Production and Characterization of Human Proliferative T-Cell Clones Specific for Human Papillomavirus Type 1 E4 Protein

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Human papillomavirus type 1 (HPV1) virions and E4 protein purified from cutaneous warts were tested in lymphocyte proliferation assays using normal individuals. Both antigens were found to be capable of eliciting good lymphoproliferative responses. Several T-cell clones specific for wart E4 protein were obtained from a donor who had consistently responded very well to E4 in these initial assays. They were maintained in culture by repeated stimulation with antigen and interleukin-2, using an autologous mitomycin-treated lymphoblastoid cell line as a source of antigen-presenting cells. Two of these clones (3F5 and 4A8), which behaved identically, have been studied in more detail. A series of overlapping synthetic peptides covering the entire E1-E4 protein sequence was used to identify a single T-cell epitope which maps to a strongly hydrophilic region spanning amino acid residues 38 to 50. We have also tested the ability of a panel of major histocompatibility complex class II-matched and -mismatched lymphoblastoid cell lines to present this peptide to the T-cell clones in proliferation assays. The study reports that the epitope is restricted through HLA-DQ7 and that it can be recognized by T cells with different T-cell receptor gene rearrangements.

Human papillomavirus type 1 (HPV1) is predominantly associated with benign papillomas (warts) occurring on the palmar and plantar surfaces of the hands and feet. With time, most skin warts are likely to undergo spontaneous regression (6), and it is generally believed that host immune mechanisms are involved in the control of HPV infection. Many studies of humoral immunity to HPV have been carried out in a variety of patients with both skin and anogenital warts (for a review, see reference 21). Despite the fact that HPV-specific immunoglobulins have been found in both human sera and cervical secretions, we still do not know whether these antibodies are the cause or effect of wart regression, nor do we understand their role in protection.

Evidence that cellular immune responses are more vitally involved in the outcome of HPV disease is more convincing, particularly with serotypes which infect skin. The incidence of nonregressing warts increases dramatically in immunocompromised patients. This general category includes individuals with the rare genetically determined disorder epidermodysplasia verruciformis, those infected with human immunodeficiency virus or with malignant disease, or patients receiving immunosuppressive therapy (reviewed in reference 2). These widespread wart infections appear to be associated specifically with depressed T-cell function and not with selective B-cell defects (20, 35). It has also been shown in both animals (36, 57) and humans (3, 28, 51, 52) that spontaneously regressing warts are characterized by lymphoid cell infiltration, a fact which further underlines the importance of cellular responses.

More specific investigations of cell-mediated immunity have also been described. Early studies looked at lymphocyte responses in vitro (27, 35, 37, 38) and at delayed-type hypersensitivity reactions after the injection of viral particles in vivo (32, 57). It was evident from these results that

T-cell-mediated responses were predominant in patients whose warts regressed spontaneously, that few individuals were positive while they were currently infected, and that weak or negative responses were obtained in patients who had warts of long duration (reviewed in reference 7). Lymphoproliferative responses to purified HPV virions and fusion proteins have also been demonstrated in small numbers of women attending colposcopy clinics (11, 12). Generally, the level of lymphocyte stimulation was low, and the results underlined the fact that well-defined antigens were required for these types of study. No attempt was made to analyze the antigen specificity of the T-cell response.

To successfully prevent or treat HPV infection by immunological means such as vaccination, we need to identify the viral epitopes recognized by the human immune system and to understand how immune responses are controlled by genes of the major histocompatibility complex (MHC). Mapping of T-cell epitopes has largely been confined to HPV16, and most studies have used proliferative assays and so detected helper T-cell determinants. Several studies have been carried out in mice following immunization with recombinant HPV proteins or synthetic peptides (10, 13, 46, 53), but some human T-cell epitopes have also been reported (1, 50). Several groups have also attempted to define the MHC restriction of these proliferative T cells to HPV antigens in vitro (1, 13, 46, 50). The profile of responses was found to vary greatly among individual haplotypes, which has important implications when one considers how well epitopes are recognized in outbred populations such as humans. There is still little information available concerning HPV-specific cytotoxic T cells although Stauss et al. (48) have recently reported use of the murine antigen-processing mutant RMA-S cell line (54) for the induction of HPV16 peptide-specific cytotoxic T cells in vitro.

