Cytokine profile of draining lymph node lymphocytes in mice grafted with syngeneic keratinocytes expressing human papillomavirus type 16 E7 protein

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Studies on the immune response to human papillomaviruses are compromised by the extreme host and tissue specificity of these viruses. To circumvent this, a mouse model system has been used in which antigen is presented via a differentiated, syngeneic keratinocyte graft expressing human papillomavirus type 16 (HPV-16) E7 protein. Using this model, previous studies have shown that animals grafted with a high cell inoculum (1 x 10^7 NEK 16 cells) exhibit a delayed-type hypersensitivity response that is E7-specific and CD4^+^-mediated, but those receiving a low cell inoculum (5 x 10^5 NEK 16 cells) are rendered unresponsive to subsequent and repeated antigen challenge. To investigate the mechanisms underlying this phenomenon, we have analysed the early changes in the cytokine profile of the graft-draining lymph node (GDLN) after high- or low-dose grafts. At 4 days post-grafting, there was a peak secretion of IL-2 associated with a decreased secretion of IL-4 by γδ-TCR^+^ cells in the group receiving 1 x 10^7 NEK 16 cells. At 5 days post-grafting, there was a peak secretion of IL-10 by CD8^+^ cells in both the high- and low-dose graft groups compared with controls. In contrast, low dose-grafted animals showed an increase in IL-4 production by CD8^+^ cells at this time-point. Low antigen challenge in this model system is associated with the appearance of a CD8^+^ population in the GDLN that secretes both IL-4 and IL-10. This population may represent a Tc2 or Ts subset that could induce further unresponsiveness.

Introduction

Human papillomaviruses (HPVs) are small, double-stranded DNA viruses that, in natural infections, cause warts (benign epithelial proliferations) of the skin and internal squamous mucosae. These viruses are classified into genotypes on the basis of nucleotide sequence homology and, at present, at least 80 distinct genotypes have been isolated from clinical lesions, each type showing a predilection for a cutaneous or mucosal surface (de Villiers, 1994). The HPV types that infect the genital tract are of intense contemporary interest, because of evidence that infection with specific genital types, principally HPV-16 or -18, is the major risk factor for the subsequent development of carcinoma of the uterine cervix in women (IARC, 1995). HPV-16 is of particular interest, since at least 50% of invasive cervical cancers contain all or part of the HPV-16 genome. Since the early region of the genome encoding the E6 and E7 oncoproteins is invariably retained and expressed in both premalignant and malignant cervical lesions, these viral proteins could therefore be potential targets for immune-based therapies for these diseases. However, before such strategies can be implemented, the nature of the immune response to these proteins in the natural infection must be characterized.

Experimental studies on the immune response to HPVs are, however, compromised by the extreme tissue and host specificity of these agents and the absence of an in vitro culture system that supports a complete infectious cycle. The target cell for infection is the keratinocyte and, as far as is known, viral genes are expressed only in keratinocytes, with the infectious cycle being totally dependent on the implementation of the complete keratinocyte differentiation programme (Stanley, 1994). A mouse model that incorporates the exclusively epithelial nature of HPV has been developed to examine the host response to the HPV-16 early proteins E6 and E7. In this model, antigen is presented via a differentiated, syngeneic keratinocyte graft that expresses viral protein, a route comparable to that employed in the natural infection. Using this system, it has been shown that animals engrafted and then challenged with a recombinant vaccinia virus expressing HPV-16 E7 exhibit a delayed-type hypersensitivity (DTH) response that is E7-specific and CD4^+^-mediated (McLean et al., 1993).
The induction of a DTH response was measured in these studies because this type of response is associated with regression of HPV-induced genital warts (Coleman et al., 1994) and, taken together, these studies suggest that a T helper type 1 (Th1)-type response is associated with virus clearance. Subsequent studies demonstrated the phenomenon of immune unresponsiveness in this model. Thus, animals engrafted with a low cell inoculum (low antigen dose) were unresponsive to a secondary priming challenge with antigen, whether this was delivered as a keratinocyte graft, protein or recombinant vaccinia virus. This unresponsiveness to antigen was maintained despite repeated antigen challenge, and was associated with graft persistence (Chambers et al., 1994). There is evidence from clinical studies that progression in HPV-associated cervical disease involves a shift from a cell-mediated, Th1-type response to an antibody-associated, Th2 response (de Gruijl et al., 1993), and this raises the possibility that, in the mouse model, different antigen loads induce either a Th1- or a Th2-type response. If this assumption is correct, the switch in the response must take place within the first days after the primary antigen encounter, and should be reflected in the cytokine profile of the lymphocytes in the nodes draining the graft. The present report presents data that examine this hypothesis.

