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Organotypic culture of HPV-transformed keratinocytes: a model for testing lymphocyte infiltration of (pre)neoplastic lesions of the uterine cervix

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Abstract The aim of our study was to establish the relevance of an in vitro model for analysing the ability of human lymphocytes to infiltrate human papillomavirus (HPV)-associated (pre)neoplastic lesions of the uterine cervix. To mimic these lesions, we have used the organotypic raft culture of HPV-transformed keratinocytes (SiHa). The SiHa organotypic raft culture was co-cultured with resting or prestimulated (IL-2 or IL-2+anti-CD3 mAb) allogeneic peripheral blood mononuclear cells (PBMC) for 24 and 72 h. The majority of infiltrating cells were T lymphocytes. Occasional NK cells were also identified. The stimulation with IL-2+anti-CD3 mAb induced the highest number of infiltrating cells, with the maximum lymphocyte infiltration observed after 24 h of co-culture. The lymphocyte infiltration was associated with an increased number of apoptotic cells in the organotypic cultures. The ability of PBMC and purified T cell and NK cell populations to lyse HPV-transformed keratinocytes was also investigated on monolayer cultures. As expected in an allogenic model, the highest cytotoxicity was mediated by NK cells activated by IL-2 or IL-2+anti-CD3 mAb. The cytotoxic activity of T cells was weak but, interestingly, increased in the presence of phytohaemagglutinin A (PHA), assuming that T cells were able to kill HPV-infected keratinocytes when a bridge between T cells and keratinocytes was provided. In conclusion, the organotypic culture of HPV-transformed keratinocytes may provide an effective in vitro model for investigating novel T cell-based immunotherapy protocols for the treatment of HPV-associated lesions.

Key words Human papillomavirus · Cervical neoplastic lesions · Organotypic culture · T lymphocytes · LAK cells

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Introduction

There is now considerable evidence that high-risk human papillomaviruses (HPV) such as HPV 16 and HPV 18 are closely associated with the malignant growth of cervical keratinocytes [55]. HPV DNA can be isolated from over 90% of cervical carcinomas and preneoplastic lesions [8, 31, 32] designated as cervical intraepithelial neoplasia (CIN) [44] or squamous intraepithelial lesions (SIL) [40]. The high frequency and rapid progression of HPV-associated SIL in AIDS patients [5] suggest that host cellular immunity controls, at least partially, the development of lesions induced by HPV. T lymphocytes are observed in HPV-associated cervical lesions [17, 22, 48, 52], but there is some evidence that they are poorly activated. For example, T lymphocytes infiltrating cervical tumour tissues rarely express the IL-2 receptor [21]. Furthermore, CTL freshly isolated from tumour specimens are often poorly cytotoxic against autologous tumour cells. In vitro activation is required to reveal the cytotoxic activity of selected clones, suggesting an inadequate intratumour activation of CTL. Activated T lymphocytes have, however, been observed in spontaneously regressing genital warts [10], and occasional therapeutic successes with autologous wart vaccines [53] have shown that HPV-specific immunity can eliminate established HPV lesions. Changes in natural killer (NK) cell and macrophage density have been reported in HPV-associated cervical SIL [1, 35, 49, 50], suggesting that nonspecific effector cells may also play a part in the outcome of HPV-related lesions.

As several studies have shown a decrease in cellular immune response in (pre)neoplastic lesions associated with HPV, therapeutic protocols using transfer of activated lymphocytes could be useful in this disease. The classic way to activate lymphocytes in vitro is to cultivate the peripheral blood mononuclear cells (PBMC) in the presence of IL-2 [23, 25, 45, 46]. The stimulated cells are designated as lymphokine-activated killer (LAK) cells and are mostly derived from activated NK cells [42]. The LAK cytotoxic activity is characterized by the lack of

major histocompatibility complex (MHC) restriction [23, 25]. In order to recruit other lymphocyte populations besides NK cells or to increase the antitumuor capacity of the activated cells, other cytokines or mitogens were used. For example, anti-CD3 monoclonal antibody (mAb) treatment induces a strong activation of T lymphocytes [51], and the stimulation with IL-2 and an anti-CD3 mAb is more effective in the generation of anti-tumour cell effectors than stimulation with IL-2 alone [3, 4, 26].

