T-cell responses to peptide fragments of the BK virus T antigen: implications for cross-reactivity of immune response to JC virus

Jongming Li, 1 Jos Melenhorst, 2 Nancy Hensel, 2 Katyoun Rezvani, 2 Giuseppe Sconocchia, 2 Yasemin Kilical, 2 Jean Hou, 3 Blanche Curfman, 3 Eugene Major 3 and A. John Barrett 2

Correspondence
A. John Barrett
barrettj@nih.gov

1 Medical Oncology Division, Thomas Jefferson University, Philadelphia, PA, USA
2 Hematology Branch, National Heart, Lung, Blood Institute, National Institutes of Health, Bldg 10 CRC, Room 3-5330, 10 Center Drive, Bethesda, MD 20892-1202, USA
3 Laboratory of Molecular Medicine and Neuroscience, National Institute of Neurological Disorders, National Institutes of Health, Bethesda, MD, USA

Infection with BK virus (BKV) induces both humoral and cellular immunity, but the viral antigens of T-antigen (T-ag) stimulating T-cell responses are largely unknown. To identify BKV-specific T cells in healthy individuals, peripheral blood lymphocytes were cultured with autologous dendritic cells (DCs) loaded with BKV lysate and T cells were screened for intracellular gamma interferon production after stimulation with an overlapping 15mer peptide library of the BKV T-ag. Among many immunogenic peptides identified, four T-ag peptides were identified as candidate major histocompatibility complex class I and II T-cell epitopes, restricted to human leukocyte antigen (HLA)-B*0702, -B*08, -DRB1*0301 and -DRB1*0901. Further, a candidate 9mer peptide, LPLMRKAYL, was confirmed to be restricted to HLA-B*0702 and -B*08. Because the polyomaviruses BKV, JC virus (JCV) and Simian virus 40 (SV40) share extensive sequence similarity in the immunogenic proteins T-ag and VP1, it was hypothesized that, in humans, these proteins contain conserved cytotoxic T-lymphocyte (CTL) target epitopes. Four HLA-restricted conserved epitopes of BKV, JCV and SV40 were identified: HLA-B*07, -B*08 and -DRB1*0901 for T-ag and -A*0201 for VP1. T cells cultured in vitro that were specific for one viral antigen recognized other conserved epitopes. CTLs generated from BKV T-ag and VP1 peptide were cytotoxic to DC targets pulsed with either BKV or JCV. Therefore, infection by one of the two viruses (BKV and JCV) could establish cross-immunity against the other. Although cross-cytotoxicity experiments were not performed with SV40, cross-recognition data from conserved antigen epitopes of polyomaviruses suggest strongly that cross-immunity might also exist among the three viruses.

INTRODUCTION

Polyomaviruses are small DNA viruses that typically establish persistent, but inapparent infection of their natural hosts. Among 12 polyomaviruses identified, two human polyomaviruses [BK virus (BKV) and JC virus (JCV)] can cause latent infections and reactivate following immunosuppression. BKV reactivation causes a number of disorders in man, including acute haemorrhagic cystitis and tubulo-interstitial nephritis. JCV reactivation can cause progressive multifocal leukoencephalopathy (PML) in immune-compromised individuals. Similarly, Simian virus 40 (SV40) is activated in monkeys suffering from simian immunodeficiency virus-induced immunosuppression. All 12 polyomaviruses share a common genome organization, consisting of an early coding block [the T antigens (T-ag)] and a late coding block (the capsid proteins VP1, VP2 and VP3) separated by a regulatory region (Pipas, 1992). During the early stage of infection, virions are taken up by endocytosis and transported to the nucleus. Inside the nucleus, the virus chromosome is transcribed by the host cell to produce large and small T-ag early mRNA. Transcription from the early-region promoter is autoregulated by the binding of large T-ag to the regulatory region of the genome. As the concentration of large T-ag builds up in the nucleus, transcription of the late genes is switched to DNA replication. Subsequently, transcription of the late genes occurs from the late promoter, resulting in the synthesis of the structural proteins VP1, VP2 and VP3. The T-ag and capsid proteins are highly immunogenic. The cellular
response to SV40 T-ag in mice has been studied extensively and several cytotoxic T-lymphocyte (CTL) epitopes have been identified (Deckhut et al., 1991). In JCV, two antigens of VP1 restricted to human leukocyte antigen (HLA)-A*0201 have also been described (Du Pasquier et al., 2004) and the presence of JCV VP1-specific T cells was demonstrated to be crucial in the prevention of PML progression (Koralnik, 2002). However, the antigens of BKV T-ag stimulating human T-cell responses are largely unknown.

