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Lymphoproliferative response to fusion proteins of human papillomaviruses in patients with cervical intraepithelial neoplasia

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SUMMARY

The cell-mediated immune response (CMI) to E6 and E4 fusion proteins of human papillomavirus type 16 (HPV-16), E6 fusion protein of HPV-18, and to control proteins similarly produced, was analysed in 29 patients with cervical intraepithelial neoplasia (CIN) and in 15 age-matched laboratory personnel using a lymphocyte proliferation assay (LPA).

Compared to controls without any added proteins, a positive response (stimulation index $> 2{\cdot}0$) to the highly purified E6 control protein was found in only one patient. Positive responses to the E4 control protein which contained β-galactosidase were noted in three patients and two controls.

With control proteins as baseline, the lymphocytes from nine patients (28%) and three laboratory personnel (20%) responded to at least one HPV fusion protein after 7 days in culture. Stimulation indices were low in both groups with a range of 2·06–4·69 and the difference in incidence of positive responses between the groups is not significant. Proliferative responses to HPV-1 and HPV-2 virion antigens were noted in 6/23 (26%) of the patients and 2/15 (18%) of the other group.

No correlation between responsiveness and degree of dysplasia or presence of koilocytes was found in the patient group. The relevance of the low proliferative responses is discussed.

INTRODUCTION

A role for human papilloma virus (HPV) in the induction of cervical cancer was first suggested by zur Hausen and his colleagues in 1975 (1). Specific papillomavirus DNA sequences, particularly of HPV-16 and HPV-18, have been present in the vast majority of premalignant and malignant cervical tumours analysed (2). It is possible that HPV may act as an 'initiator' of malignancy as appears to be the case in the transformation of alimentary papillomas to carcinomas by bovine papillomavirus type 4 (3), or as a 'promoter' following

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initiation by some other event such as infection with herpes simplex virus or the carcinogenic effects of smoking (4).

It is becoming increasingly evident, however, that the immune status of the patient is an important element in the outcome of HPV infection. The rare disease of epidermodysplasia verruciformis, in which there is an underlying defect of cell-mediated immunity (CMI) (5), is associated with widespread and persistent flat warts and plaque-like lesions which frequently progress to malignancy especially in sun-exposed areas (6). Similarly patients, immunosuppressed therapeutically after renal allograft transplantation, develop multiple persistent warts resistant to treatment, some of which may progress to skin carcinomas (6–8). An increase in cervical intraepithelial neoplasia (CIN) in renal allograft recipients and in patients immunosuppressed as a result of underlying malignancy (9, 10) has been recognized for some time, and in some cases at least these lesions have been shown to be HPV-associated (11).

In vitro assays of specific systemic cellular responses to HPV infection were described more than 10 years ago (12–14) from which it was evident that, in patients whose warts regressed spontaneously, a specific T-cell mediated response could be observed. The responses were, however, low and relatively short-lived. In an attempt to investigate the immune response to HPV infection in patients with CIN, we described a lymphoproliferation assay (LPA) using several PV antigens including SDS-disrupted and glycine-extracted bovine papillomavirus type 1 (BPV-1), HPV-1 and HPV-2 virus preparations (15). No correlation was found between positive responses and the presence in cervical biopsies of koilocytes (pathognomonic for HPV infection) nor between positive responses and the degree of dysplasia present. However, positive T-cell responses did correspond with a history of past or present hand and/or foot warts. There exists the possibility that papilloma antigens prepared from skin warts, even when treated to reveal group specific antigens, do not contain the correct epitopes to screen for T lymphocytes responding to the HPV types found in the cervix.

It has recently been shown that cervical carcinomas and cultured cell lines of cervical carcinoma origin, although negative for virion antigens, do contain early proteins, particularly coded by the E6/E7 segment of the HPV genome (16–18). Fusion proteins containing E6 from HPV-16 and HPV-18 have been produced following the insertion of the entire E6 open reading frame (ORF) into pAS plasmid vector (19). In addition, β-galactosidase fusion proteins have been made containing the HPV-16 E4 ORF. Thus it is now possible to look for memory T cells recognizing some early antigens of HPV types relevant to cervical infections. The results of such a study are given in this paper.

MATERIALS AND METHODS

Subjects

Venous blood, totalling 10–20 ml was collected in preservative-free heparin from 29 patients (with a mean age of $31{\cdot}6 \pm 7{\cdot}0$ years) attending the Lothian Colposcopy Clinic for investigation of 'suspicious' or abnormal cervical smears. Colposcopically-directed cervical biopsies were taken at the same time for histopathology. Blood was also obtained from 15 healthy female members of staff

(mean age 30·1 ± 7·6 years). All subjects were asked if they had skin warts or ha[illegible] had them in the past.