Methods

- **Animals.** Female BALB/c mice, 8–12 weeks of age, were obtained from Charles River. Mice were anaesthetized and a 22 mm coverslip was inserted under the skin in either flank. Seven days later, mice were anaesthetized again, the coverslips were removed and a grafting chamber was inserted in the same site. Mice in the experimental groups received either 1 × 10⁶ or 5 × 10⁶ NEK 16 cells in the chamber. Control mice received PBS. NEK 16 cells have been described previously as mouse neonatal keratinocytes transfected with the early region of HPV-16 (McLean et al., 1993). Mice were sacrificed at different time-points: 3, 4, 5 and 7 days after grafting. Experiments at 3 and 7 days after grafting were repeated twice, but once only at 5 days after grafting. Three experiments were set up for 4 days after grafting. The number were repeated twice, but once only at 5 days after grafting. Three experiments at 3 and 7 days after grafting

- **Lymphocyte isolation and culture.** Mice were sacrificed and the lymph nodes draining the graft (GDLN) from each mouse were isolated and processed individually. Single-cell suspensions were prepared by pressing the lymph node cells through a mesh (cell strainer Falcon 2340) with the help of a sterile syringe plunger. Cells were collected on complete medium (CM) containing RPMI 1640 (Gibco) supplemented with 10% heat-inactivated foetal calf serum (Gibco), 20 mM HEPES, 0.05 mM mercaptoethanol and antibiotics (penicillin, streptomycin). To induce cytokine synthesis and to facilitate its retention within the cytoplasm, a published protocol was followed (Openshaw et al., 1995), with slight modifications. Cells were resuspended at 1 × 10⁶ cells/ml and 2 ml suspension was deposited in a well of a 24 well plate and stimulated

with phorbol myristate acetate (50 ng/ml) (Sigma) plus ionomycin (500 ng/ml) (Sigma) for 4 h at 37 °C. Next, brefeldin A (Epicentre Technologies) was added at 10 pg/ml and the cells were incubated for a further 2 h. After the incubation period, cells were ready for the determination of surface markers and intracytoplasmic cytokines.