Animal vaccination studies are well under way in cattle (30, 31), and the results have shown that immunity to bovine papillomaviruses is strictly type specific. In addition, it has

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been demonstrated in mice that HPV16 E7 can serve as a target for a tumor-specific rejection response, the first report suggesting a role for an HPV-encoded protein as a tumor rejection antigen (8). Inferential evidence that the MHC is involved in the control of HPV infection has also been reported. It has been suggested that there is an association between the MHC class II antigen HLA-DQw3 and susceptibility to cervical carcinoma (58). In addition, using rabbits infected with the Shope cottontail rabbit papillomavirus, Han et al. (25) were able to find strong linkage between wart regression and a DR α gene fragment and between malignant transformation and a DQ α fragment. This finding indicates that wart evolution may in part be controlled by genes in the class II region of the MHC.

Overall, the evidence suggests that there is an immune response to HPV, but there may be important differences between the HPV types with respect to their immunogenicities at various sites and exactly which viral proteins are targeted by the immune system. This study looks at human proliferative (helper) T-cell responses to purified HPV1 proteins. Relatively large amounts of virus and E4 protein can be obtained from HPV1-induced warts (16, 17), which therefore represent a useful system in which to begin immunological investigations. We have also carried out a more detailed analysis of the human T-cell response to HPV1 E4 with respect to antigen specificity and HLA restriction.

MATERIALS AND METHODS

Preparation of viral antigens. Skin parings obtained from individual verrucae were collected from local chiropodists and stored at -20°C . HPV1-induced lesions were identified, and HPV1 virions were purified from the tissue according to methods described by us previously (49). HPV1 E4 was prepared from tissue pellets obtained after the first round of centrifugation during virion purification. The pellets were homogenized in phosphate-buffered saline (PBS), and the E4 protein was affinity purified by using the HPV1 E4-specific monoclonal antibody (4.37) described by Doorbar et al. (17). The HPV1 E4 protein expressed in insect cells by using the baculovirus expression system (39a) was purified from cell extracts by the same procedure. All viral antigen preparations were dialyzed extensively against PBS at 4°C and then filter sterilized prior to use in tissue culture.

Preparation of peripheral blood mononuclear cells. Venous blood samples (60 ml) were taken from normal healthy donors and placed in heparinized (20 U/ml) RPMI 1640 medium (GIBCO), and unfractionated mononuclear (UM) cells were separated by isopycnic centrifugation on lymphocyte separation medium (Flow Laboratories). Purified UM cells were washed three times in PBS and were used as required or cryopreserved in liquid nitrogen.

Short-term proliferation assays. UM cells were seeded in 96-well round-bottom plates at 2×10^5 cells per well in RPMI 1640 containing 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg of gentamicin sulfate per ml, and 10% (vol/vol) HPV1-seronegative human serum (complete medium). Various amounts of the appropriate viral antigen were added to the well in quadruplicate, and the cultures were incubated at 37°C for 7 days, with the addition of 1 μCi of [^3H]thymidine ([^3H]TdR; Amersham International) per well over the last 24 h. Control wells which contained only medium and cells but no antigen were also prepared. Lymphocyte proliferation was assessed by harvesting the cells onto glass fiber filters and measuring [^3H]TdR incorporation, using the LKB Beta-plate system (Pharmacia).