In view of the fact that the cellular immune response is important for the control of BKV-associated diseases (Braun, 2003), we decided to study the peptide sequence of the BKV T-ag to identify major histocompatibility complex (MHC) class I and II peptide epitopes that elicit CD4+ and CD8+ T-cell-mediated immunity against BKV. Because the BKV, JCV and SV40 genomes demonstrate 70% sequence similarity (Pipas, 1992), it is possible that the T-ag of BKV, JCV and SV40 might contain conserved CTL target epitopes restricted through common HLA alleles. Because of this, a cross-immunity in humans might exist against these three viruses. Indeed, previous studies have shown that T-cell cross-recognition of VP1 epitopes exists between BKV and JCV (Krymskaya et al., 2005; Chen et al., 2006). In this study, we identified several such conserved T-ag and VP1 antigen epitopes among these viruses. Further, we demonstrated that cross-cytotoxicity exists between BKV- and JCV-pulsed targets following in vitro stimulation of peripheral blood mononuclear cells (PBMCs) with BKV T-Ag peptide.

**METHODS**

**Study population.** Subjects studied were healthy blood donors (HD) at the National Institutes of Health. All individuals gave written informed consent to donate lymphocytes and monocytes by apheresis. All were seropositive for BKV by ELISA (Hamilton et al., 2000). Cell collections were frozen according to standard operating procedures and stored in liquid nitrogen until use.

**BKV peptides and viral lysates.** A BKV T-ag overlapping peptide library (171 15mers), with a 4 aa shift between each peptide and up to 70% sequence similarity (Pipas, 1992), was purchased from Biosynthesis Inc. (Fig. 1). After the identification of MHC-restricted antigens, purified peptides (>90% purity) were obtained from the same source to confirm the results from the peptide library, Polymavirus VP1 peptides ATTEVECLF (BKV) and SITEVECLF (JCV) were reported previously (Du Pasquier et al., 2004; Krymskaya et al., 2005; Chen et al., 2006). BKV and JCV lysates were prepared by freeze-thaw lysis of a BKV- or JCV-infected embryonic kidney (HEK) cell line.

**Preparation of dendritic cells (DCs) pulsed with BKV or JCV.** DCs were generated from PBMCs as described previously (Comoli et al., 2003). Elutriated monocytes (40 million to 60 million) were suspended at a concentration of 1 × 10^6 ml⁻¹ in complete medium (CM) consisting of RPMI 1640 medium (Biofluids) containing 10% heat-inactivated normal AB serum (Atlanta Biologicals), in a volume of 50 ml per 75 cm² culture flask. After 90 min incubation at 37°C, non-adherent cells were discarded. The adherent cells were washed three times with warm PBS. Afterwards, human recombinant interleukin-4 (rIL-4; R&D Systems) at a final concentration of 500 U ml⁻¹ and human recombinant granulocyte–monocyte colony-stimulating factor (GM-CSF; Sandoz Pharmaceuticals) at a final concentration of 800 U ml⁻¹ were added. After 5 days incubation, floating cells were collected and transferred to one well of a six-well plate. These immature DCs were then pulsed with BKV or JCV lystate. After 5 days culture, they were ready as antigen-presenting cells (APCs) in the CTL assay or to generate polyclonal, BKV-specific CTLs.

**Generation of polyclonal, BKV-specific CTLs.** BKV-specific T-cell lines were obtained by priming autologous PBMCs with BKV-pulsed DCs or directly with peptide. PBMCs containing 50 × 10⁶ lymphocytes in 50 ml CM were added to BKV lystate-pulsed DCs or peptide (final concentration 5 μM) in 75 cm² flasks. On day 7, the co-cultures were restimulated with 5 × 10⁶ autologous BKV-pulsed DCs or elutriated monocytes pulsed with peptide as described above. Also, on day 7 of culture and every 3 days thereafter, recombinant human IL-2 (rhIL-2; Tecin, Roche), final concentration 20 U ml⁻¹ was added to expand the proliferating T cells. On day 14, the frequency of antigen-specific T cells was determined by flow cytometry.