- **Flow cytometric simultaneous analysis of surface markers and intracellular cytokines.** After culture, cells were resuspended by pipetting and a 100 μl aliquot was deposited in each well of a V-bottomed 96 well plate (Costar). Plates were spun down and cells were resuspended in 100 μl PBS/2% BSA containing brefeldin A. Fifty μl CM containing the directly conjugated antibody (raised against mouse cell surface markers), prepared at the optimal dilution, was added to each well. Plates were incubated for 30 min at 4 °C in the dark. Plates were spun down and cells were washed twice with PBS/2% BSA. Cells were resuspended in 100 μl PBS/2% BSA/brefeldin A and 100 μl 4% paraformaldehyde, pH 7.4, was added. Cells were incubated for 20 min at room temperature. Plates were then spun down, cells were resuspended in 100 μl PBS/2% BSA/0.5% saponin and incubated for 10 min at room temperature to permit pore formation. Next, 50 μl CM, containing the directly conjugated antibody (raised against mouse cytokines) at the appropriate dilution, was added. Plates were incubated for 30 min at room temperature before being spun down. Cells were washed twice in PBS/2% BSA/0.5% saponin and once in PBS/2% BSA. Cells were resuspended in 2% paraformaldehyde, pH 7.4, and kept at 4 °C in darkness until analysed in a FACScan. Results were analysed with Lysis II software. All samples were run in the same conditions for forward and side scatter, FL1, FL2 and FL3. Lymphocytes were gated according to their scatter characteristics. In all experiments, unstained cells and cells stained separately with each fluorochrome were included to optimize compensation settings. A minimum of 10000 gated events was collected; in some experiments, 20000 or 30000 gated events were collected. Samples were gated on CD4⁺, CD8⁺ or γδ-TCR⁺ cells and the percentage of cells producing cytokines within the new gate was determined. To calculate the percentage of cells producing cytokines, the cursor was set up to exclude the non-specific binding produced by the respective directly conjugated isotype control. The individual results from each mouse, from different experiments, at the same time-point were added together to calculate means and SEM.

The directly conjugated antibodies used in this study were as follows: FITC–anti-mouse IPN-γ, FITC–anti-mouse IL-2, FITC–anti-mouse IL-4, FITC–anti-mouse IL-10, phycoerythrin (PE)–anti-mouse CD8 and FITC–anti-rat IgG1 were from Pharmingen, PE–anti-mouse IL-4, FITC–anti-mouse IL-10, Tricolor–anti-mouse CD4, Tricolor–anti-mouse CD8, Tricolor–anti-mouse γδ-TCR, FITC–anti-rat IgG2a and FITC–anti-rat IgG2b were from Caltag.

Statistical analysis was performed by ANOVA, considering a difference at the level of P < 0.05 to be significant. Groups showing statistical differences were reanalysed by using Student’s two-tailed t-test; the exact level of significance is shown in each case. Data are presented as means ± SEM.

Results

**Percentage of T cells in the GDLN after culture.**

The distribution of CD4⁺, CD8⁺ and γδ-TCR⁺ cells was studied after culturing GDLN cells obtained at days 3, 4, 5 and 7 after grafting. The percentage of CD4⁺ cells recovered from the cultures at days 3 and 4 after grafting did not differ between groups. However, at day 5 post-grafting, a higher percentage of CD4⁺ cells was present in mice engrafted
Cytokine profile and HPV-16 E7 skin grafts

Fig. 1. Percentage of T cells in the GDLN after culture. Animals received PBS (○, dashed line) or 5 x 10^5 (●, dotted line) or 1 x 10^7 (□, solid line) NEK 16 cells. Cells were cultured and analysed as described in Methods. The percentage of CD4^+ (a), CD8^+ (b) and γδ-TCR^+ (c) cells was determined at days 3, 4, 5 and 7 after grafting. Data are presented as means ± SEM.

with NEK 16 cells compared with sham-grafted controls; 40.48 ± 2.88 (n = 5) and 34.49 ± 0.68 (5) vs 29.14 ± 1.61 (5) (P < 0.026 and P < 0.032, respectively) (Fig. 1a). At day 7 after grafting, mice that received 1 x 10^6 NEK 16 cells had a lower percentage of CD4^+ cells after culture than mice that received 5 x 10^5 NEK 16 cells [39.60 ± 1.17 (11) vs 45.58 ± 1.24 (10); P < 0.003] (Fig. 1a).

There were no differences between the experimental groups in the percentage of CD8^+ cells recovered after culturing GDLN at days 3, 4 and 7 after grafting. At day 5 after grafting, the percentage of CD8^+ cells recovered after culture was higher in the group that received 1 x 10^6 NEK 16 cells when compared either with those that received 5 x 10^5 NEK 16 cells [10.98 ± 0.31 (5) vs 9.19 ± 0.55 (5); P < 0.034] or with the control group [10.98 ± 0.31 vs 8.82 ± 0.33; P < 0.005] (Fig. 1b). The percentage of γδ-TCR^+ cells recovered after culturing GDLN at the different time-points mentioned above did not change for the length of this study in the different experimental groups (Fig. 1c).