The generation of an effective protective response is poorly understood and may be influenced by the cytokines produced by infected keratinocytes and cells infiltrating the transformation zone of the cervix, where most SIL and cancers develop. Because different patterns of synergism or antagonism may be observed between cytokines and immune effectors, preliminary studies of immunotherapeutic manipulations should be performed in models approximating the in vivo environment of the tissue of origin. The organotypic (raft) culture system has been increasingly used to examine the effects of viral or biochemical therapeutic agents on a variety of malignant keratinocytes [12, 14, 29]. The raft technique permits cell proliferation and differentiation at an air–liquid interface on a dermal equivalent support. Normal keratinocytes stratify and fully differentiate in a manner similar to the normal squamous epithelial tissues, while HPV-immortalized (late passage) and established squamous carcinoma cell lines exhibit dysplastic morphologies similar to those of high-grade lesions seen in vivo [7, 33, 36]. Because of the superior tissue morphology produced in organotypic cultures, this culture system was adapted to study the ability of allogeneic unstimulated or stimulated (IL-2 and IL-2+anti-CD3 mAb) PBMC to migrate into epithelial sheets mimicking high-grade intraepithelial lesion of the cervix.

Two mechanisms could be used by T cells and NK cells to kill tumour or infected cells [6, 15]. The classical first mechanism depends on the exocytosis of toxic material (perforin, granzyme) by the cytotoxic cells [30]. The cytotoxic cells may also induce programmed cell death (apoptosis) by the interaction between Fas and Fas ligand [47]. We examined the presence of apoptotic cells in organotypic cultures, and classical ^{51}Cr-release assays were used to quantify the lysis of malignant keratinocytes in the presence of PBMC, either unstimulated or stimulated with anti-CD3 mAb and/or IL-2. To enhance the contact between keratinocytes and effector cells, we also used a lectin-dependent cell-mediated cytotoxicity (LDCC) assay.

Materials and methods

The SiHa cell line is a tumorigenic cervical carcinoma-derived keratinocyte cell line containing one copy of integrated HPV 16 DNA [18, 41]. Cultures of normal cervical keratinocytes were established from hysterectomy specimens.