T-cell cloning. T cells were cloned by limiting dilution according to the method described by Lamb et al. (34). Seven-day in vitro-stimulated UM cultures were set up initially in 24-well plates (2×10^6 cells per well), using optimal concentrations of antigen. Proliferating T cells were isolated from the cultures by centrifugation through lymphocyte separation medium and collection of the enriched blast cell fraction from the interface. Briefly, the T cells were then seeded at densities of 30, 3, and 0.3 cells per well in Terasaki plates with the autologous mitomycin-treated lymphoblastoid cell line (LCL) (10^4 cells per well) as a source of antigen-presenting cells (APCs). The complete medium was supplemented with interleukin-2 (IL-2)-containing supernatant from the MLA 144 cell line (39) to a final concentration of 20% (vol/vol). After 8 to 12 days, wells containing proliferating cultures were harvested and expanded into flat-bottom 0.2-ml wells and then 2-ml wells. The cells were fed twice weekly with IL-2-containing medium and restimulated every 7 days with fresh antigen and APCs (5×10^5 cells per ml). Surface marker analysis of T-cell clones was performed by standard indirect immunofluorescence with the monoclonal antibodies OKT3 (anti-CD3), OKT4 (anti-CD4), and OKT8 (anti-CD8; Ortho Diagnostics).

The LCLs used as APCs during cloning and subsequent proliferation assays were generated from HLA-typed donors by transformation of normal B cells with the B95.8 strain of Epstein-Barr virus and were maintained in RPMI 1640 medium containing 2 mM L-glutamine and 10% fetal calf serum.

Proliferation assays on T-cell clones. The proliferative specificity of the T-cell clones was assessed under various conditions. Assays were carried out in 96-well flat-bottom plates at the end of a 7-day passage in culture so that the cells were not rapidly proliferating in response to the IL-2 used for feeding. T cells (2×10^4 cells per well) were assayed against specific antigens or peptide, using mitomycin-treated APCs (10^5 cells per well) in complete medium containing no IL-2. Control wells containing T cells alone or T cells and APCs alone were also prepared. Cultures were set up in triplicate, pulsed with [^3H]TdR (1 μCi per well) after 3 days, and harvested on the 4th day.

In some experiments, monoclonal antibodies to HLA-DP, -DQ, and -DR (Becton Dickinson) were tested for the ability to inhibit antigen presentation to T cells. In this case, APCs were seeded as described above in complete medium containing the appropriate concentrations of the monoclonal antibody and incubated for 1 h at 37°C prior to the addition of T cells.

DNA analysis of T-cell clones. High-molecular-weight DNA was extracted from T cells (1×10^7 to 2×10^7 cells), using the procedure described by Lahiri and Nurnberger (33).

For Southern blot analysis, DNA was digested with either *Bam*HI or *Eco*RI, which differentiates T-cell receptor (TCR) gene rearrangements from the germ line configuration. DNA was size fractionated on an 0.8% agarose gel and transferred to Hybond N+ nylon filter (47). Filters were hybridized to ^{32}P -labeled probes (a 0.7-kb *Hind*III DNA fragment specific for the J γ region and a 0.7-kb *Hind*III-*Eco*RI fragment specific for the C β region), using appropriate conditions, and exposed for up to 14 days. DNA from cultured human fibroblasts was used as a germ line control.

T-cell DNA was also analyzed by the polymerase chain reaction (PCR), which was performed essentially according to the method of Saiki et al. (44). Reaction mixtures (50 μl) contained 100 μg of DNA, 0.25 μg of each 5' and 3' oligonucleotide primer, 0.2 mM each dATP, dCTP, dGTP,

TABLE 1. Lymphoproliferative responses to purified HPV1 virions and HPV1 E4 protein

Donor	SI		Antibody status to HPV1 virions ^a	Wart history
	Virions	E4		
1	3.5	2.0	—	No history of skin warts
2	4.1	1.6	+++	
3	0.9	NT ^b	+++	
4	1.4	2.9	+++	Had warts at time of sampling
5	10.9	NT	+	
6	1.5	NT	NT	
7	10.4	9.1	+++	Had warts treated
8 ^c	10.4	26.4	++	
9	11.9	18.6	NT	
10	0.9	0.9	+	
11	1.0	8.3	NT	
12	1.6	1.2	NT	
13	NT	1.3	+	
14	4.3	2.4	NT	Had warts which had regressed
15	6.8	7.9	NT	
16	9.6	18.7	++	
17	1.2	1.0	++	

^a Assessed several months previously by immunoprecipitation of ¹²⁵I-labeled HPV1 virions (49).

