Immunophenotypes of lymphocytes in prospectively followed up human papillomavirus lesions of the cervix

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SUMMARY From 1981 286 women were prospectively followed up for a mean (SD) of 16 (14) months for established infection with human papillomavirus (HPV) with or without coexistent cervical intraepithelial neoplasia (CIN). The in situ immunocompetent cell infiltrates in 263 cervical punch biopsy specimens from these women were phenotypically identified by the avidin-biotin peroxidase complex (ABC) technique using monoclonal antibodies Leu-10, OKT-3, OKT-4, and OKT-8. Leu-10⁺ B lymphocytes far outnumbered the OKT-3⁺ T lymphocytes in all types of HPV lesions (flat, inverted, and papillomatous condylomas of the cervix). The ratio of OKT-4⁺ to OKT-8⁺ (T helper to T suppressor cells) was slightly reduced in HPV lesions with more severe CIN and correlated positively with the intensity of the immunocompetent cell infiltrate. The ratio of OKT-4⁺ to OKT-8⁺ cells was highest in the 47 (28.8%) patients with HPV lesions that regressed during follow up, somewhat lower in the 85 (52.1%) with persistent lesions, and lowest in the 31 (19.1%) with lesions showing clinical progression. The results are discussed in terms of the proposed immune surveillance functions attributed to immunocompetent cells in situ according to the mucosal associated lymphatic tissue (MALT) concept. The conclusion drawn is that a dynamic balance between the immunoregulatory cells and their subtypes is a prerequisite for the proper handling of intracellular infections of the mucosa, including that with HPV.

Introduction

The concept that human papillomavirus (HPV) caused entirely innocuous human tumours (squamous cell papillomas and warts) has recently been reappraised.¹⁻⁶ Pertinent new data have been provided by the recent reports on the common association of HPV lesions of the uterine cervix (the flat, inverted, and papillomatous condylomas) with cervical intraepithelial neoplasia (CIN),^{1 3 7 9-11 13} the malignant transition of HPV lesions elsewhere in the body,^{1 4 6} the epidemiological risk factors shared by cervical infection with HPV and squamous cell carcinoma of the cervix (both of which have been shown to be sexually transmitted),^{1 4 6 12} the immunohistochemical detection of HPV structural proteins (or viral antigens) in CIN lesions,^{1 2 5} and finally by the DNA hybridisation experiments showing HPV 16

and 18 DNA in invasive cervical cancer.⁸ Despite evidence suggesting HPV as a causative agent of squamous cell carcinomas of the cervix and other sites in man, the final proof is still awaited because of the lack of prospective data on the natural history of the HPV, such as the formation of cancer from a pre-existing HPV lesion.^{6 7 14} In the same way, the factors that determine whether an HPV lesion will regress, persist, or progress are poorly understood.⁶

HPV lesions, particularly warts, are recognised as being more common in immunosuppressed patients than in healthy people.¹⁵⁻²⁰ Furthermore, cutaneous HPV lesions are also known to disappear spontaneously, that is, they regress and show an intense inflammatory cell infiltration.^{16 17 21-24} This observation led to the conclusion that immune reactivity of the host would appreciably contribute to infectivity and the clinical course of infections with HPV in man.¹⁷ Indeed, both humoral and cell mediated immunity have been shown in a variety of infections with HPV.^{16 17 22 25-28}

In HPV lesions of the uterine cervix, these immune

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mechanisms have so far been poorly identified, although some recent data suggest that local immune reactivity against HPV infections of the cervix might also exist,²⁹⁻³¹ and some preliminary analyses of the in situ immunocompetent cell infiltrates have been published.^{14 29 31 32} According to our tentative results of immunophenotyping the T cell subsets in 65 HPV lesions of the cervix followed up for a shorter period, some changes in the ratio of OKT-4⁺ to OKT-8⁺ (T helper to T suppressor cells) occurred in lesions with different clinical courses.¹⁴

The report published here is a direct extension of these studies and gives data on the phenotypic identification (with monoclonal antibodies Leu-10, OKT-3, OKT-4, and OKT-8) of the immunocompetent cells in situ in HPV lesions of the cervix in 286 women studied prospectively for a mean (SD) of 16 (14) months.