**Purification of antigen-specific T cells for CTL assay.** After PBMCs were primed with BKV-pulsed DCs or peptide of interest, antigen-specific T cells were purified with a novel solid-phase T-cell purification method that was developed in our laboratory (Li et al., 2005). This method exploits a layer of monocytes pulsed with peptide (9 mer for CD8+ T cells and 15mer for CD4+ T cells), which adhere to a solid surface spontaneously as APCs to select antigen-specific T cells. The underlying hypothesis is that antigen-specific T cells recognize their cognate antigens and bind to them faster than non-antigen-specific T cells. After removing non-adherent cells, antigen-specific T cells were subsequently ‘concentrated’ on the surface because of immunological-synapse formation. These activated T cells
were then expanded with addition of low-dose IL-2 (20 U ml⁻¹). This combination of selection and expansion enables us to increase the purity of antigen-specific T cells by up to 3 logs in 1 week. More than 50% purity of antigen-specific T cells can usually be achieved after first-round selection and expansion (cell count > 10⁸), whilst >90% purity of antigen-specific T cells can be achieved after second-round selection and expansion. Briefly, 6 × 10⁶ elutriated monocytes were thawed and added immediately to one well of a non-tissue-culture-treated six-well plate ( Falcon 351146; Becton Dickinson) in 3 ml CM containing DNase I (960 U) (Boehringer Mannheim). After 60–90 min at 37°C (5% CO₂), the medium was removed. Warm (37°C) PBS (6 ml) was added for gentle washes and this was repeated up to three times. After removing PBS, 3 ml warm CM was added. The adherent cells were washed with 5 μM selected viral peptide for 2 h at 37°C. The medium was removed again and the adherent cells were washed three times in warm PBS. Primed lymphocytes at a concentration of 20 × 10⁶–40 × 10⁶ ml⁻¹ in 3 ml warm CM were then added. After 3 min (for CD4+ T cells) or 5 min (for CD8+ T cells), non-adherent cells were removed with a transfer pipette while holding the plate at a 30–45° angle. The remaining adherent cells were washed gently three times with warm CM and again after 15–30 min incubation. After 24 h, adherent cells were removed by gentle agitation with a pipette and transferred to a culture flask in 100 ml CM and rhIL-2 (final concentration, 20 U ml⁻¹). Medium with rhIL-2 was exchanged every 2–3 days. On day 7–10, cultured cells were analysed by flow-cytometric intracellular cytokine assay (ICC) or used as effectors in the CTL assay.

Detection of peptide-specific gamma interferon (IFN-γ)-producing CD8+ /CD4+ T cells. IFN-γ production was determined by using a technique established previously for cytomegalovirus-antigen-specific T cells (Hensel et al., 2002). Briefly, 2 × 10⁶ lymphocytes and approximately 2 × 10⁸ autologous monocytes were co-incubated for 6 h (37°C, 5% CO₂) in the presence of BKV T-ag peptides in a final volume of 2 ml CM. Included with each experiment was an unstimulated control (no antigen) and a positive control, staphylococcus endotoxin B superantigen (Sigma). After 2 h, 10 μg brefeldin A ml⁻¹ (Sigma) was added and cultures were incubated for a further 4 h. Cells were pelleted, transferred to fluorescence-associated cell sorting (FACS) tubes (Falcon 352235; Becton Dickinson) and labelled with CD3-APC, CD4-phycocerythrin (PE) and CD8-PerCP (Becton Dickinson). Then the cells were fixed in FACS Lyse, permeabilized with FACS Perm2 and labelled with anti-IFN-γ-fluorescein isothiocyanate (all from Becton Dickinson) according to the intracellular-staining procedure protocol supplied by the manufacturer. Acquisition was performed by using a FACScalibur (Becton Dickinson) flow cytometer, equipped with a 488 nm argon and 635 nm red diode laser. Data files containing >30,000 CD8+ events within a lymphocyte gate (forward scatter versus side scatter) were analysed. Frequencies of IFN-γ+ positive cells within the CD4+ and the CD8+ populations were determined by using CellQuest software (Becton Dickinson). The frequency determined for the unstimulated control was subtracted from the frequency for the BKV antigen-stimulated population to determine the true BKV-specific T-cell frequency.