^b NT, not tested.

^c The donor whose E4-specific T cells were cloned.

and dTTP, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, and 1 U of *Taq* DNA polymerase (Boehringer). The amplification was performed with two sets of nested TCR- γ primers, outer (V γ [5'-GTGCTTCTAGCTTTCCTGTCTC-3'] and J γ [5'-CACCTTGTTGTGAACAACAGTGTC-3']) and inner (V γ [5'-GAGTACGCTGCCTACAGAGAGG-3'] and J γ [5'-TTCTTTGAGAAACCGTCACC-3']), in a PCR processor (LEP Scientific). In the first round of amplification, 25 cycles were performed (94°C for 45 s, 58°C for 1.5 min, and 72°C for 1 min); in the second round, 35 cycles (94°C for 45 s, 58°C for 1.5 min, and 72°C for 1 min) were performed. After amplification, the products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Proliferative responses of unfractionated mononuclear cells to HPV1 virions and purified HPV1 E4 protein. HPV1 virions and E4 protein purified from clinical material were tested for the ability to stimulate UM cells from 17 laboratory donors in initial proliferation assays. The results in each case were expressed as a stimulation index (SI), calculated as follows: SI = cpm obtained in presence of antigen/cpm obtained in absence of antigen. A SI of greater than 2 is generally considered significant. Both antigens were capable of eliciting very good lymphoproliferative responses in a number of cases, with 56% of individuals giving an SI of greater than 2 against purified HPV1 virions and 64% giving an SI of greater than 2 against purified HPV1 E4 (Table 1). Proliferation was mostly seen to both proteins in responding individuals and was generally higher in response to purified E4. Both antigens were tested over a range of concentrations (0 to 50 μ g/ml), and the SI shown is the maximum obtained in the presence of optimal amounts of protein (usually 30 to 40 μ g/ml).

FIG. 1. Proliferative response of T-cell clone 3F5 after stimulation with overlapping synthetic peptides (1 to 23) covering the entire HPV1 E1^{E4} molecule. The peptides were added to a final concentration of 10 μ g/ml in each well, and proliferation was assessed by [³H]TdR labeling over the final 24 h of a 4-day culture period. The first two control columns represent the background counts per minute obtained in the absence of peptide, using T cells alone and T cells with APCs. The autologous mitomycin-treated LCL was used as a source of APCs. Error bars represent the standard deviation of triplicate wells.

Although the numbers tested in this assay were low, it appears that individuals who had had warts treated generally gave the highest SIs to both HPV1 virions and E4 protein. In most cases, the antibody status of the donors had been determined by using ¹²⁵I-labeled HPV1 virions in an immunoprecipitation assay several months previously (49), but this did not correlate with the lymphoproliferative responses obtained here (Table 1).

Cloning of E4-specific T cells. An enriched population of T lymphoblasts present in 7-day E4-stimulated cultures of UM cells from donor 8 (Table 1) was obtained by centrifugation through lymphocyte stimulation medium and cloned by limiting dilution. Outgrowth of T cells was observed in 30 of 288 cultures seeded at 30 cells per well and in 1 of 288 cultures seeded at 3 cells per well, and these colonies were expanded by using the protocol described in Materials and Methods. Such populations are here designated as clones by virtue of their derivation from limiting dilution cultures. Direct proof of monoclonality was obtained subsequently by DNA analysis of TCR gene rearrangements (see Fig. 6).

