CASE REPORTS

Patient 9 (A3, A11, B51, B39, DR4, DR16) is a 51-year-old white man, seropositive for HHV-8, who underwent an orthotopic liver transplantation (OLTx) for posthepatitis B cirrhosis and hepatocellular carcinoma (HCC) on June 2004. His donor (A1, A11, B51, DR11, DR14) was HHV-8 negative. Initial immunosuppressive treatment consisted of tacrolimus (FKT) (Prograf, Astellas Pharma) 0.10 mg/kg at days 0 and 1 and prednisone (PDN) 1 g at reperfusion. The maintenance immunosuppression consisted of FKT with blood trough levels 8 to 13 ng/mL and PDN, slowly tapered from 20 mg/day until discontinuation by month 3. Because of an episode of acute cellular rejection (ACR) PDN was maintained at 12 mg/day. In December 2004, 6 months after OLTx, the patient showed multiple (>10), firm, purple plaques, and nodules of the lower limbs, with edema and erythema (Fig. 1E). Histologic and immunohistochemical examination (see Methods section) showed Kaposi sarcoma (KS) at nodular stage (Fig. 1G and not shown). Staging examinations, including oesophago-gastro-duodenoscopy, colonoscopy, laringoscopy, and total body computed tomography (tBCT) resulted negative for visceral or lymph nodes involvement. FKT and PDN were withdrawn, and the immunosuppressive regimen was switched to sirolimus (SRL) (Rapamune, Wyeth, Ayerst) with blood trough levels 6 to 10 ng/mL. In April 2005, gradual improvement of edema and erythema of the legs presented regression. In July 2005, changes in color, initial reduction in size and number, and flattening of the skin lesions have been recorded; peripheral edema and erythema of the legs presented regression. In July 2005, few residual tan maculae persisted. In September 2005, histologic or the immunohistochemical examinations of the three most representative lesions showed complete remission (CR) of KS (Fig. 1F and 1H, and not shown). The staging screening for visceral involvement of KS resulted negative.

Patient 10 (A2, A30, B51, B62, DRB1*11, DRB3, DQB1*03) is a 43-year-old white woman, seronegative for HHV-8, with idiopathic liver cirrhosis, who underwent an OLTx on November 2005, because of a traumatic liver injury (with hemoperitoneum) during the insertion of a transjugular intrahepatic porta-systemic shunt. Her donor (A3, A68, B7, B53, DR1, DR7, DQ1, DQ2, DRB1*01, DRB1*07) was HHV-8 positive. Preoperative complications included acute renal failure by prerenal etiology; thus, the conditioning immunosuppressive regimen consisted of daclizumab (Zenepax, Roche) at 2 mg/kg at day 0 and 1 mg/kg at day 7; cyclosporine A (CsA) (Neoral, Novartis) 2.5 mg/kg two times per day; mycophenolate-mofetil (MMF) (Cell-Cept, Roche) 1 g three times per day; and PDN 1 g/day at reperfusion. The renal function returned normal in the post-Tx, and the patient was maintained with CsA (blood trough levels 150–200 ng/mL), MMF 500 mg three times per day, and PDN slowly tapered from 20 mg/day until discontinuation by month 3. Four months later, on March 2006, the patient presented multiple purple small nodular lesions and plaques in the upper part of lower limbs. Neither peripheral edema nor erythema was present. Histologic and immunohistochemical examinations showed KS at plaque stage. Neither lymph nodes nor visceral involvement was found at staging examination. CsA and MMF were withdrawn. An immunosuppressive regimen with SRL was undertaken with blood trough levels 6 to 10 ng/mL. In April 2006, gradual improvement in color, size, and number of the cutaneous lesions was recorded. In July 2006, the clinical regression of all the existing nodules was evident, with few lesions persisting as pigmented maculae. The biopsy of two of the most representative lesions demonstrated CR of KS, on morphologic or immunohistochemical examination (see Methods section). The staging screening for visceral involvement of KS resulted negative.