Cytotoxicity assay. The potential of IFN-γ-producing CD8+ lymphocytes to kill relevant targets was determined by using a modified carboxyfluorescein diacetate succinimidyl ester (CFSE)-based assay (Jedema et al., 2004). The assay uses CFSE (Vibrant CFDA SE Cell Tracer kit; Molecular Probes) to label the target-cell population with the addition of fluorescent microbeads [FlowCount Beads (FCBs); Beckman Coulter] to determine target-cell death. Briefly, the BKV-pulsed autologous monocytes (targets) were washed with PBS and resuspended at 2 × 10⁶/ml. Control targets included non-infected autologous monocytes and monocytes with no shared HLA alleles. An aliquot of 2 × 10⁶ cells was washed in PBS, pelleted and resuspended. One millilitre of CFSE (1 μM) was added to the cells, mixed and incubated for 15 min at 37°C in a water bath. The reaction was stopped by adding an equal volume of fetal calf serum (FCS; Atlanta Biologicals) followed by a 2 min incubation at room temperature. After two washes with 10% FCS in RPMI 1640 medium, the labelled targets were resuspended in CM and used immediately. The cell concentration was adjusted to 5 × 10⁶ cells ml⁻¹, and 100 μl per tube was added in triplicate to 12 × 75 mm FACS tubes. CTLs were counted and adjusted to 2–5 × 10⁷ ml⁻¹ in CM. Aliquots (50 μl) were added to the targets at different effector:target (E:T) ratios. Tubes were mixed and placed in a humidified 37°C, 5% CO₂ incubator for 4 h. Then, 250 ml cold 0.5% BSA (MP Biomedicals) in PBS was added to each tube. Tubes were kept in wet ice until acquisition was complete. A measured amount (positive-displacement technique) of FCBs (usually 10,000) was added to each tube immediately before acquisition. At the same time, 10 μl To-Pro-3 (0.5 μM; Molecular Probes) was added to label dead cells. For each sample, 5000 FCB events were acquired (LSRII; Becton Dickinson), allowing the calculation of absolute numbers of target cells. The absolute number of target cells was determined by calculation of the ratio between the number of cells and the number of beads. Percentage survival was calculated as follows: survival (%) = [absolute no. viable CFSE target cells (t = 4 h)]/[absolute no. viable CFSE target cells (t = 0)] × 100.

T-ag peptide library and epitope-identification strategy. Just for HLA-A*0201 epitopes in the T-ag sequence, the SYFPEITHI epitope-prediction program (http://www.syfpeithi.de) identified over 84 potential peptides. Therefore, we chose to use an overlapping peptide library to search for candidate peptide epitopes in the T-ag as a more inclusive and unbiased strategy than the prediction program. Of the 171 BKV T-ag library 15mer peptides, 88 were solubilized successfully. Peptides were divided into 13 pools with six to nine peptides per pool. Lymphocytes primed by 14 day exposure to BKV lysate-pulsed DCs were stimulated separately with each of the 15 pools. Pools inducing significant IFN-γ production (>2SD above mean control) were then selected for further screening of the individual 15mers from that pool. After screening individual peptides from the pool, those that elicited significant IFN-γ production were candidates for further study to identify their MHC class I or II restrictions. Candidate MHC class I (9mer)-restricted peptide sequences were selected by using the SYFPEITHI epitope-prediction program from 15mers that elicited significant IFN-γ production and synthesized (>90% purity). Elutriated peptide-pulsed monocytes were used as APCs to define the HLA restriction. Positive controls were monocytes sharing only one HLA allele with the responder and negative controls were monocytes sharing no HLA alleles with the responder. One hour after thawing, the monocytes were pulsed with 1 μM of the peptide of interest or with an irrelevant peptide. Two hours after pulsing, monocytes were washed at least twice with warm CM. Then, 10⁶ monocytes per well were used as APCs to stimulate equal numbers of responder lymphocytes. IFN-γ production was measured by flow cytometry. Finally, selected MHC class I peptides showing HLA-restricted T-cell responses were studied for their ability to generate peptide- and BKV-specific cytotoxic T cells.

RESULTS

Screening of peptides for BKV-specific polyclonal T-cell lines by flow cytometry

PBMCs from 17 healthy donors were screened directly by flow cytometry to detect T-cell responses to the 15 BKV T-ag peptide library pools. No significant IFN-γ production was identified in any individual (data not shown). In subsequent
experiments, we prepared DCs from seven healthy donors. These DCs were then pulsed with BKV to prime responders for 7–14 days before screening for peptide-specific IFN-γ production using the 15 peptide pools and, subsequently, individual peptides from the positive pools. Among these seven healthy donors, lymphocytes raised by autologous DCs pulsed with BKV recognized multiple peptide epitopes of the T-ag. Twelve peptides were found to be able to stimulate significant IFN-γ production (nine stimulating HLA class I responses and 12 stimulating HLA class II responses) (Table 1). In five of seven donors, CD8+ T cells recognized nine peptides that were recognized by CD4+ cells. Further, CD4+ and/or CD8+ T cells from multiple donors recognized P7 (GNLPLMRKAYLRKCK) and P154 (TFSRMKYNCMGKCI).