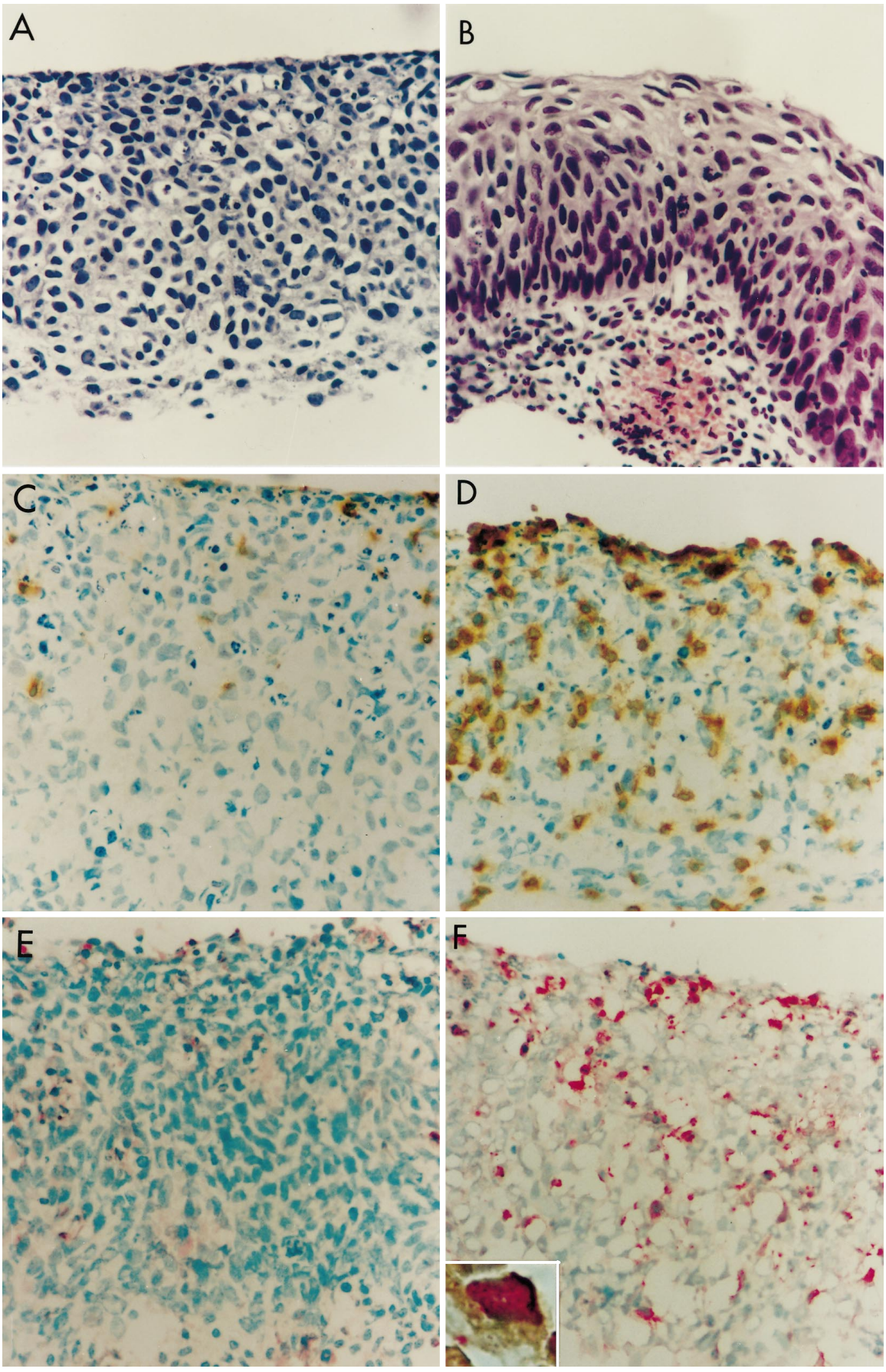
Organotypic cultures were generated using procedures slightly modified from those described previously [7, 9, 12, 33, 36]. Dermal equivalents were produced by using collagen I according to the manufacturer's instructions (cellagen solution AC-5, type collagen I; ICN Biomedicals, Asse-Relegem, Belgium). Briefly, 32 mg of collagen was mixed on ice with 1 ml of chilled 10-fold-concentrated Hank's buffer supplemented with phenol red, NaOH 1 N to give a pH of 7.2, and 10^5 normal human fibroblasts in 1 ml of decomplemented fetal calf serum (Gibco-BRL, Gent, Belgium). The 1 ml of the gel solution was layered onto 24-well plates (Nunc) and allowed to solidify at 37°C for 30 min to 1 h. After gel equilibration with 1 ml of DMEM at 37°C overnight, 25–30 10^4 keratinocytes were suspended in 1 ml of growth medium; 1:3 mixture of HAM F12 (Gibco-BRL)/Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 0,5 μg/ml hydrocortisone (Sigma, Bornem, Belgium), 10 ng/ml EGF (Sigma), 10% decomplemented fetal calf serum (Gibco), 1% L-glutamine 200 mM (Gibco-BRL), 10 mM HEPES (Gibco-BRL), 1 μg/ml fungizone (Gibco-BRL), 1 mM sodium pyruvate (Gibco-BRL), 3000 U/ml penicillin-streptomycin (Gibco-BRL), 10^{-10} M cholera toxin (Sigma), 5 μg/ml insulin (Sigma), 20 μg/ml adenine (Sigma), 5 μg/ml human transferrin (Sigma), 15 10^{-4} μg/ml 3,3′,5-triiodo-L-thyronin (Sigma). They were then seeded on top of the gels and kept submerged for about 24 h. Rafts were then raised onto a stainless metal grid and allowed to grow at the air–liquid interface for 10 days.