Patients, materials, and methods

We investigated 286 consecutive women who were currently included in a prospective study of HPV lesions of the cervix, which was started in 1981. These women were invited to participate in the prospective study if their routine cervical Papanicolaou (Pap) smears, evaluated using the criteria outlined recently,^{3-6 11} showed cytopathic changes induced by HPV. All patients were studied at the outpatient department of gynaecology and obstetrics, Kuopio University Central Hospital, Kuopio, Finland, at six month intervals, as described recently.¹⁴ At each attendance, the patient had a complete gynaecological examination including colposcopy; a directed punch biopsy specimen was taken if concomitant CIN lesions (HPV-CIN) had been identified from the primary Pap smear or in subsequent biopsies, or both.¹⁴ Thus two groups of patients were formed, those with HPV but not CIN (HPV-NCIN) who were studied by colposcopy and Pap smears, and those with HPV-CIN who

underwent additional punch biopsies. Table I shows the age distribution of these patients; the peak was between 20 and 24 years, and 61.9% of the women were aged under 30. The mean (SD) follow up time was 16.0 (14.2) months.

Whenever a punch biopsy was indicated, three samples of cervical tissue were removed; one was processed for routine light microscopy, one for electron microscopy, and one was frozen immediately for immunohistochemical analysis of local immunocompetent cell infiltrates.^{14 32} In sections stained with haematoxylin and eosin, the morphology of the HPV lesions was analysed using the criteria outlined previously,^{3-6 11} and each lesion was classified as being in one of three categories: flat, inverted, or papillomatous condyloma. The grade of CIN was assessed using the commonly accepted criteria for CIN I, II, and III. On special occasions carcinoma in situ (CIS) was included as a separate category.

The fresh tissue samples were immediately frozen in isopentane cooled with liquid nitrogen, and were stored at -70°C until processed further. Cryostat sections 6 µm thick were cut, and stained for B lymphocytes and T lymphocyte subsets using the monoclonal antibodies and the avidin-biotin peroxidase complex (ABC) method. The following monoclonal antibodies were used: Leu-10 (Becton-Dickinson, Sunnyvale, California, USA), which is known to define B lymphocytes; OKT-3 (Ortho Immunobiology Ltd, Raritan, New Jersey, USA), which is known to react with all peripheral T lymphocytes; OKT-4 (Ortho) for T inducer or helper cells; and OKT-8 for T suppressor or cytotoxic cells.³³ Briefly, the 6 µm cryostat sections were fixed in cold (4°C) acetone for 10 minutes and dried at room temperature. The endogenous peroxidase activity was blocked by methanol-hydrogen peroxide solution. The sections were then incubated sequentially with appropriate dilutions of the following antisera: each of the monoclonal antibodies Leu-10, OKT-3, OKT-4, and OKT-8 for 15 minutes; biotinylated rabbit anti-mouse IgG (Vector Laboratories, Burlingame, California, USA) for 15 minutes; and avidin-horseradish peroxidase (Vector Laboratories) for another 15 minutes. The peroxidase reaction was developed for five minutes using diaminobenzidine (DAB) as substrate. The primary antibody was omitted from one negative control, and DAB substrate alone was applied to another to show the residual endogenous peroxidase activity.

Positive reactions for each monoclonal antibody were shown as a membrane or granular staining of lymphocytes in the subepithelial immunocompetent cell infiltrates and within the squamous epithelium (figs 1 and 2). The percentages of Leu-10⁺, OKT-3⁺, OKT-4⁺, and OKT-8⁺ cells in the lesions were

TABLE I Ages of 286 women studied prospectively for infection with human papillomavirus (HPV) and cervical intraepithelial neoplasia (CIN)

Age (years)	No (%) of women
15-19	37 (12.9)
20-24	88 (30.8)
25-29	52 (18.2)
30-34	41 (14.3)
35-39	25 (8.7)
40-44	13 (4.5)
45-49	10 (3.5)
50-54	6 (2.1)
55-59	9 (3.2)
60-64	2 (0.7)
65-69	3 (1.1)

FIG 1 Low-power view of cervical HPV lesion stained with Leu-10 monoclonal antibody for B lymphocytes. Most cells stain positive in both the stromal (S) infiltrate and within the squamous epithelium (E) (avidin-biotin peroxidase complex (ABC) technique with Leu-10, original magnification $\times 100$).

FIG 2 HPV lesion adjacent to the transformation zone stained with OKT-8 monoclonal antibody for T suppressor or cytotoxic cells. Dense cluster of OKT-8+ cells present in stromal infiltrate (open arrow), and scattered OKT-8+ cells found within squamous epithelium (solid arrow) (avidin-biotin peroxidase complex (ABC) technique with OKT-8, original magnification $\times 250$).

established by counting 200 cells at random in different sections of each lesion. The ratio of OKT-4⁺ to OKT-8⁺ (T helper to T suppressor cells) was established in each specimen. The cell counting was completed in a blind manner by one of us (KS), who was not aware of the identity of the specimens at this stage of the study.