**Identification of HLA class I restriction of peptide epitopes**

A panel of monocyte APCs matched or mismatched with the responder for HLA-B alleles were used to define the MHC restriction of P7. Lymphocytes from HLA-B*0702 and -B*08 individuals showed significant IFN-γ production with P7, but not with an irrelevant peptide (Fig. 2). Similarly, P15 (TLYKKMEQDVKVAHQL) and P154 were shown to have HLA restriction at HLA-DRB1*0301 and -DRB1*0901, respectively (data not shown).

**9mer sequence of P7**

By using the SYFPEITHI epitope-prediction program, a 9mer sequence LPLMRKAYL of P7 (gnLPLMRKAYLrkck) was predicted to bind to HLA-B*0702 and -B*08. The peptide was synthesized to a purity of >90%. The 9mer sequence induced IFN-γ production by responder CD8+ cells comparable to that induced by the full P7 sequence, indicating that this was the epitope recognized by the CTLs (data not shown). Moreover, we were able to generate CD8+ T cells specific to 9mer sequence LPLMRKAYL of P7 from another four healthy donors' PBMCs (with HLA-*B0702) and two healthy donors' PBMCs (with HLA-*B08) separately. Typically, after 7 days, a single stimulation of peptide-loaded PBMCs generated low frequencies (<1%) of IFN-γ-producing T cells. A restimulation with the same peptide increased the frequencies to 5% (Fig. 3a).

**Identification of the cross-reactive epitopes**

The following conserved peptide epitopes of BKV, JCV and SV40 were identified: LPLMRKAYL (BKV T-ag), IPVMRKAYL (JCV T-ag) and IPLMRKAYL (SV40), which were HLA-B*07- and -B*08-restricted, and AITEVECFL (BKV VP1), SITEVECFL (JCV VP1) and

<table>
<thead>
<tr>
<th>Healthy donor</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-DRB1</th>
<th>HLA-DQB1</th>
<th>Peptides (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HLA class I</td>
</tr>
<tr>
<td>HD1803</td>
<td>0202</td>
<td>68</td>
<td>41</td>
<td>53</td>
<td>0901</td>
</tr>
<tr>
<td>HD32603</td>
<td>0201</td>
<td>0101</td>
<td>07</td>
<td>14</td>
<td>07</td>
</tr>
<tr>
<td>HD5803</td>
<td>0201</td>
<td>0201</td>
<td>15</td>
<td>44</td>
<td>0901</td>
</tr>
<tr>
<td>HD4903</td>
<td>0301</td>
<td>68</td>
<td>18</td>
<td>35</td>
<td>01</td>
</tr>
<tr>
<td>HD1804</td>
<td>0301</td>
<td>31</td>
<td>08</td>
<td>47</td>
<td>0301</td>
</tr>
<tr>
<td>HD11133</td>
<td>29</td>
<td>32</td>
<td>15</td>
<td>44</td>
<td>04</td>
</tr>
<tr>
<td>HD12023</td>
<td>0201</td>
<td>32</td>
<td>08</td>
<td>40</td>
<td>0301</td>
</tr>
</tbody>
</table>

Fig. 2. HLA-B restriction of peptide 7 (GNLPLMRKAYLRKCK). (a) T cells from patient HD32603 (HLA-B*07) were raised with peptide 7. Positive controls were allogeneic monocytes (only share HLA-B*07, cells 1, 2 and 3) pulsed with peptide 7 or peptide 10 (KCKEFHPKGGDEDK). Negative control was allogeneic monocytes (HLA-B*07 neg) pulsed with peptide 7. (b) T cells from HD1804 (HLA-B*08) were raised with peptide 7. Positive controls were allogeneic monocytes (only share HLA-B*08, cells 1 and 2) pulsed with peptide 7 or peptide 11 (FHPDKGGDEDKMKRM). Negative control was allogeneic monocytes (HLA-B*08 neg) pulsed with peptide 7.

Table 1. HLA type of healthy donors and immunogenic T-ag fragments (15mer)
SFTEVECFL (SV40 VP1), which were HLA-*A0201-restricted.

Cross-recognition of BKV, JCV and SV40 conserved epitopes of T-ag and VP1

The following scheme shows the general procedure to test T-cell cross-reactivity towards conserved epitopes of polyomavirus T-ag and VP1.