HPV fusion proteins

E6 proteins of HPV-16 and HPV-18 were synthesized in *Escherichia coli* AR120 using pAS constructs (20) and purified by superose 12 fast protein liquid chromatography as described by Banks and co-workers (19). A control protein preparation from *E. coli* containing no HPV sequences was similarly prepared. HPV-16 E4-β-galactosidase fusion protein was expressed in *E. coli* as follows: a 2·6 kb *Bal* I fragment from HPV-16 containing the C-terminal portion of E4, but lacking the putative N-terminal portion, had *Bgl* II linkers attached, was cut with *Bgl* II and ligated into *Bam* HI/*Bgl* II cut pUR288 (21). Ampicillin-resistant colonies were screened for fusion protein on SDS PAGE following induction with 100 μg/ml isopropyl-β-D-thiogalactoside. E4 β-galactosidase fusion protein and control β-galactosidase were purified by gel filtration on sepharose CL-4B (Pharmacia). The fusion proteins were suspended in a buffer of 0·02% SDS, 50 mM Tris-HCl pH 7·5 and 5% glycerol at protein concentrations of 25 μg/ml. They were aliquoted and stored at −70 °C and, immediately prior to use, diluted from 10^{-1} to 10^{-4} in cell culture medium.

Lymphocyte proliferation assay

The assay was carried out as described by Cubie and Norval (15) except that the culture period was 7 instead of 8 days which gave more consistent results between replicate wells. Briefly, washed peripheral blood mononuclear cells (PBM) were suspended in culture medium (RPMI-1640) containing 15% autologous plasma and seeded into wells of microtitre plates in quintuplicate (2×10^5 cells/well). Five microlitres of antigen was added to each well and the plates incubated in a humidified atmosphere of 5% CO_2. The day before harvesting 0·75 μC_i [^{3}H]methyl thymidine was added to each well. Harvesting and counting were carried out as previously described. Stimulation indices (SI) were calculated from the ratio:

$$\frac{\text{cpm of cultures in the presence of control or HPV protein}}{\text{cpm of cultures in the presence of culture medium or control protein}}.$$

SI greater than two were considered positive.

Controls of the test system were included as follows: the mitogen, concanavalin A, was added to quintuplicate wells in all assays, a virion antigen preparation of purified HPV-2 or HPV-1 (15) was included whenever sufficient lymphocytes were available, and occasionally a glycine-extract of Vero cells infected with herpes simplex virus type 1 (HSV-1) (22).

RESULTS

The lymphoproliferative responses to control proteins of HPV-16/18 E6 and HPV-16 E4-β galactosidase were assessed against a baseline of culture medium as mock antigen. Only one patient (out of 29) and none of the laboratory controls (out of 15) responsed to the control E6 protein after 7 days in culture. No positive responses were noted after 3 or 5 days in culture in those specimens with sufficient

Table 1. *Patients with CIN and laboratory personnel with positive lymphoproliferative responses to control proteins of HPV-16/18 and HPV-16 E4*

Control protein	Patient number	Days in culture	Lymphoproliferative response to Culture medium $\bar{x}$ cpm	Culture medium S.E.	Control protein $\bar{x}$ cpm	Control protein S.E.	SI
E6	P31	7	1305	298	3087	748	2·37
E4	P12	7	328	8	835	111	2·55
	P31	7	1305	298	5546	608	4·25
	P33	7	270	35	601	67	2·23
	C5	7	706	113	1432	165	2·03
	C13	3	261	6	541	36	2·07
	C13	5	540	51	1255	128	2·32

P, patient; C, laboratory personnel; $\bar{x}$ cpm, mean counts per min; S.E., standard error; SI. stimulation index.

PBM for testing at earlier time points. Three patients and two laboratory controls responded to the control E4–β galactosidase protein (Table 1). SI ranged from 2·03 to 4·25 and were observed after 7 days in culture in patients, and at differing time points in the laboratory controls.

The lymphoproliferative response to HPV specific fusion proteins were then assessed by comparison with the control proteins. After 7 days in culture, 7 patients (24%) and 3 laboratory controls (20%) responded to HPV-16 E6 and/or HPV-18 E6 and a further patient responded to HPV-16 E4-β galactosidase (Table 2). SI were low, ranging from 2·06 to 4·34, and standard errors (SE) were variable although most were below 25% and any results with SE greater than 30% were excluded. The difference in responsiveness between patients and laboratory controls is not significant by the χ^2 test.

No correlation was found in the patient group between SI and the degree of dysplasia or presence of koilocytes (data not shown). Similarly there was no correlation between SI and a past or present history of skin warts in either the patient or laboratory group. Responses to purified HPV-1 and HPV-2 as antigens were noted in 6/23 (26%) of patients, a similar proportion to that obtained in colposcopy patients in our earlier study (15). Two of 11 laboratory personnel also responded to HPV-2 virion antigens. Proliferative responses to concanavalin A with SI > 10 were present in all specimens and both negative and positive responders to HSV-1 were found in both the patient and control groups. In a separate study we have shown that in patients who are seropositive for HSV, the SI is always greater than 3; those with recrudescences had a median SI of 5·7 outwith the time of an attack rising to a median value of 10·6 in the weeks following the lesion, while those without recrudescences had a median SI of 4·9 (23). These results lend weight to the specificity of the responses obtained with HPV fusion proteins.