Of the 31 clones obtained from donor 8, 8 were shown to proliferate specifically when stimulated with the native wart E4 protein and also with the baculovirus-expressed E4. A more detailed examination of the antigen specificity and the possible HLA restriction of antigen recognition was carried out with two of these E4-specific T-cell clones (3F5 and 4A8), which were shown to be CD3⁺ CD4⁺ CD8⁻ by immunofluorescence surface staining (data not shown). These two clones behaved identically in all of the assays performed, and so only the results obtained from 3F5 will be reported here.

Peptide specificity of T-cell clone 3F5. To identify which part of the E4 protein was being recognized by clone 3F5, overlapping synthetic peptides covering the entire sequence of the E1^{E4} molecule were made. The resulting 23 peptides (15-mers; overlapping by 10 amino acids) were used in

FIG. 2. (a) Peptides A to J synthesized on the basis of the results shown in Fig. 1. Peptides A, B, and C are identical to peptides 7, 8, and 9 from the first set (1 to 23), and D comprises the overlapping 10-mer between B and C. E to J represent nested sets of peptides derived from D. (b) Stimulation of clone 3F5 with peptides A to J at a final concentration of 10 μ g/ml. The results show the proliferation of T cells alone and of T cells and APCs (autologous mitomycin-treated LCL) and also their responses to whole protein (baculovirus-expressed E4) and IL-2. Proliferation was assessed by using the [3 H]TdR uptake assay previously described after 4 days in culture. Error bars represent the standard deviation of triplicate wells. (c) Nested set of peptides K to N derived from the overlapping 10-mer between B and C working toward the N terminus of peptide B. (d) Proliferative response of clone 3F5 to peptides D, K to N, and B used at 10 μ g/ml. Additional columns show the background counts per minutes for T cells or T cells and APCs and also the T-cell responses to native wart E4 or IL-2. APCs were obtained by using the autologous mitomycin-treated LCL, and the same [3 H]TdR uptake assay was used after 4 days in culture to measure proliferative responses. Error bars represent the standard deviation of triplicate wells.

proliferation assays with T cells from clone 3F5. Reactivity to peptides 8 and 9 was clearly and consistently seen (Fig. 1). The sequence covered by these two peptides spans amino acid residues 36 to 55 on the E1 Δ E4 molecule and represents a strongly hydrophilic region containing several putative phosphorylation sites (23). To confirm these peptide specificities, and in an attempt to more precisely define the epitope, several more peptides (A to J) were made (Fig. 2a). Peptides A, B, and C were identical to peptides 7, 8, and 9 from the first set. D was the overlapping 10-mer between peptides 8 and 9, and the remaining (E to J) were smaller peptides derived from this overlapping sequence. When this set of peptides was tested with clone 3F5, only peptides B and C (identical to peptides 8 and 9) were recognized once again. Neither the overlapping 10-mer nor any of the peptides derived from it were reactive (Fig. 2b). One further set of peptides (K to N) was synthesized, working from the overlapping sequence toward the N terminus of peptide 8, which was where the predominant reactivity appeared to lie (Fig. 2c). Clone 3F5 responded to peptides M and N as well as did the original peptide 8 (Fig. 2d). The smallest sequence

recognized, or the minimal epitope seen by clone 3F5, therefore corresponds to peptide M, which is a 13-mer covering amino acids 38 to 50 of the E1 Δ E4 protein (Fig. 3). The addition of either one or both of the threonine residues at the N terminus (peptide M or 8, respectively) did not appear to greatly affect T-cell recognition. These experiments were carried out several times and consistently gave the same results.