PBMCs were stimulated with a T-ag or VP1 peptide→T-cell IFN-γ production was confirmed by ICC→T cells were exposed to T-ag, VP1 conserved epitope peptides and DCs pulsed with BKV or JCV→T-cell IFN-γ production was measured by ICC.

T cells from four donors (+ HLA-B*07) were raised with the polyomavirus T-ag peptides restricted to HLA-B*07. T-cell cross-recognition experiments of conserved T-ag epitope peptides and DCs pulsed with BKV or JCV lysate were performed three times. A representative experiment is shown in Fig. 3. Cross-recognition experiments were also performed similarly with T-ag peptides restricted to HLA-B*08, BKV T-ag peptide 139 (IYLRKSLQNSEFLE), 140 (KSLQNSEFLEKRI), 154 and VP1 peptides restricted to HLA-A*0201 (Figs 4, 5 and 6).

Cross-reactive cytotoxicity

PBMCs from donor 72104 (HLA-A*0201) were primed with the BKV VP1 peptide AITEVECFL. After two stimulations, the primed T cells were tested for IFN-γ production by ICC and then used in a CTL assay to assess their ability to lyse DC targets pulsed with either JCV or BKV lysate. The frequency of CD8+ IFN-γ+ cells was 5.6% for BKV VP1 peptide and 5.1% for JCV VP1 peptide. At a 20:1 E:T ratio, 61% of BKV lysate-pulsed DCs and 69% of JCV lysate-pulsed DCs were killed, whereas non-pulsed monocytes were not killed (Fig. 7a).

PBMCs from donor 33004 (HLA-B*07) were primed with the BKV T-ag peptide LPLMRKAYL. After selection and expansion, 70% of the CTLs were specific to peptide LPLMRKAYL (data not shown). DCs were prepared from HD32603 (+ HLA-B*07) and were pulsed with BKV or JCV as targets. Unmanipulated monocytes from HD32603 (+ HLA-B*07) and HD4903 (− HLA-B*07) were used as negative control. CTLs specific for BKV T-ag peptide...
LPLMRKAYL showed HLA-restricted cytotoxicity toward DCs pulsed with BKV or JCV, but none toward unpulsed monocytes (Fig. 7b).

**DISCUSSION**

We chose to study the T-ag from BKV for several reasons. First, polyomavirus T-ag appears to be a potent inducer of T-cell responses in murine studies. Second, there have been no studies reporting CTL peptide epitopes in humans. In addition, polyomavirus T-ag has been implicated in many human malignancies (Bergsagel *et al.*, 1992; Carbone *et al.*, 1994, 1996; Testa *et al.*, 1998; Shivapurkar *et al.*, 2002; Vilchez *et al.*, 2002). The strategy that we used to identify T-ag peptide antigens relied on the ability to detect low frequencies of BKV-specific T cells in healthy

---

**Fig. 4.** CTL cross-recognition of T-ag epitopes (restricted to HLA-B*08) of polyomaviruses by ICC. (a) T cells (HD90402) raised with BKV T-ag peptide recognized conserved epitopes of BKV, JCV and SV40. The control was lymphocytes without stimulation. (b) T cells (HD90402) raised with JCV T-ag peptide recognized conserved epitopes of JCV and BKV. The control was lymphocytes without stimulation. Data shown here are representative of three similar experiments.