Patient 11 (A2, A26, B38, B51, DR11, DR16, DRw51, DRw52) is a 34-year-old white man, seropositive for HHV-8, who underwent an orthotopic renal transplantation for idiopathic glomerulonephritis, on March 2004. His donor (A2, A3, B39, B51, DR 11, DR 16) was HHV-8 negative. Induction immunosuppressive treatment consisted of basiliximab (Simulect, Novartis) (20 mg at days 0 and 4), FKT (0.1 mg/kg/two times per day for 4 days) and PDN (1 mg/kg for 4 days). The maintenance immunosuppression consisted of FKT with blood trough levels 8 to 10 ng/mL and PDN 16 mg/day. On October 2004, the patient presented a firm purple-brownish papular lesion of the left calf. Histologic and immunohistochemical studies revealed KS in plaque stage. Neither tumor-associated edema nor erythema was present. Staging procedures showed neither visceral nor nodal involvement. The immunosuppressive regimen was reduced, consisting of FKT with blood trough levels at 5 to 6 ng/mL and PDN at 8 mg/day. In September 2005, because of stable disease, the immunosuppressive regimen has been switched to SRL with blood trough levels 6 to 10 ng/mL and PDN 8 mg/day. In February 2006, the lesion showed improvement in color, slightly in size and presented flattening of the borders, and judged in partial remission on the basis of ACTG criteria (11). The treatment protocol, based on SRL conversion, has been approved by our institutional ethics committee, and all three patients have given written informed consent.

METHODS

PCR and Immunohistochemistry for HHV-8

DNA was isolated from 5×10⁶ peripheral blood mononuclear cells (PBMCs) by use of the Easy-DNA kit (Invitrogen, Milan, Italy) according to manufacturer’s instructions. The HHV-8 viral load was determined by real-time PCR with forward primer 5′-CCA ACG GAT TTG ACC TCG TG-3′ and reverse primer 5′-CGG CCG ATA TTT TGG AGT AGA T-3′, which yielded a 104 bp product of ORF 26, as described (5). Results were normalized to account for the total input cellular DNA in the PCR, and the lower limit of detection was 1 to 5 copies/μg DNA. Immunohistochemical analysis with a monoclonal mouse antibody to the HHV-8 latency-associated nuclear antigen (LANA-1) (Novoceastra Laboratories, Newcastle, UK) was performed, as described (6), on the paraffin-embedded KS biopsy tissue from patients 9 to 11, and on three BM biopsy tissues from three HHV-8 negative subjects, as controls. PCR for EBV and CMV has been performed as described (16, 17).

Elispot for HHV-8 Latent and Lytic Antigens

Peptides were synthesized by Sigma-Genosys, Pampisford, Cambs, UK. Identified A2-restricted HHV-8 peptides were derived from the latent protein K12 (LLNGWRWRL) (7) and from the following lytic proteins: gB (LMWYELSKI) (8), gH (FLNWQNLLNV) (9), and orfK8.1 (gp35/37) (LVL1-LYLTV) (7,10). Peptides spanning the entire orf K8.1 (228aa, 22 peptides) and K12 (60aa, 5 peptides) amino acid sequences were synthesized as 20-mer peptides, overlapping by 10 aa. Peptide sequences were based on the HHV-8 sequence from Accession numbers AAC63270 and AAN64668. Full-length recombinant K8.1 was produced in Escherichia coli whereas full-length recombinant ORF73 was produced in baculovirus-infected insect cells.
PBMCs from the 11 HHV-8 positive patients or the three HHV-8 negative control subjects were separated by use of Ficoll-Hypaque gradient centrifugation (Linaris, Bettingen am Main, Germany) and then cultured in a 96-well polyvinylidene difluoride-bached plate coated with anti-IFN-γ monoclonal antibody (Mabtech, Nacka Strand, Sweden). A total of 2×10^5 cells/well were stimulated with single peptides (10–12 μg/mL) and phytohemagglutinin (PHA) (Sigma-Aldrich, Milan, Italy) (5 μg/mL), and CEF (CMV, EBV, and influenza virus peptide pool) (Mabtech) (2 μg/mL), in 10% FBS/RPMI1640 (Invitrogen, Milan, Italy), and cultured for 16 hrs. All test conditions were carried out in triplicate and results were considered positive if the number of spot forming cells (SFC)/10^5 cells in HHV-8 antigens-stimulated wells was 2-fold higher than that in control wells (non-HHV-8 antigens-stimulated cells) and there were at least 20 spots. The assay was performed by one of the authors (PB), blinded to the patients’ clinical histories and personal identifiers, using an automated ELISPOT counter (AID-GmbH, Strassberg, Germany). The single peptides and peptide pools for CMV or EBV, contained in CEF preparation and described by Currier et al. (11, in supplemental information), were used separately in ELispot assays to study the specific T-cell responses against each herpesvirus.