Percentage of CD4^+ GDLN cells that produced IFN-γ, IL-2, IL-4 and IL-10

The percentage of CD4^+ cells that produced IFN-γ, IL-2, IL-4 and IL-10 in the GDLN was studied at days 3, 4, 5 and 7 after grafting. No significant differences were observed after comparing the experimental groups at the different time-points studied (Fig. 2).

Percentage of CD8^+ GDLN cells that produced IFN-γ, IL-2, IL-4 and IL-10

No differences in the percentage of CD8^+ GDLN cells that produced IFN-γ and IL-2 were observed in the different experimental groups at any time-point after grafting (Fig. 3a,
Fig. 2. Percentage of CD4+ cells in the GDLN that produced IFN-γ (a), IL-2 (b), IL-4 (c) and IL-10 (d). Animals received PBS (○, dashed line) or 5 × 10^5 (△, dotted line) or 1 × 10^7 (□, solid line) NEK 16 cells. Simultaneous staining for surface markers and cytoplasmic cytokine was performed after culturing cells at days 3, 4, 5 and 7 after grafting.

There was no difference in the percentage of CD8+ GDLN cells that produced IL-4 at days 3, 4 and 7 after grafting. However, there was a significant increase in the percentage of CD8+ cells that produced IL-4 in the group that received 5 × 10^5 NEK 16 cells at day 5 after grafting when compared with the control group [0.47 ± 0.08 (5) vs 0.24 ± 0.02 (5); P < 0.05] (Fig. 3c).

The percentage of IL-10-producing CD8+ GDLN cells was not significantly different at days 3, 4 or 7 after grafting, but a significant increase in the percentage of CD8+ GDLN cells that produced IL-10 was observed at day 5 after grafting in the group that received 1 × 10^7 NEK 16 cells (Fig. 3d). This difference was significant when compared with the group that received 5 × 10^5 NEK 16 cells [4.15 ± 0.71 (5) vs 1.81 ± 0.39 (5); P < 0.032] and when compared with the control group [4.15 ± 0.71 (5) vs 0.73 ± 0.13 (5); P < 0.007].

Percentage of γδ-TCR+ GDLN cells that produced IFN-γ, IL-2 and IL-4

The percentage of γδ-TCR+ GDLN cells that produced IFN-γ did not differ between the experimental groups at any of the time-points studied (Fig. 4a). The percentage of γδ-TCR+...
GD LN cells that produced IL-2 did not differ between groups at days 3 and 5 after grafting. However, at day 4 post-grafting, IL-2 production was significantly increased in the group that received $1 \times 10^7$ NEK 16 cells when compared with the group that received a smaller number of NEK 16 cells $[12.57 \pm 0.7 (5) \text{ vs } 9.53 \pm 0.4 (5); P < 0.0096]$ (Fig. 4b). At day 7 after grafting, the percentage of γδ-TCR$^+$ GD LN cells that produced IL-2 was lower in the group that received a larger number of NEK 16 cells than in the control group $[5.52 \pm 0.68 (11) \text{ vs } 8.85 \pm 0.51 (7); P < 0.004]$. This pattern of IL-2 secretion by γδ T cells in the high antigen dose group is comparable to that reported to occur in trinitrochlorobenzene-induced contact sensitivity (Dieli et al., 1998).