Student's *t* test was applied for the statistical calculations where they are shown separately.

Results

Table II shows the percentages of B and T lymphocytes (Leu-10⁺ and OKT-3⁺) as well as T cell subsets (OKT-4⁺, OKT-8⁺) in different types of HPV lesions. Only small fluctuations were found in the proportions of both B and T cells between the three types of condylomata, the ratio of T helper to T

TABLE II B lymphocytes and T cell subsets in different types of cervical human papillomavirus (HPV) lesions in 163 women

Type of HPV lesion	No of patients	Mean (SE) No of cells reactive with:				Mean (SE) ratio of OKT-4 to OKT-8 cells
		LEU-10	OKT-3	OKT-4	OKT-8	
Flat	173	53.2 (1.08)	19.7 (0.67)	8.6 (0.38)	10.5 (0.52)	1.12 (0.08)
Papillomatous	10	52.9 (2.56)	18.3 (2.06)	9.5 (1.46)	12.2 (2.03)	1.03 (0.19)
Inverted	8	48.0 (5.88)	21.9 (3.75)	11.3 (1.87)	13.9 (2.62)	1.05 (0.29)
Normal	72	50.3 (2.03)	20.6 (1.12)	9.3 (0.67)	10.6 (0.86)	1.05 (0.07)

TABLE III Percentages of B lymphocytes and T cell subsets related to the grade of cervical intraepithelial neoplasia (CIN) associated with human papillomavirus (HPV) infection in 163 women

Grade of CIN associated with HPV	No of biopsy specimens	Mean (SE) No of cells reactive with:				Mean (SE) ratio of OKT-4 to OKT-8 cells
		LEU-10	OKT-3	OKT-4	OKT-8	
HPV-NCIN	166	52.6 (1.23)	19.9 (0.71)	8.9 (0.41)	10.5 (0.57)	1.14 (0.08)
HPV-CIN I	62	53.2 (1.72)	20.1 (1.19)	9.0 (0.70)	10.6 (0.83)	1.04 (0.12)
HPV-CIN II	20	50.1 (2.81)	18.5 (1.58)	8.3 (0.91)	12.5 (1.40)	0.91 (0.21)
HPV-CIN III	12	46.2 (4.52)	22.6 (2.07)	10.4 (1.11)	11.5 (1.25)	1.04 (0.13)
HPV-CIS	3	55.0 (2.52)	18.3 (4.42)	6.0 (1.53)	12.7 (6.68)	0.67 (0.16)

HPV-NCIN = patients with HPV but not CIN.
CIS = carcinoma in situ.

suppressor cells being almost identical. The same held true of the biopsies showing a normal epithelium—that is, those in which viral changes had disappeared.

Table III shows the percentages of immunocompetent cells related to the grade of HPV-CIN. Only minor changes in percentages of B and T cells, and of their subsets were found between the different grades of HPV-CIN. There seemed, however, to be a slight tendency for the ratio of OKT-4⁺ to OKT-8⁺ cells to decrease parallel with increasing severity of CIN, reaching the lowest values in HPV-CIS lesions ($p < 0.01$ between HPV-NCIN and HPV-CIS).

Table IV shows the relative numbers of different cells in relation to the intensity of the immunocompetent cell infiltrate. The percentage of B cells increased in parallel with the increasing intensity of the infiltrate. The same held true of the relative numbers of T helper cells, leading to a rise in the ratio of OKT-4⁺ to OKT-8⁺ cells in moderate and intense infiltrates.

Table V summarises the percentages of types of immunocompetent cells in various age groups. The figures show that no clear cut age dependence exists for any of these cell types.

Table VI shows the percentages of the different types of immunocompetent cells related to the clinical course of the lesions. The relative numbers of OKT-8⁺ T cells rose in lesions that had progressed, when compared with those that had regressed or persisted. Thus the ratio of OKT-4⁺ to OKT-8⁺ cells seemed to be lowest (the only ratio below 1) in HPV lesions that had progressed clinically, and highest in those that had regressed, but the difference was not significant.

Discussion

Recent evidence from studies on the organisation of human lymphatic tissues in different anatomical sites suggests a unifying concept of skin associated lymphoid tissue (SALT) and mucosal associated lymphoid tissue (MALT) to explain the complex interplay of immune functions required to cover the special demands of continuous antigenic stimuli.^{34 35} According to the SALT/MALT concept, the local microenvironment is capable on its own of accepting, processing, and presenting the diversity of antigens conditioned by the keratinocytes, Langerhans cells, and immunocompetent lymphocytes, including their subsets.^{34 35} The dynamic regulatory balance between the different cell types and their subsets is a prerequisite of an appropriate immune reactivity in situ, which is designed to provide the skin and mucosal sites with immune surveillance against developing neoplasms and infections with intracellular pathogens, such as viruses.