---

**Fig. 5.** CD4^+^ T-cell cross-recognition of T-ag epitopes by ICC. (a) The frequency of peptide 154-specific CD4^+^ T cells (HD1803, HLA-DRB1*0901) increased to 94% after purification. Eighty-eight per cent of these T cells recognized DCs pulsed by JCV. The control was lymphocytes without stimulation. (b) T cells (HD40704) raised with peptide 139 and 140 recognized both BKV-pulsed monocytes (mono.) and JCV-pulsed monocytes. The control was lymphocytes without stimulation. Data shown here are representative of three similar experiments.
BKV-seropositive individuals. Although no BKV-specific T cells were detected by flow cytometry of unmanipulated PBMCs from 17 healthy donors, it was possible after 7–14 days to expand T-ag peptide-specific T cells to detectable levels. As BKV antigen-specific T cells were also elicited directly with peptides using PBMCs as non-professional APCs, we presume that the T-cell responses described here were derived from circulating memory T cells rather than from naïve T cells, but the low frequencies in the blood precluded study of their memory-effector phenotype. By priming PBMCs, we could define the MHC class I and II HLA restriction of candidate peptides from a T-ag library and thereby define likely 9mer sequences binding to MHC class I antigens. Because of difficulties in solubilizing T-ag peptides, it was not possible to identify all possible peptide sequences provoking a T-cell response. However, the selection of peptides tested spanned the entire T-ag sequence (Fig. 1) and the number of positive responses obtained with the 88 peptides tested corresponded with previous predictions for MHC-binding specificity (Yewdell & Bennink, 1999). Because the library-screening approach makes no prior assumptions of HLA restriction, it was a more promising strategy than to search directly for peptides predicted by the SYFPEITHI database. The limited MHC class I and II allele information in SYFPEITHI probably overlooks significant epitopes. Furthermore, only about 30% of predicted peptides in fact bind to the selected MHC (Gulukota et al., 1997). After screening the peptide pools with a panel of cells, candidate peptides with HLA class I and II restrictions were identified. These epitopes could be confirmed by further screening of 15mer peptides and SYFPEITHI-predicted 9mers by using a panel of HLA-relevant and -irrelevant individuals. By using peptide-stimulated PBMCs, we found an abundance of CD4+ T-cell responses, making it difficult to dissect out CD8+ T-cell responses. We therefore generated DCs pulsed with BKV or JCV lysate to process and present T-ag epitope peptides naturally to both CD8+ and CD4+ T cells. We do not believe that the BKV or JCV lysate contains a significant amount of T-ag or VP1 protein or fragments thereof to be processed and presented by DCs directly, because there was no T-cell recognition of DCs pulsed with BKV or JCV lysate until day 4 after viral lysate was added to immature DCs (data not shown). This strategy allowed us to discover both CD4+ and CD8+ T-cell viral antigens with the 88 peptides studied. Several 15mer peptides from the T-ag peptide library contained sequences that bound to both MHC class I and II antigens. This property may contribute to the immunodominance of certain peptides, which can also recruit CTLs along with T-cell help. Notably, P7 (GNLPLMRKAYLRKCK) was recognized by T cells from several healthy donors in the context of HLA-B*0702 and -B*08. Further studies demonstrated that the derived 9mer peptide P27-35, LPLMRKAYL, was a super-motif for HLA-B*0702 and -B*08. Thus the peptide LPLMRKAYL represents an excellent candidate for monitoring BKV-specific T-cell responses in vivo, due to its ability to induce CTLs capable of killing BKV-pulsed DCs and the high frequency of both HLA-B*0702 and -B*08 in most populations.

T cell-mediated viral cross-reactivity has been an increasingly recognized phenomenon. This cross-reactivity has been observed not only within the subtypes of a specific virus, but also between different viruses. For example, conserved CTL epitopes within Epstein–Barr virus (EBV) LMP2 have been reported regardless of EBV type and isolates from different regions in the world (Lee et al., 1993, 1997). These conserved CTL epitopes usually present one or
et al. (2001). Similarly, the sequence similarity is 90, 84 and 82 % between BKV and SV40, and JCV and SV40, respectively (Imperiale, 1999). Because the lowest similarity is observed in the regulatory region, this focuses attention on the sequence similarities of VP1 and T antigen. The sequence similarity of the genomes of human polyomaviruses and the monkey polyomavirus SV40 are approximately 70 % similar (Butel & Oldstone, 1987). Among the 12 polyomaviruses identified, three polyomaviruses, we expect that the cross-immunity should exist.

In this study, we also examined CD4 T-cell cross-recognition between BKV and JCV T-ag. We found that three peptides of BKV T-ag, P139 (IYLKSLQNSELLE), P140 (KSLSQNFLEKRL) and P154 (TFSRMKYNICMGKCI), were immunogenic and their expression by APCs was confirmed in our studies (Fig. 5). Their counterparts in JCV T-ag were P138 (AYLRKSLQSELLE), P139 (KSLSQNFLEKRL) and P153 (TFSTMKVNGMRPI) with three, four and six amino acid substitutions of BKV peptides, respectively. Previous studies have established a threshold-of-activation model for T-cell recognition and signalling (Sloan-Lancaster & Allen, 1996). The capacity of an extracellular stimulus to trigger activation to different thresholds is probably dictated by the amount of T-cell receptor (TCR) cross-linking induced and the affinity of the ligand for the TCR. Thus, as a peptide analogue becomes more structurally similar to the agonist ligand, activation to higher thresholds will be accomplished and more effector functions will be stimulated (Evavold & Allen, 1991; Evavold et al., 1993; Sloan-Lancaster et al., 1993). Because of limited information about MHC structure and primary and secondary contact points related to these three 15mer peptides, we could only speculate how much T-cell function, including cytotoxicity, could be triggered with these homologous peptides, other than noting strong IFN-γ production upon stimulation.