PBMC were isolated from buffy coat of healthy donors (Centre de Transfusion de Liège) by centrifugation on Lymphoprep (Nycomed, Oslo, Norway), washed three times and counted. Culture medium consisted of RPMI 1640 (Gibco) supplemented with 1% of nonessential amino acids (Gibco), sodium pyruvate (1 mM, Gibco), 30 U/ml penicillin-streptomycin (Gibco) and 5% pooled heat-inactivated human AB serum. For stimulation, cells were cultivated (1.25×10^6 cells/ml) in the presence of 10 ng/ml of anti-CD3 mAb kindly provided by Dr. Kurrle Behringwerke (Marburg, Germany) and/or 50 U/ml of human rIL-2 kindly provided by the Glaxo Institute for Molecular Biology (AG, Geneva, Switzerland) for 4 days. After the culture, 10^6 PBMC were washed in RPMI medium and resuspended in 50 μl of keratinocyte culture medium. The cell suspensions were seeded at the top of 10-day organotypic cultures.

Frozen sections 8 μm thick were fixed in 4% paraformaldehyde and stained with monoclonal antibodies specific for CD3, CD4, CD8, CD16, CD45, CD56 (Becton Dickinson, Erembodegem, Belgium) and intercellular adhesion molecule-1 (ICAM-1) (Immunotech, Marseille, France). Positive cells were detected using a peroxidase-based detection system. Control sections were made in which the primary antibody was omitted. The evaluation of immunostaining in organotypic cultures was based on the density of positive cells assessed by counting the number of stained cells in the reconstituted epithelium whose area was estimated with a computerized system of image analysis (IBAS) following a previously described method [12, 13]. Cell densities were assessed in triplicate in each culture, and the means were taken by considering values obtained from four separate experiments. Statistical analysis was performed using the Mann-Whitney test or the Kruskal-Wallis nonparametric ANOVA test followed by the Dunnet's multiple comparison test (Instat Mac 2.01 software; GraphPad Software, San Diego, Calif.).

Apoptosis was identified in tissue sections by using a procedure slightly modified from that described previously [24]. Briefly, cryosections of organotypic cultures (5–6 μm) prepared at –20°C were fixed in paraformaldehyde (4% in cacodylate buffer, 0.1 M) for 20 min at room temperature. After washing in TBS, sections were treated with 0.1% Triton -X100 in 0.1% sodium citrate for 10 min at 37°C, washed in TBS, dehydrated and air-dried.

Fig. 1A–F SiHa organotypic culture as a model of high-grade squamous intraepithelial lesions (SIL) and lymphocyte infiltration. **A** Haematoxylin/eosin-stained sections of an organotypic culture of SiHa cells in the absence of PBMC; **B** biopsy specimen of highgrade cervical SIL; **C**, **D** CD45-immunolabelled sections of organotypic cultures of SiHa cells infiltrated by **C** resting PBMC or **D** PBMC stimulated with IL-2+anti-CD3; **E**, **F** in situ demonstration of apoptosis in organotypic cultures of SiHa cells infiltrated by **E** resting PBMC or **F** PBMC stimulated with IL-2+anti-CD3, *insert*: apoptotic cell positive for EMA. **A–F** ×400, *insert* ×1,000 ▶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TUNEL (TdT-mediated dUTP nick end labelling) was carried out using 50 ml of labelling mix per section. In situ Cell Death Detection Kit AP containing α-dUTP-FITC (Boehringer Mannheim, Brussels, Belgium) was used. Sections were incubated at 37°C for 60 min. The reaction was terminated by washing sections three times in distilled water and then in TBS. Incorporated α-dUTP-FITC was detected by incubating the section with converter AP (anti-fluorescein antibody, Boehringer Mannheim) for 30 min at 37°C. Sections were washed three times (5 min each) in TBS, and alkaline phosphatase activity was detected by incubation in the dark with New Fuchsin Substrate System (Dako, Golstrup, Denmark) for 10 min. A double staining (apoptotic and epithelial cells) was also performed. The TUNEL assay was first realized (except the alkaline phosphatase revelation). The cells were then labelled with a mouse anti-EMA (epithelial membrane antigen) mAb (Dako). Apoptotic and epithelial cells were finally visualized, respectively, by an alkaline phosphatase-based and a peroxidase-based detection system, as described previously.