According to the SALT/MALT concept, it seems feasible to assess whether (and how effectively) such local immune mechanisms can resist and modify the course of infections with HPV. Reports on the rejection of cutaneous warts conditioned by the in situ immunocompetent cells^{17 21-24 28} are possible indicators of such effectiveness. On the other hand, depletion of cell mediated immune mechanisms and immunosuppression seem to be associated with generalised infections with HPV and often with malignant transformation.^{15-20 26 27 36} Morphologically different patterns of reaction found in plane and common skin warts (due to different types of HPV) in regression^{17 21 23 24 36} show some

TABLE IV Numbers of B cells and T cells subsets related to intensity of immunocompetent cell infiltrate in 163 women

Intensity of infiltrate	No of biopsy specimens	Mean (SE) No of cells reactive with:				Mean (SE) ratio of OKT-4 to OKT-8 cells
		LEU-10	OKT-3	OKT-4	OKT-8	
Weak	116	50.3 (1.29)	19.2 (0.81)	8.2 (0.47)	9.5 (0.59)	1.00 (0.05)
Moderate	82	52.7 (1.54)	20.2 (0.98)	9.5 (1.54)	11.9 (0.74)	1.15 (0.14)
Intense	65	52.8 (1.91)	20.1 (1.08)	9.9 (0.68)	11.6 (0.91)	1.17 (0.12)

TABLE V Age dependence of the percentages of B cells and T cell subsets in 163 women with cervical human papillomavirus (HPV) lesions

Age (years)	No of patients	Mean (SE) No of cells reactive with:				Mean (SE) ratio of OKT-4 to OKT-8 cells
		LEU-10	OKT-3	OKT-4	OKT-8	
15-19	18	46.1 (3.99)	23.9 (2.78)	10.4 (1.58)	10.9 (1.58)	1.19 (0.22)
20-24	51	53.2 (1.77)	20.1 (1.12)	9.1 (0.69)	10.4 (0.75)	0.98 (0.08)
25-29	33	55.0 (2.55)	20.0 (1.61)	9.5 (0.86)	8.9 (1.00)	1.16 (0.08)
30-34	18	57.5 (2.79)	17.9 (1.72)	7.0 (0.68)	10.6 (1.39)	0.87 (0.14)
35-39	17	49.9 (3.01)	22.8 (2.65)	7.1 (1.03)	10.6 (1.89)	0.86 (0.11)
40-44	8	52.8 (6.27)	19.4 (3.49)	12.1 (2.67)	16.5 (3.54)	1.58 (0.92)
45-49	3	53.7 (3.85)	18.0 (1.00)	4.7 (1.77)	9.3 (2.19)	0.49 (0.12)
50-54	5	61.8 (7.94)	19.6 (4.57)	9.2 (2.49)	11.2 (2.87)	0.87 (0.15)
55-59	8	58.8 (2.91)	19.6 (1.73)	7.5 (1.05)	12.3 (2.95)	0.84 (0.19)
60-64	1	40.0	21.0	12.0	19.0	0.63
65-69	1	56.0	24.0	12.0	12.0	1.00

evidence that the type of local reaction might depend on the type of HPV.¹⁷ Practically no such data are available on HPV lesions of the cervix, which are mostly caused by HPV 6, 10, and 11, although a certain percentage of them seem to regress even within a short period of follow up.¹⁴

In the healthy adult uterine cervix, Langerhans cells and B and T lymphocytes have been found in the subepithelial infiltrate, as well as within the squamous epithelium.³⁰ T suppressor cells outnumbered T helper cells within the epithelium in these biopsy specimens (from a total of six patients only), the reverse being true in the subepithelial infiltrates.³⁰ Squamous epithelium infected by HPV contained fewer T cells and Langerhans cells than were found in healthy cervical epithelium,³¹ although no data were given on B cells and T cell subsets in such infiltrates associated with HPV infections.³¹ Such an infiltrate containing both OKT-4⁺ and OKT-8⁺ T cells was, however, recently reported in the three genital warts included in a study of skin warts.²⁹ This agrees with the data provided by the first systematic study on HPV lesions of the cervix using the histochemical acid α -naphthyl acetate esterase (ANAE) technique to define B and T lymphocytes and mononuclear phagocytes (MPS cells).³² In that study, B lymphocytes far outnumbered T cells and MPS cells both in infections with HPV and in CIN lesions. This was confirmed by the study published here, in which Leu-10⁺ B cells predominated over OKT-3⁺ T cells in all types of infection with HPV (table II).