The percentage of γδ-TCR$^+$ GD LN cells that produced IL-4 did not differ between the experimental groups at days 3 and 5 after grafting (Fig. 4c). At day 4 after grafting, the percentage of γδ-TCR$^+$ GD LN cells that produced IL-4 was lower in both groups that received NEK 16 cells when compared with the control group $[1.93 \pm 0.17 (5) \text{ vs } 3.53 \pm 0.39 (5); P < 0.01; \text{ and } 2.14 \pm 0.11 (5) \text{ vs } 3.53 \pm 0.39 (5); P < 0.015]$. At day 7 after grafting, the percentage of γδ-TCR$^+$ GD LN cells that produced IL-4 in the group that received $5 \times 10^6$ NEK 16 cells...
was lower than in the group that received \(1 \times 10^7\) NEK 16 cells \([0.82 \pm 0.08 (10)\) vs \(1.23 \pm 0.14 (11); P < 0.028]\) and in the control group \([0.82 \pm 0.08 (10)\) vs \(1.59 \pm 0.13 (7); P < 0.0002]\).

**Discussion**

We have used a mouse model that mimics the natural route for presentation of HPV-16 E7 antigen (McLean et al., 1993; Chambers et al., 1994) in order to investigate the early changes in the cytokine profile of GDLN lymphocytes after challenge with high and low doses of antigen. The rationale for this study was based upon previous findings that showed that the number of keratinocytes expressing HPV-16 E7 inoculated in the first graft was associated with either a strong DTH response or unresponsiveness to subsequent challenge with a priming antigen dose (Chambers et al., 1994). These observations led to the hypothesis that the cytokine profile induced by different antigen loads would be different and could determine the nature of the ensuing immune response. The possibility that the initial exposure to antigen could determine the response to subsequent antigen challenge could be of clinical importance. In HPV-associated cervical disease, neoplastic progression seems to be associated with virus persistence and a failure to clear and resolve HPV infection, despite an immune response to early viral proteins (de Grujil et al., 1996; Nimako et al., 1997).

The data presented in Figs 2, 3 and 4 allow us differentiate the general time-dependent pattern of cytokine secretion in our experimental model from the differences amongst groups at the different time-points analysed. The cytokine profile of
CD4+ cells suggested that different numbers of CD4+ cells were engaged in cytokine production at different time-points. This pattern was time-dependent and not group-dependent. Moreover, no difference was found at any time-point studied in the percentage of CD4+ cells that produced IFN-γ, IL-2, IL-4 and IL-10 amongst CD4+ GDLN cells from mice that received different numbers of NEK 16 cells. These data indicate that the hypothesis that there would be a clear difference in the induction of a Th1- or Th2-type response, depending on the number of cells grafted, was not tenable.

The production of IFN-γ and IL-2 by CD8+ GDLN cells also failed to discriminate between groups at any time-point studied. However, γδ-TCR+ GDLN cells from mice that received a larger number of NEK 16 cells showed an increase in the percentage of cells that produced IL-2 associated with a decrease in IL-4 production at day 4 after grafting. The production of IL-4 and IL-10 by CD8+ cells showed striking differences at day 5 after grafting. CD8+ cells from mice that received $5 \times 10^5$ NEK 16 cells produced more IL-4 and IL-10 than the control group. Moreover, CD8+ cells from mice grafted with $1 \times 10^7$ NEK 16 cells produced more IL-10 than CD8+ cells from mice that received $5 \times 10^5$ NEK 16 cells or PBS. These data suggest that the essential differences in the cytokine profiles of the two experimental groups were (i) the induction, in animals that received a priming antigen dose, of a peak secretion of IL-2 by γδ-TCR+ cells 4 days after grafting and (ii) a peak secretion of IL-10 by CD8+ cells 5 days after grafting, together with a simultaneous up-regulation of IL-4 and IL-10 production by CD8+ cells at day 5 in animals that received a low antigen dose.

The evidence of McLean et al. (1993) showed clearly that the DTH response to E7 in this model is mediated by CD4+ cells, since treatment of animals with anti-CD4 antibodies abolished the E7-specific DTH response. Nevertheless, the results described above suggest that this is not a conventional Th1 response. This is not surprising, since several events are occurring in the grafts. The grafting procedure itself induces an inflammatory response, and this will change the cytokine milieu. Furthermore, not only is an E7-specific DTH response engendered by the priming grafts, but these grafts are then recognized as foreign and rejected by the hosts, usually by day 14–16 post-grafting. The complex cytokine profile of the GDLN, with the secretion of IL-10 by CD8+ cells, reflects the complexity of these events.