HLA restriction of peptide responses. Monoclonal antibodies to HLA class II molecules were used in experiments to block antigen presentation in an attempt to define the MHC restriction of the T-cell clones (Fig. 4). At low concentrations, these antibodies tended to stimulate T-cell proliferation to a greater extent than when peptide alone was used, and all of them were inhibitory when added in large amounts. Although these results were not totally conclusive, they did suggest that the restriction was through the HLA-DQ locus. It can be seen that the anti-DR antibody did not appear to inhibit peptide presentation appreciably (Fig. 4c), whereas the anti-DQ antibody was far more inhibitory at much lower

Met Ala Asp Asn Lys Ala Pro Gln Gly Leu Leu Gly Leu Leu Gln
M A D N L A P Q G L L G L L Q

Tyr Thr Pro Thr Thr Gln Pro Tyr Pro Arg Val Thr Pro Pro Ser Asp
Y T P T T Q P Y P R V T P P S N

Arg Arg Pro Ser Thr Thr Pro Asn Ser Gln Asp Arg Gly Arg Pro
R R P S T T P N S Q D R G R P

Arg Arg Ser Asp Lys Asp Ser Arg Lys His Leu Tyr Ala Asp Gly
R R S D K D S R K H L Y A D G

Leu Thr Asp Gly Glu Asp Pro Glu Val Pro Glu Val Glu Asp Glu Glu
L T D G E D P E V P E V E D E E

Lys Glu Asn Gln Arg Pro Leu Gly His Pro Asp Leu Ser Leu Leu Arg
K E N Q R P L G H P D L S L L R

Glu Thr Leu Glu Val Tyr Thr Gln Arg Leu Lys Arg Asp Ile Leu Gln
E T L E V Y T Q R L K R D I L Q

Asp Leu Asp Asp Phe Cys Arg Lys Leu Gly Ile His Pro Trp Ser Val
D L D D F C R K L G I H P W S V

FIG. 3. Amino acid sequence of the HPV1 E1/E4 protein showing the region covered by peptide M. This 13-mer was the shortest sequence recognized by clone 3F5 (Fig. 3) and spans residues 38 to 50, which represent a strongly hydrophilic region of the molecule.

concentrations than was the anti-DP antibody (Fig. 4a and b).

Panels of allogeneic LCLs were used to confirm and further define the HLA antigen involved in the response of clone 3F5 to peptide 8. These LCLs were treated with mitomycin and used in proliferation assays as APCs in exactly the same way as were the autologous LCL. Initially, a number of cell lines matched and mismatched at the HLA-DR locus were investigated (Fig. 5a). The donor was DR3,4, and LCLs matched at one, neither, or both of these alleles were tested for the ability to present peptide in comparison with the autologous line. The results strongly suggested that the HLA restriction of this peptide response was not through DR. Identical experiments examining DQ restriction were then carried out (Fig. 5b). In this case, antigen presentation to the 3F5 clone was observed with the autologous (DQw2,7) LCL, with an LCL matched at both alleles and with several lines sharing only the DQ7 allele. None of the complete mismatches or those LCLs sharing only the DQw2 antigen could present peptide in these assays. In contrast, therefore, the data in Fig. 5b showed quite convincingly that the response of clone 3F5 (and 4A8 [data not shown]) to peptide 8 was restricted through HLA-DQ7.

DNA analysis of T-cell clones. The possibility existed that clones 3F5 and 4A8 were not unique but derived from a single T-cell population. To investigate this possibility and to examine status of these clones, Southern blotting and PCR studies were carried out as reported below.

EcoRI digests of DNA from T-cell clones 3F5 and 4A8 were electrophoresed, blotted, and hybridized to a probe specific for the C β region of the TCR gene. The germ line control DNA gave two bands (4 and 12 kb), and both T-cell clones showed rearrangements (Fig. 6a). When *Bam*HI-digested DNA was probed with a fragment specific for the J γ region of the TCR gene, only clone 3F5 showed rearrangement in one of the germ line alleles, whereas clone 4A8 remained in the germ line configuration (results not shown).