In previous studies, the increase in proportions of T cells paralleled an increasing grade of HPV-CIN,^{14,32} which was attributed to a relative increase in the numbers of T suppressor cells.³² This was partly confirmed by the results presented here (table III), which showed the highest percentage (22.6%) of OKT-3⁺ T cells in HPV-CIN III lesions, although the difference from that in HPV-NCIN lesions (19.9%) was not significant. In the same way, the percentage of OKT-8⁺ cells was lowest in HPV-NCIN lesions and highest in HPV-CIS lesions. This seems to confirm the previous suggestions concerning the relative increase of T suppressor cells in HPV-CIN lesions,³² which led to a lower ratio of OKT-4⁺ to OKT-8⁺ cells in HPV-CIS compared with that in HPV-NCIN ($p < 0.01$).

The increase in relative proportions of B cells paralleled the increasing intensity of the local infiltrate (table IV). The same is true of the relative increase of T helper cells, which led to a parallel rise in the ratio of OKT-4⁺ to OKT-8⁺ cells. This agreed with the observations made in the normal cervix, in which dense subepithelial infiltrates were shown to have a preponderance of OKT-4⁺ cells over OKT-8⁺ cells,³⁰ thus reflecting an enhanced humoral immune reactivity in these dense infiltrates. Consistent with the data on many human malignancies that an intense stromal inflammatory cell reaction is a sign of favourable outlook, the increased ratio of OKT-4⁺ to OKT-8⁺ cells in this study could also be regarded as a favourable sign, as the highest ratio of OKT-4⁺ to OKT-8⁺ cells was found in HPV lesions that

TABLE VI Percentages of B lymphocytes and T cell subsets related to the clinical course of cervical (HPV) lesions in 163 women

Clinical course	No (%) of patients	Mean (SE) No of cells reactive with:				Mean (SE) ratio of OKT-4 to OKT-8 cells
		LEU-10	OKT-3	OKT-4	OKT-8	
Regressed	47 (28.8)	55.5 (2.37)	21.1 (1.51)	8.5 (0.79)	9.2 (0.95)	1.07 (0.08)
Persisted	85 (52.1)	52.5 (1.41)	20.1 (0.99)	9.1 (0.56)	11.0 (0.71)	1.04 (0.10)
Progressed	31 (19.1)	52.5 (2.26)	21.2 (1.42)	8.9 (0.78)	11.2 (1.09)	0.93 (0.09)

regressed during follow up (table VI).

Of the 163 cervical HPV lesions from which data on biopsies and ABC staining were available, 47 (28.8%) regressed, 85 (52.1%) persisted, and 31 (19.1%) progressed during the mean (SD) follow up period of 16 (14) months. The results presented here show only minor fluctuations of the numbers of immunocompetent cells in lesions with different clinical courses (table VI). There seemed, however, to be a relative increase in OKT-8⁺ cells in order of regressing, persistent, and progressing lesions, which had also been shown in the preliminary study based on a small number of patients and a short follow up.¹⁴ Thus it seems that in HPV lesions of the cervix the shift in local immunoregulatory cell balance in favour of OKT-8⁺ T cells is associated with clinical progression of the lesions. Consistent with the data available on other immunologically conditioned disorders (including AIDS) the lowered ratio of OKT-4⁺ to OKT-8⁺ cells is probably due to an inappropriate increase of T suppressor cells in relation to the number of T helper cells. The latter, in fact, seemed to remain at a relatively constant level irrespective of the clinical course of the HPV lesions in this study. Until we have means available to distinguish between T suppressor and T cytotoxic cells (both OKT-8⁺), however, this interpretation is made with caution.

In conclusion, the present study lends support to the concept that a local immunocompetent cell infiltrate is common in cervical HPV lesions. The constituents of this infiltrate probably participate in the immune reactions to infection with HPV, as suggested by the fluctuations in their numbers related to the grade of CIN associated with HPV and to the intensity of the infiltrate proper, but not related to the age of the patients. Furthermore, the ratio of OKT-4⁺ to OKT-8⁺ cells seems to be subjected to changes related to the clinical behaviour of HPV lesions, progression being associated with an inappropriate increase of OKT-8⁺ T suppressor cell activity. The value of these observations will be shown by the extension for many years of the current follow up study.

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