The chromium-51 release cytotoxic assays were performed in 96-well V-bottom plates using 5000 ^{51}Cr-labelled tumour targets per well. Six serial dilutions of effector cells were made (effector cell:target ratios, E:T*, 20:1–0.75:1). Plates were centrifuged for 6 min at 400 *g* to ensure cell contact and incubated for 4 h at 37°C. Then 100 µl of supernatant was then recovered from each well and the radioactivity was measured in a γ-counter (Cobra auto-gamma Packard, Downers Grove, Ill.). Maximum chromium release was obtained by adding 100 µl of detergent (RBS 10%, Chemical Products, Belgium) to 100 µl of target cell suspension, and spontaneous release was given by incubation of target cells without effector cells. The percentage of target lysis was calculated as previously described [26]. For some experiments, PHA (0.8%, Difco) was added during the cytotoxic assay.

The magnetic cell separation process has been described elsewhere [26]. Briefly, the cells were incubated in PBS (Gibco) with a biotinylated anti-CD3 mAb F(ab)'2 (Zymed, Calif.) for 20 min. In a second step the cells were incubated with streptavidin-FITC (Boehringer Mannheim). Finally, the cells were incubated in the presence of 100 µl of 1/100 diluted biotinylated magnetic beads (Miltenyi Biotec, Germany) for 5 min. Cell sorting was realized with the MACS instrument [37] (Becton Dickinson). The purity of the positive and negative cell fractions was respectively checked by flow cytometry (FACStar, Becton Dickinson). When needed, further purification was performed by flow cytometry (FACStar).

Results

By using the organotypic culture model, we tested the ability of activated allogeneic PBMC to infiltrate an in vitro-formed epithelium reminiscent of high-grade SIL observed in vivo. After 1–2 weeks of culture, the SiHa keratinocyte cell line grown on a collagen gel and at the interface between the air and the liquid culture medium produced an epithelial sheet more than 10 cells in thickness that closely resembled a high-grade cervical lesion (Fig. 1A, B). After 10 days of culture, resting or stimulated PBMC were laid down at the top of the culture. To activate the lymphocytes, PBMC were cultivated in the presence of IL-2 (50 U/ml) or IL-2+anti-CD3 mAb (BMA030, 10 ng/ml) for 4 days. The PBMC infiltration in SiHa organotypic cultures was assessed at 24 and 72 h. After 24 h of co-culture, mononuclear cells (detected by anti-CD45 mAb) were observed in the epithelial sheet (Table 1). The majority of these cells were T lymphocytes, as shown by the anti-CD3 labelling (Table 1). Fewer NK cells (detected by anti-CD56 mAb and anti-CD16 mAb) were also found (data not shown). The density of IL-2+anti-CD3 mAb-stimulated T cells (212±79 cells) infiltrating the epithelial sheet was higher than that of unstimulated (26±19 cells) or IL-2-stimulated T lymphocytes (39±8 cells; Fig. 1C, D). Moreover, the maximum lymphocyte infiltration was observed after 24 h, whereas the density of infiltrating unstimulated or IL-2-stimulated lymphocytes increased from 24 h to 72 h of culture (Table 1). The proportions of infiltrating $CD4^+$ and $CD8^+$ T cells did not change as we observed 60±10% of $CD4^+$ T cells and 35±7% of $CD8^+$ cells under all culture conditions (data not shown).

Together with an increased number of infiltrating cells, we observed an increased number of apoptotic cells. Because of the granular staining pattern of apoptotic cells, it was not possible to determine the respective number of epithelial or lymphoid apoptotic cells (Fig. 1E, F). We therefore performed a double staining for apoptotic and epithelial cells to determine whether

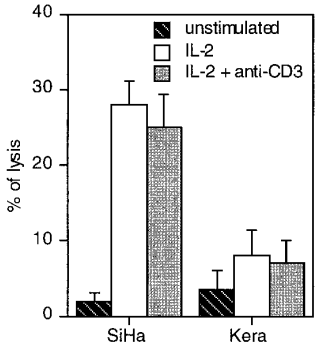


Fig. 2 Cytotoxic activity against HPV-transformed cell line (*SiHa*) and normal keratinocytes (*Kera*). The cytotoxic activity of PBMC cultivated in medium alone, in the presence of IL-2 (50 U/ml) or IL-2+anti-CD3 mAb (10 ng/ml) was evaluated by a chromium release assay after 4 days of culture. The effector cells/target cells ratio = 20/1 (means±SEM; n = 10 for SiHa cells and n = 2 for normal keratinocytes)