PCR was also performed on DNA from the two T-cell

FIG. 4. Monoclonal antibody blocking of the proliferative response of clone 3F5. The results show the response of T cells to peptide 8 (10 μ g/ml) in the presence of various amounts of monoclonal antibody (anti-DP [a], anti-DQ [b], or anti-DR [c]). Control columns in each case show the background counts per minute obtained for T cells or T cells and APCs (autologous LCL) alone and also the results when an irrelevant monoclonal antibody was used. The [3 H]TdR uptake assay was used to measure proliferative responses after 4 days in culture. Error bars represent the standard deviation obtained from triplicate cultures.

FIG. 5. Presentation of peptide 8 to clone 3F5, using autologous and allogeneic LCLs as APCs. The LCLs were obtained from HLA-typed laboratory donors and were matched or mismatched at either the HLA-DR (a) or the HLA-DQ (b) locus. The degree of matching between T cells and APCs is as indicated. Results show the proliferation of T cells when cultured with the APCs in the presence (□) and absence (■) of peptide 8 (10 µg/ml), and responses were measured by [3 H]TdR uptake after 4 days in culture. Error bars represent the standard deviation obtained from triplicate wells.

clones, using primers designed to look for TCR- γ gene rearrangements. With clone 3F5, a band of approximately 450 bp was seen, whereas DNA from clone 4A8 and the germ line control was not amplified (Fig. 6b). These results agreed with the Southern blot analysis in that the TCR- γ gene appeared to be rearranged in clone 3F5 but not in clone 4A8.

In summary, the DNA analysis suggested that the two clones 3F5 and 4A8 were different, with 3F5 showing rearrangement of both the TCR- β and - γ genes and clone 4A8 showing rearrangement of only TCR- β .

DISCUSSION

The lymphocyte proliferation assay is useful as an in vitro guide to the existence of T cells already sensitized to a particular antigen in vivo. During this study using native antigens, several individuals responded very well to both HPV1 virions and E4 purified from cutaneous warts. The blastogenic responses were generally quite good, particularly when E4 protein was used (Table 1). This finding is in contrast with those of earlier human studies using native proteins, in which the SIs obtained were low (11, 35).

When HPV1 virions were used as antigens in these assays, we cannot be absolutely sure about the actual target for the proliferative responses, since the viral particles consist of both the L1 and L2 structural proteins (18). The use of the baculovirus-expressed HPV1 L1 and L2 in insect cells (14)

FIG. 6. (a) *Eco*RI digests of DNA from T-cell clones 3F5 and 4A8 hybridized to a probe specific for the C β region of the TCR gene. Control DNA was obtained from a human fibroblast cell line and showed the germ line configuration of two bands at 4 and 12 kb. (b) PCR using primers to identify TCR- γ gene rearrangements in T-cell clones 3F5 and 4A8. Germ line DNA from human fibroblasts was used as a control.

should enable us to delineate these results and to determine whether both of these proteins can be targets of the T-cell response.

Work has also been carried out to look for proliferative T-cell responses to HPV16, but because virus production is too low to enable preparation of sufficient amounts of viral antigen (22), these studies have relied on the use of recombinant proteins and/or synthetic peptides for initial immunological studies in both the mouse (10, 13, 46, 53) and humans (1, 12, 50). Unfortunately, positive responses in these systems could not be checked against native bona fide proteins.

The analysis of human T-cell function is difficult because of the heterogeneity of the T-lymphocyte population, and it is essential to obtain antigen-specific monoclonal T cells for detailed study. Although other groups have obtained human lines and clones following stimulation with HPV peptides (1, 50), this is the first report describing human HPV-specific T-cell clones raised against native viral protein, an approach which greatly increases the chances of obtaining biologically relevant information. Our study shows that for the HPV1 E4 protein, the baculovirus system produces a papillomavirus protein which can maintain the proliferation of T-helper cells to the same extent as can the clinically derived protein. Not all of the T-cell clones obtained from the short-term UM cultures showing HPV1 E4-induced proliferation were in fact E4 specific, and there is clearly involvement of other activated CD4 $^{+}$ T cells in the polyclonal short-term response. This report concentrates on two E4-specific clones (3F5 and 4A8) from donor 8 (Table 1) which behaved identically throughout the study.