In this study, we have identified a super-motif of BKV T-ag that was restricted to HLA-B*0702 and -B*08. We also demonstrated that cross-recognition of polyomaviruses existed in both CD4+ and CD8+ T cells. Future studies will be focused on identifying more conserved CTL epitopes that are restricted to common HLA alleles, in addition to demonstrating cross-cytotoxicity between BKV or JCV and SV40.

**Fig. 7.** Cross-cytotoxicity experiments of BKV and JCV. (a) BKV VP1 9mer peptide-primed T cells (HD72104) kill viral lysate-loaded DCs. Autologous DCs prepared from monocytes were pulsed with either BKV lysate or JCV lysate. PBMCs primed and stimulated with BKV VP1 HLA-A*0201-restricted peptide recognized and killed DCs expressing viral antigen, but not unpulsed autologous monocytes. (b) CTLd (HD33004, HLA-*B0702; 70% specific for BKV T-ag peptide LPLMRKAYL) were used as effectors to kill BKV- and JCV-pulsed targets. The targets were DCs from HD32603 (+HLA*B07) that were pulsed with BKV or JCV. The negative-control targets were unpulsed monocytes from HD32603 and HD4903 (–HLA*B07). Each point represents the mean of data from triplicate experiments.

two mutations among the amino acid sequences and this cross-reactivity could be the consequence of extensive sequence similarity between the epitopes. Recently, CTL-mediated cross-reactivity between Hepatitis C virus and Influenza A virus has also been reported because of extensive sequence similarity between the two viral epitopes (Wedefeyer et al., 2001). Interestingly, little or no sequence similarity between epitopes could also result in cross-recognition (Loftus et al., 1997; Hemmer et al., 1999; Acierno et al., 2003). This phenomenon is more consistent with the molecular-mimicry model of cross-reactivity (Oldstone, 1987). Among the 12 polyomaviruses identified, the genomes of human polyomaviruses and the monkey polyomavirus SV40 are approximately 70 % similar (Butel & Lednicky, 1999). Because the lowest similarity is observed in the regulatory region, this focuses attention on the sequence similarities of VP1 and T antigen. The sequence similarity of VP1 protein is 87, 88 and 85 % between BKV and JCV, BKV and SV40, and JCV and SV40, respectively (Imperiale, 2001). Similarly, the sequence similarity is 90, 84 and 82 % between BKV and JCV, BKV and SV40 and JCV and SV40, respectively, for T-ag protein (Imperiale, 2001). Therefore, it is not surprising that studies carried out with BKV-, JCV- and SV40-transformed cell lines reported cross-reactivity between CTL epitopes of T-ag encoded by these viruses (Campbell et al., 1983; Tevethia et al., 1998). In this study, we showed that this cross-reactivity between CTL epitopes of T-ag and VP1 also exists in the human cellular response to polyomavirus infection. CTL cross-reactivity was demonstrated both with synthetic peptides and in DCs pulsed with BKV and JCV lysates, indicating that these epitopes were processed and presented naturally by APCs (Figs 3 and 6). CTL cross-reactivity was apparently the result of extensive sequence similarity of both VP1 and T-ag among the three polyomaviruses. Our study shows that, in the case of HLA-B*07- and -B*08-restricted T-ag epitopes, human cellular cross-immunity exists between BKV and JCV. In this study, we did not perform cross-cytotoxicity experiments between BKV or JCV and SV40. However, based on CTL cross-reactivity between peptides of conserved epitopes of the three polyomaviruses, we expect that the cross-immunity should exist.
ACKNOWLEDGEMENTS

The authors have no commercial or other association that might pose a conflict of interest. Financial support: Department of Health and Human Services, National Institutes of Health. Presentation of some of the data included in the article: abstract no. 105, ISCT, May 2005, Vancouver, BC, Canada: ‘Identification of human CD4 and CD8 T cell responses to HLA-restricted peptide epitopes of the BK virus’. The findings and conclusions presented in this article are those of the individual authors and do not necessarily reflect the views of the Department of Health and Human Services.

REFERENCES


Testa, J. R., Carbone, M., Hirvonen, A. & 7 other authors (1998). A multi-institutional study confirms the presence and expression of