Table 1 Number of lymphocytes in SiHa organotypic culture after 24 and 72 hours of co-culture[a]

	24 h		72 h	
	$CD45^+$	$CD3^+$	$CD45^+$	$CD3^+$
Unstimulated lymphocytes[b]	26±19	20±13	47±28	37±24
IL-2-stimulated lymphocytes	37±8	33±10	165±70	140±57
IL-2 + anti-CD3-stimulated lymphocytes	212±79	196±43	232±56	206±50

[a] Results are expressed as mean of positive cells±SEM per mm^2, n=4
[b] Before addition on SiHa organotypic culture, the PBMC were cultivated for 4 days without stimulation, in the presence of IL-2 (50 U/ml) or IL-2 + anti-CD3 mAb (10 ng/ml)

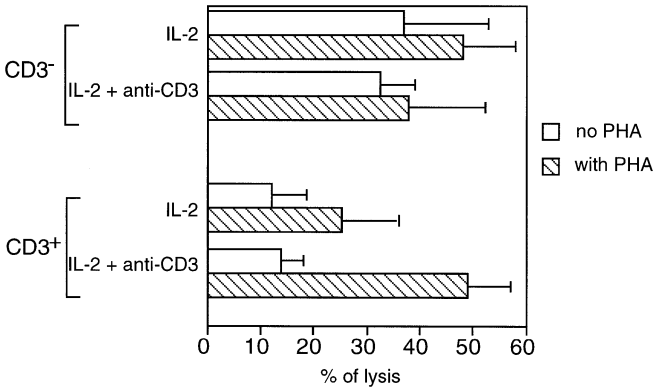


Fig. 3 Cytotoxic activity of $CD3^+$ and $CD3^-$ against SiHa cells in the presence of PHA. After 4 days of culture in the presence of IL-2 or IL-2+anti-CD3 mAb, PBMC were sorted in CD3+ and CD3– cell population. The cytotoxic activity was evaluated by a chromium release assay in the presence or absence of PHA. The effector cells/target cells ratio = 20/1 (mean±SEM; $n = 3$)

keratinocytes undergo apoptosis in the presence of activated lymphocytes. Many cells exhibited the double staining, demonstrating that keratinocytes died by apoptosis. The insert in Fig. 1F shows an epithelial cell (EMA^+) positive for the TUNEL staining.

The ability of activated PBMC to kill HPV-transformed keratinocytes was studied using a cytotoxic assay against the SiHa cell line and normal keratinocytes. Unstimulated lymphocytes induced only a low cytotoxic activity against SiHa cell lines (Fig. 2). By contrast, stimulated lymphocytes were able to lyse the SiHa cell line. No significant difference was observed between the two modes of stimulation (IL-2 and IL-2+anti-CD3 mAb). Neither method of stimulation increased the cytotoxic activity against normal keratinocytes (less than 12% of cytotoxic activity; Fig. 2).