We now know that T cells recognize protein antigens in the form of processed peptide fragments bound to molecules encoded by the MHC. Most of the amino acid differences that distinguish MHC allelic variants lie in the peptide-binding cleft, and different allelic forms of MHC molecules bind distinct peptides (4, 5). This is true of both class I and class II molecules, and the information available so far suggests that the situation is comparable in both mouse and human systems. MHC class I- and class II-binding peptides are

similar in that those which have been isolated so far appear to have characteristic amino acid residues at certain anchor positions, and they exhibit allele-specific structural motifs (9, 19, 26, 29, 42, 43, 56). One major difference, however, concerns their variation in size. Peptides eluted from class I molecules have a defined length of 8 or 9 amino acids (29, 41, 45, 55), whereas class II peptides can be anywhere between 13 and 18 amino acid residues in length (9, 26, 42, 43). It appears that the same class II-binding peptide with an identical core sequence can exhibit both N- and C-terminal variation. We have obtained similar results with the class II-restricted E4-specific human T-cell clones. By using synthetic peptides, the T-cell epitope was mapped to a hydrophilic region on the E1/E4 molecule spanning amino acid residues 38 to 50 (Fig. 2 and 3). The minimal T-cell epitope that we have defined is 13 amino acid residues long, and we have also observed variability at the N terminus. The addition of one or two threonine residues does not appear to affect T-cell recognition of the peptide to any great extent (Fig. 2). This sequence that we have defined does not fit the early predictive algorithms for T-cell epitopes published by DiLisi and Berzofsky (15) or Rothbard and Taylor (40).

The MHC restriction of immune responses to HPV proteins is an important consideration for the design of prophylactic and therapeutic vaccines. In a series of antigen presentation experiments using allogeneic LCLs and class II-specific monoclonal antibodies, we have shown that our T-cell epitope is restricted through the HLA-DQ7 allele (Fig. 4 and 5). This is interesting because HLA-DQ7 is actually a subtype of HLA-DQw3 which has been reported to increase susceptibility to cervical carcinoma (58). It should be pointed out, however, that these workers did not mention any involvement of HPV16. Analysis of DNA from the two clones 3F5 and 4A8 by Southern blot and PCR to identify TCR gene rearrangements has shown that they are monoclonal and also unique, despite the fact that they appear to recognize the same epitope with the same MHC restriction (Fig. 6). This result has important implications as it suggests that there is more than one clone of T cells within the same individual capable of recognizing the same peptide-MHC complex. Similar findings with T-cell clones in other systems are just becoming apparent (33a). Perhaps this situation would not be too surprising since it seems likely that the T-cell repertoire capable of responding to a particular antigen needs to be maximized in order to recognize what must be a fairly limited number of peptide-MHC complexes.

Although the immunological responses to many HPV proteins are now being characterized, we still need to establish the precise role that these immune responses play in the natural history of the infection. This will involve an understanding of how these epitheliotropic viruses are actually seen and controlled by the immune system and of which viral proteins, if any, are involved in tumor regression. In the future, we should be looking at all aspects of the immune response in relation to how HPV infection is controlled. We may also need to consider HPV immunity at a more local level in infected epithelia, which may include a role for γ , δ intraepithelial T cells. These relatively static T cells are different from the α , β T-cell clones described in this work and appear to survey the cell membranes of epithelial cells with which they are in contact (24). It is possible that HPV-infected cells are susceptible to surveillance and lysis by these specialized T cells which, at least in vitro, have cytotoxic capabilities.

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