had the histology of early malignancy, viral particles were also observed by electron microscopy of the tissue extracts, but not of the ultrathin sections.

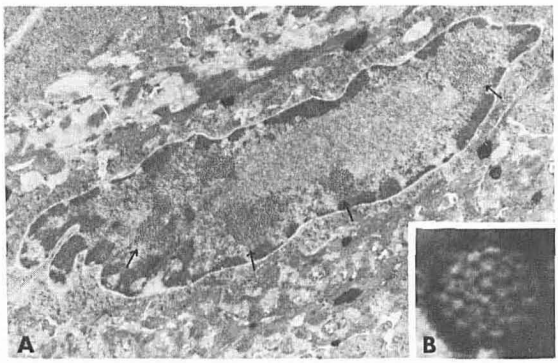

FIG. 7. Viral particles in a typical lesion (case 1, hand). *A:* Cell of the stratum granulosum; *arrows* indicate crystalline arrangements (× 8,000). *B:* Negatively stained tissue extract (× 240,000).

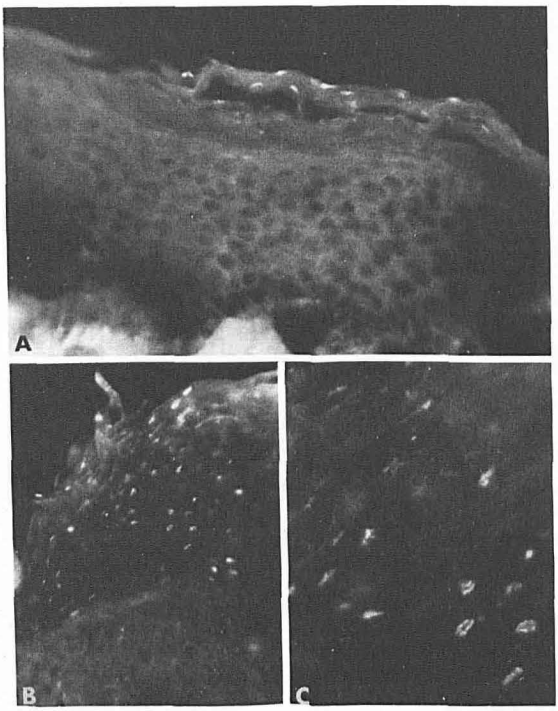

FIG. 8. Fluorescent photomicrographs of benign lesions stained with the antiserum against purified wart virus (case 4). *A:* A flat lesion on the forehead, showing specific fluorescence in the stratum corneum and the stratum granulosum (× 215). *B:* A slightly elevated lesion on the forehead, showing specific fluorescence in the stratum corneum and the stratum granulosum (× 160). *C:* Higher magnification of *B*, showing the form and intranuclear localization of specific fluorescence (× 420).

Search for Viral Particles and Antigens in Lesions of Advanced Malignancy

Four advanced malignant lesions, 2 primary ulcerated invasive squamous cell carcinomas (Fig. 3) and 2 squamous cell carcinomas recurring after the surgical removal of the primary carcinomas, were similarly studied. In none of these lesions, were viral particles or viral antigens detected.

DISCUSSION

The antiserum used for the fluorescent antibody study was prepared by injecting purified human wart virus. In the immunodiffusion test, this antiserum contained no antibody to normal skin antigens. The antiserum stained only nuclear sites of cells of the upper layers of verrucae vulgares where viral particles were observable by electron microscopy. Thus, it appears that the antiserum used for the fluorescent antibody staining was specific to the virion antigen of the virus of common human warts. Fluorescent antibody staining with this antiserum stained the nuclear site of cells in the upper layers of the skin lesions of e.v. where viral particles were also observed by electron microscopy. The viral particles observed in the lesions of e.v. appeared morphologically, even in their surface structure, identical to the virus of common human warts. These results suggest that the virus of e.v. is related to that of common human warts not only morphologically but antigenically, though this does not necessarily mean the two viruses are identical.