Table 2. *Patients with CIN and laboratory personnel with positive lymphoproliferative responses to fusion proteins of HPV-16/18 E6 and HPV-16 E4*

Antigen	Patient number	Histological findings	Days in culture	Lymphoproliferative response to: Control protein $\bar{x}$ cpm	Control protein S.E.	HPV protein $\bar{x}$ cpm	HPV protein S.E.	SI
HPV-16 E6	P12	N, K^-	7	224	17	581	88	2·60
	P31	CIN I, K^+	7	3087	748	7558	835	2·45
	P43	CIN II, K^+	7	1343	463	2911	637	2·17
	P44	CaCx	7	440	45	958	82	2·18
	P48	CIN II, K^+	7	503	65	1146	266	2·28
	C5	—	7	313	36	694	114	2·22
HPV-18 E6	P36	N, K^-	7	316	45	1373	366	4·34
	P43	CIN II, K^+	7	1343	463	3846	728	2·86
	P45	CIN III, K^+	7	1074	275	2215	186	2·06
	C2	—	7	240	46	656	89	2·73
	C5	—	5	350	86	928	58	2·65
	C5	—	7	313	36	765	193	2·44
	C7	—	5	261	31	831	118	3·18
HPV-16 E4	P36	N, K^-	5	251	14	600	60	2·39
			7	527	155	1199	347	2·27

P, patient; C, laboratory personnel; K^+, koilocytes reported; K^-, no koilocytes reported; N, normal; CIN I, II, III, grade of cervical intraepithelial neoplasia; CaCx, carcinoma of the cervix.

DISCUSSION

Positive but small proliferative responses were observed in 8/29 patients with CIN and 3/15 apparently healthy laboratory staff to HPV-16 E6 or HPV-18 E6 fusion proteins when examined 7 days after stimulation of peripheral blood lymphocytes *in vitro*. The E6 gene has been shown to be involved in BPV cell transformation (24, 25) and the major transcripts in cervical carcinoma cell lines and in biopsies of cervical carcinoma are from the E6–E7 region of viral DNA (16, 26). Protein products of E6 and E7 have been found in cervical tumours and in CaSki and SiHa cell lines which contain HPV-16 DNA and in HeLa cells which contain HPV-18 DNA (18, 19, 27), although they are not always detected (28, 29). Recent work suggests that the 20 kDa E7 protein is abundant but has a half-life of about 1 h (27), while the 16·5 kDa E6 polypeptide may be expressed only at very low levels (19). Although such investigations have been limited to transformed cell lines and a few malignant biopsies, it may be that expression is equally low in cervical HPV infection with or without associated dysplasia. This would explain why immune responsiveness to E6 proteins was rare and weak. Responses to E7 proteins may yield more conclusive results.

While the protein product of E4 is abundantly expressed in HPV-1 warts (30, 31) and appears to be associated with virion release by altering the keratin skeleton (32, 33), its significance in other HPV infections is unknown. A 10 kDa

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E4 protein was reported to be produced in CaSki cells (18). However, little evidence was found in the current study for immune responsiveness to HPV-16 E4. The presence of β-galactosidase gene in the fusion protein induced proliferation in three patients and two control subjects, but only one patient (P36, Table 2) responded specifically to HPV-16 E4 after 5 and 7 days in culture. It is essential to use highly purified fusion proteins in investigations of the cellular immune response to HPV products if specific results are to be obtained.

No correlation was noted between lymphoproliferative responses to any of the fusion proteins and the degree of dysplasia or the presence of koilocytes. In addition, there was no correlation with a past or present history of skin warts. This is in contrast to the results we obtained using purified disrupted HPV-1 or HPV-2 as antigens in a similar LPA where a correlation was found between a positive response and a history of skin warts, but not with CIN or koilocytosis (15). It is thus crucial to use the correct viral antigens in testing CMI in wart infections caused by different HPV types and it seems likely that the immune response generated by these types may vary.

In those people who had positive responses to E6 proteins, it would be interesting to see which T-cell subset had been stimulated. It is possible that some of the HPV types found in the cervix may induce a preferential T suppressor response depending on the interaction of the virus with the local antigen presenting cells. It is interesting to speculate further whether the low responses may be due to the very low production of any HPV-specific antigen to which the immune system could respond, to the sequestration of HPV antigens in the upper layers of the epidermis away from immune effector cells or perhaps to a virally-induced lack of contact of the virus with antigen presenting cells in the cervix. This latter suggestion is supported by increasing evidence that Langerhans cells, the major antigen presenting cells of the skin, are depleted from areas of HPV infection as well as surrounding areas (34, 35).

The occurrence of positive responses to HPV-16/18 in apparently normal women as well as in patients with CIN is not unexpected. It has been shown increasingly in the last few years that HPV DNA can be detected in normal cervices (summarized in reference 36) particularly when sensitive methods such as the polymerase chain reaction (36, 37) are used. It would seem likely that certain HPV types can persist for long periods while generating minimal detectable immune response, until some additional factor(s) upsets the balance and enables clinically evident lesions to develop. Tests of immune responsiveness of peripheral cells may be unimportant in what is essentially a confined local infection.

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