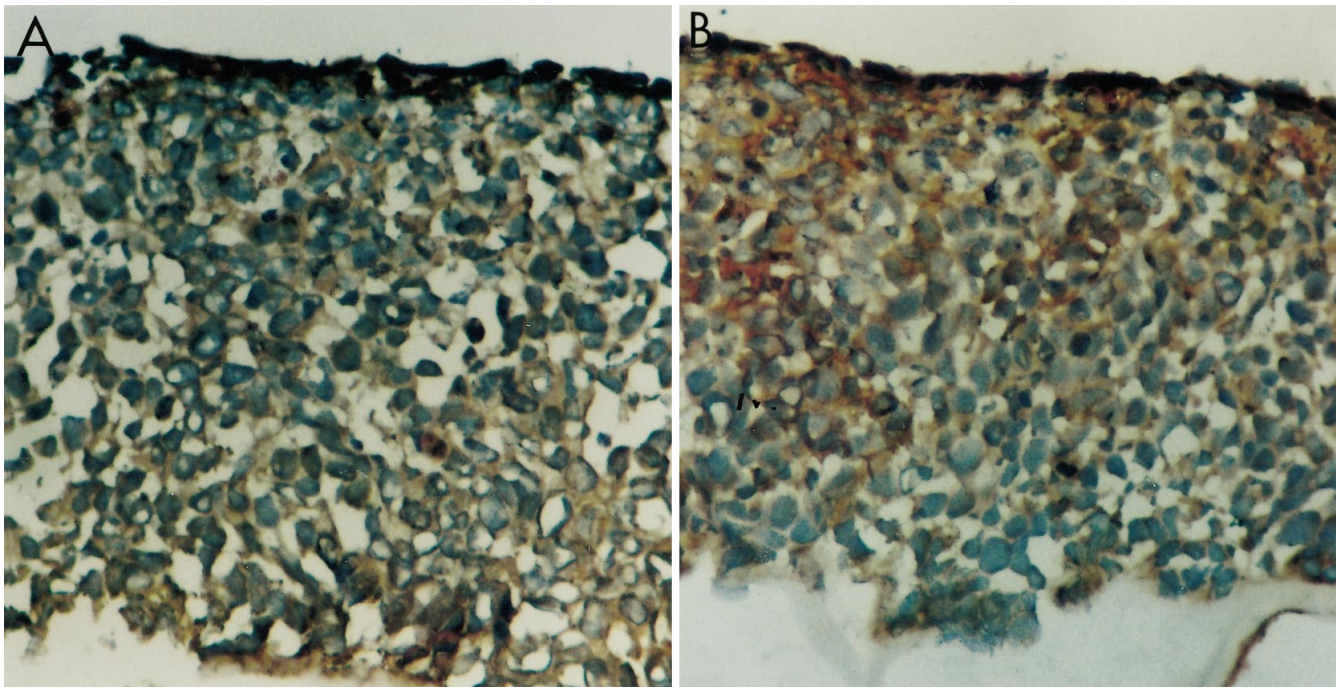
As the majority of infiltrating lymphocytes were T cells, we tested the cytotoxic activity of T cells ($CD3^+$) and non-T cells ($CD3^-$) against SiHa. The cells were sorted by a magnetic cell sorter (MACS). The majority of $CD3^-$ cells obtained by this procedure were NK cells [26]. The main cytotoxic activity was a classical LAK activity mediated by NK cells ($CD3^-$ cells; Fig. 3). The cytotoxic activity of $CD3^+$ cells was weak, but this activity increased in the presence of PHA, especially after activation by IL-2+anti-CD3 (Fig. 3).

The cytotoxic activity could also be mediated by IFNγ and TNFα produced by lymphocytes. These cytokines are directly cytotoxic, but they can also up-regulate adhesion molecules that are important for cell killing, such as ICAM-1. To test whether the production of IFNγ and TNFα by lymphocytes is sufficient to up-regulate the expression of ICAM-1 on SiHa cells, organotypic culture sections were stained with anti-ICAM-1 mAb. In the absence of infiltrating lymphocytes, we observed that SiHa cells constitutively expressed a low amount of ICAM-1 (Fig. 4A). This expression was increased when activated (IL-2+anti-CD3) lymphocytes were present (72 h) in the organotypic cultures (Fig. 4B).

Discussion

Since intraepithelial T lymphocytes have been shown to be depleted in cervical SIL [48] (unpublished observations) and to increase in spontaneously regressing HPV-

Fig. 4A, B ICAM-1 expression in SiHa cell organotypic cultures. **A** ICAM-1-immunolabelled sections of an organotypic culture of SiHa cells in the absence of PBMC and **B** infiltrated by activated (IL-2+anti-CD3) PBMC. Counterstained with haematoxylin, ×400



associated genital warts [9], the cell-mediated immune response probably plays a crucial part in host defence against HPV infection and associated (pre)cancerous lesions. The establishment of an in vitro model allowing the investigation of the factors contributing to the presence and function of immunocompetent cells within a dysplastic epithelium is therefore relevant. Since tissue cells cultivated on plastic are in an environment that does not prevail in vivo, organotypic cultures present a very promising in vitro model for testing the ability of immune cells to infiltrate a preneoplastic epithelium.