In all the benign lesions of e.v. tested, varying amounts of viral particles and/or viral antigens were observed by electron microscopy of ultrathin sections and/or tissue extracts and by fluorescent antibody staining with the antiserum to human wart virus. In a lesion from the forehead of one case which showed the histology of early malignancy, viral particles, though much fewer than those usually observable in benign lesions, were observed in cells of the stratum granulosum and the stratum corneum covering the deeper layers undergoing downward malignant growth. In another lesion which showed the histology of early malignancy, viral particles were observed by electron microscopy of the tissue extract, but not of ultrathin sections. In advanced malignant lesions, primary ulcerated invasive carcinomas, and carcinomas recurring after the removal of the primary ones, neither viral particles nor viral antigens were detected.

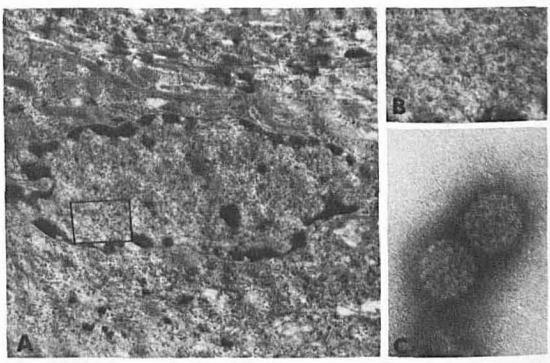

FIG. 9. Viral particles in a lesion showing early malignancy (case 1, forehead). *A:* A cell in the stratum granulosum, containing a few viral particles in the nucleus (× 7,800). *B:* Portion of the cell shown in *A* (× 21,000). *C:* Viral particles in a negatively stained tissue extract (× 130,000).

In deeper layers of e.v. lesions, neither viral particles nor viral antigens were detected. This fact might suggest that, as inferred in rabbit viral papillomas [28,29], the virus exists in the proliferating cells in a non-antigenic state. In animal tumors induced by polyoma virus, SV40, and adenoviruses, viral particles and viral antigens are usually not observed, but, instead, virus-specific nonvirion antigens such as T-antigen and surface antigen have been demonstrated [30–35]. Similar antigens have been observed also in viral papillomas of the rabbit [36]. Pass, Janis, and Marcus [37] and Pass and Marcus [38] have reported on a nuclear and a cell surface antigen in human wart tissue, which are not structural components of human wart virus but exist in concentrated extracts of normal skin. To study the role of virus in the oncogenesis in e.v., the presence of such antigens in the skin lesions of e.v., particularly in the malignant lesions, must be studied also.

In 4 of 6 lesions showing early stages of malignancy, viral particles and/or antigens were not observed. Even in benign lesions, the number of cells containing viral particles or antigens and the amount of viral particles or antigens in a cell varied, and, in 2 benign lesions, viral particles were so few that they were observed only in the partially purified, condensed tissue extract. Hence, the absence of viral particles in 4 of 6 lesions of early malignancy might be due to the fact that there were too few viral particles in the original lesions, although the possibility still remains that these virus-negative early malignant lesions might be induced by the malignant transformation of virus-uninfected skin by sunlight or other factors.

Viral particles or antigens were most frequently observed in the stratum granulosum and the lower layer of the stratum corneum. In a few lesions, however, degenerating nuclei of the desquamating parakeratotic cells were packed with viral particles. These would be a source for the effective spread of the virus.

The authors are grateful to Drs. K. Tanioku, N. Nobara, H. Yoshida, S. Ohmori, J. Arata, and S. Fujita for permitting us to investigate their patients; to Drs. H. Asagoe, M. Hattori, R. Hirowatari, H. Kodama, T. Masuda, K. Matsuo, T. Takaiwa, M. Takenaka, K. Tsukinoki, and H. Wake for supplying verrucae vulgares; to Miss A. Miyake for technical assistance; and to Miss N. Kataoka for preparation of manuscript.

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