Our data also suggest a complex collaboration between γδ-TCR+ cells and CD8+ cells in the GDLN at the inductive phase of the immune response against NEK 16 cells. These data are intriguing, since a similar cross-talk and temporal relationship has been described in contact sensitivity (Dieli et al., 1998). In this study, it was shown that, while the contact sensitivity reaction towards the hapten tri-nitrochlorobenzene persists for up to 21 days in the host, it can only be transferred successfully into naive hosts at days 4 and 5 after immunization. Importantly, it was further demonstrated that IL-2-producing γδ-TCR+ cells obtained from the draining lymph nodes at days 4 and 5 after immunization were strictly required for a successful transfer (Dieli et al., 1998). There is little information on the role of γδ T cells in the immune response to papillomavirus infections. Spontaneous regression of bovine papillomavirus-induced warts is associated with a significant γδ T cell infiltrate in the lesions (Knowles et al., 1996). However, this phenomenon is not observed in spontaneous regression of HPV-6/-11-induced genital warts (Coleman et al., 1994) or oral warts induced in the dog by the canine oral papillomavirus (P. K. Nicholls, personal communication). Further studies on γδ T cell function are needed in both experimental and natural papillomavirus infections.

In the original view of the Th1/Th2 dichotomy, IL-10 is secreted by Th2-type cells that inhibit cytokine secretion by Th1-type cells, switching the immune response towards a Th2 type (Howard & O’Garra, 1992). However, IL-10 can enhance IL-2-mediated induction of CTL differentiation (Chen & Zlotnik, 1991) and human IL-10 is a chemoattractant for CTLs (Jinquan et al., 1993). Furthermore, IL-10 stimulates human NK cell function (Carson et al., 1995) and IL-10 secreted by transfected tumour lines enhances anti-tumour activity by activating NK and CTL cells (Giovarelli et al., 1995). Hence, the presence of CD8+ cells that secrete IL-10 in mice grafted with a priming dose of NEK 16 cells could favour the recruitment of E7-specific CTL and/or NK cells.

The generation of a CD8+ population that secretes IL-4 and IL-10 in the GDLN of mice grafted with $5 \times 10^5$ NEK cells could be associated with the induction of a conventional type 2 response mediated via CD8+ cells. There is evidence for a dichotomy amongst CD8+ cells. This phenomenon is well illustrated in lepromatous leprosy (Salgame et al., 1991), where CD8+ and CD4+ clones were isolated from both lesions and blood from patients. CD8+ clones produced IL-4 and suppressed the proliferation of CD4+ clones in vitro. Recently, it has been shown that low-dose tolerance to chemical allergens applied on the skin is mediated by CD8+ cells with a type 2 cytokine profile (Steinbrink et al., 1996). These authors have shown that the induction of tolerance is strictly dependent on the amount of antigen used. This model closely resembles our system, since in both cases the access of the immune system to the antigen is very limited and in both experimental models the final outcome of the immune response depends strictly on the antigen load.

In conclusion, in our experimental model, when animals were challenged locally by a high antigen dose via a graft of keratinocytes expressing HPV-16 E7, two distinctive events were observed in the lymphocytes of the GDLN: an increased secretion of IL-2 by γδ lymphocytes at day 4 post-grafting; and, at day 5, a peak secretion of IL-10 but reduced secretion of IL-4 by CD8+ cells. In contrast, in the GDLN of animals challenged with a low antigen dose, the response at day 5 was mediated by CD8+ cells (Tc2 or Ts) that secreted both IL-4 and IL-10, a scenario that could induce further unresponsiveness.
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References


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