To evaluate the feasibility of this model, as a first step we have studied the infiltration of resting and activated allogeneic PBMC into an organotypic culture of HPV-transformed keratinocytes (SiHa cell line). The PBMC were activated by IL-2 and IL-2+anti-CD3 to allow the generation of LAK cells, as already used in immunotherapeutic protocols [46]. The activation in the presence of anti-CD3 mAb induced an increase in the proportion of T lymphocytes, as previously shown [27]. Although unstimulated and stimulated lymphocytes were able to penetrate SiHa organotypic cultures, the stimulated lymphocytes infiltrated the cultures more rapidly and in higher numbers**,** especially after activation with IL-2+anti-CD3 mAb. Most of the infiltrating lymphocytes were T cells. These results highlight the potential interest of organotypic cultures of HPV-transformed keratinocytes as a model for studying the ability of lymphocytes to migrate into a (pre)neoplastic epithelium. Other models are, however, necessary to analyse the ability of lymphoid cells to cross the basement membrane, such as those using a three-dimensional collagen matrix [19, 43].

As the lymphocyte infiltration was associated with an increased number of apoptotic cells in the SiHa organotypic cultures, we also studied the cytotoxic activity of PBMC against SiHa cell monolayer cultures. As already reported for other HPV-immortalized cell lines [20], unstimulated lymphocytes induced only a low cytotoxic activity against SiHa cell line. Stimulation of PBMC with IL-2 or IL-2+anti-CD3 mAb resulted in a dramatic increase in the lysis of HPV-transformed keratinocytes, as already shown for other tumour cell types [27, 46]. Interestingly, SiHa cells exhibited a higher lysis in the presence of activated PBMC than did normal keratinocytes. This difference could be related to the loss of MHC class I expression on SiHa cells; these molecules have been shown to inhibit the LAK cytotoxicity (see [28] for review) and to be deleted in cervical carcinomas [11].

As expected, the cytotoxic activity of stimulated T cells was low in an allogeneic model, except when the cytotoxic assay was performed in the presence of PHA. This lectin acts as a bridge between effector and target cell. These data indicate that activated T cells could be useful in the elimination of HPV-infected keratinocytes when a target cell bridge is provided. The development of antibodies with a double specificity enables the effector cell to redirect its cytotoxicity specifically to the target cells [16]. Bispecific antibodies with CD3 and HPV-transformed keratinocytes binding sites (i.e. RL67 laminin receptor [2] or the epithelial growth factor receptor (EGFR) [34]) may play the part of a bridge between T effector cells and HPV-infected keratinocytes.

In addition, the low cytotoxic activity of T cells detected in a 4-h chromium-51 assay could also be explained by the use of a mechanism requiring more than 4 h, such as the cytotoxicity induced by IFNγ and TNFα. Stimulated PBMC produce IFNγ and TNFα [38]. The stimulation with IL-2+anti-CD3 mAb induced a larger amount of IFNγ and TNFα than the stimulation with IL-2 alone, and a higher tumour inhibition of other epithelial cell lines was observed when these cell lines were co-cultivated during 7 days with PBMC activated by IL-2+anti-CD3 mAb (unpublished data). We have previously demonstrated that IFNγ, especially when associated with TNFα, has a direct antiproliferative effect on HPV-transformed cell lines [12]. Moreover, IFNγ has been shown to inhibit HPV gene transcription [39, 54] and to up-regulate the expression of ICAM-1 in HPV-transformed keratinocytes [9]. This up-regulation could explain the greater infiltrative ability of IL-2+anti-CD3 mAb-stimulated lymphocytes, as these cells express high levels of ICAM-1 ligand (LFA-1) [27].

In summary, stimulated allogeneic PBMC were able to infiltrate an in vitro-formed epithelium reminiscent of a high-grade SIL observed in vivo. Infiltrating cells were T lymphocytes and, to a lesser degree NK cells. Together with an increase in the proportion of infiltrating cells, we observed increased in situ staining for apoptosis. By using a standard ^{51}Cr-release cytotoxic assay, we showed that the stimulation of PBMC induced LAK activity against HPV-transformed cell lines. This activity was mediated mainly by NK cells, but T cells also killed these target cell lines when PHA was added to the cytotoxic assay. This study demonstrated that HPV-transformed keratinocytes grown in organotypic cultures can be co-cultivated with immunocompetent cells. Our culture system may serve as a model for investigating novel immunotherapy protocols for the treatment of HPV-associated lesions.

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