**T-Cell Phenotype Analysis**

The phenotype of circulating lymphocytes was analyzed by flow cytometry. A total of 1×10^6 PBMC were stained with the following mAbs (BD PharMingen, San Diego, CA) according to manufacturer’s instructions: CD3 allophycocyanin (APC), CD4 peridinin chlorophill A protein (PerCP), CD27 fluorescein isothiocyanate (FITC), CD28 phycoerythrin (PE), CD45RA FITC, CD62L PE, CD25 FITC, CD28 phycoerythrin-cyanin (APC), CD4 peridinin chlorophill A protein (PerCP), with K8.1 or K12 peptide pools (1 μg/mL of each peptide), respectively, or recombinant proteins (orf K8.1 and orf73 proteins) (10 μg/mL) and phytohemagglutinin (PHA) (Sigma-Aldrich, Milan, Italy) (5 μg/mL), and CEF (CMV, EBV, and influenza virus peptide pool) (Mabtech) (2 μg/mL), in 10% FBS/RPMI1640 (Invitrogen, Milan, Italy), and cultured for 16 hrs. After 16 hrs all test conditions were carried out in triplicate and results were considered positive if the number of spot forming cells (SFC)/10^5 cells in HHV-8 antigens-stimulated wells was 2-fold higher than that in control wells (non-HHV-8 antigens-stimulated cells) and there were at least 20 spots. The assay was performed by one of the authors (PB), blinded to the patients’ clinical histories and personal identifiers, using an automated ELISPOT counter (AID-GmbH, Strassberg, Germany). The single peptides and peptide pools for CMV or EBV, contained in CEF preparation and described by Currier et al. (11, in supplemental information), were used separately in ELispot assays to study the specific T-cell responses against each herpesvirus.

**Cytokine Production**

To analyze the cytokine secretion profile of circulating lymphocytes, 1×10^5 PBMC were seeded in 96-well plates and stimulated with 25 ng/mL phorbol myristate acetate (Sigma) and 1 μg/mL ionomycin (Sigma). After 2 hrs, 10 μg/mL brefeldin A (Sigma) was added for additional 4 hrs. Cells were then stained with anti-CD3 APC and anti-CD4 PerCP and fixed with 1% paraformaldehyde at 4°C for 10 min. Cells were then stained with anti-IFN-γ FITC, anti-IL-2 PE, and anti-IL-4 PE (PharMingen) for 20 min at room temperature in PBS 2% FBS containing 0.05% saponin (Sigma). Samples were analyzed with FACS Calibur flow cytometer.

**Analyses of Memory T Lymphocytes Specific for K8.1 and K12 HHV-8 Antigens**

A total of 2×10^6 PBMC were stimulated for 6 hrs in 96-well plates in 200 μL of medium containing 10% of FBS with K8.1 or K12 peptide pools (1 μg/mL of each peptide), in the presence of anti-CD28 (2 μg/mL) and anti-CD49 (1 μg/mL) antibodies. After 1 hr, 10 μg/mL brefeldin A was added to allow cytosolic accumulation of cytokines. Cells were then incubated with anti-CD3 Cy7, anti-CD4 PerCP, anti-CD45RA FITC, anti-CD62L PE to identify central memory CD4 and CD8 lymphocytes. Cells were then fixed and permeabilized, and finally stained with anti-IFN-γ APC or anti-IL-2 APC antibodies as described above. Samples were analyzed by Cyan ADP flow cytometer (DAKO, Milan, Italy).

**Expansion of Peptide Specific T Cells**

To expand peptide-specific T cells, PBMCs from patients were cultured for 12 days in the presence of K8.1 or K12 peptide pools as follows: 1×10^5 cells/well were seeded in 96 round-bottom plates. Peptide pool (at 1 μg/mL of each peptide) was added on day 0 and then after 6 days IL-2 was added at 25 U/mL. On day 12, cells were harvested and restimulated for 6 hrs using the protocol described above.

**Spectratype Analysis of TCR Vbeta Families**

RNA from 1×10^6 PBMC was extracted using TRIzOL (Invitrogen) according to manufacturer’s protocol. Briefly, 1×10^6 cells were lysed in 100 μL of Trizol, followed by the addition of chloroform to separate the organic and aqueous phases. RNA was precipitated from the aqueous phase with isopropanol, followed by a wash with 70% ethanol. The resulting RNA pellet was resuspended in sterile water, and the yield of RNA and purity were determined by spectrophotometry. Complementary DNA was generated from 1 μg of RNA in a 20–μL reaction using oligo dT primers for reverse transcription with reverse transcriptase (Superscript, Gibco, Paisley, UK). Each of 22 functionally rearranged BV gene superfamilies was amplified across the C/V junctions using the 24 BV family-specific primers described previously by Maslanka et al. (12), and a fluorescent dye (FAM, Perkin Elmer)-(conjugated) BC region specific primer. Some of the BV primers amplify short, and others longer PCR products. Short and long BV primers were combined in multiplex PCRs to amplify two BV families in one reaction as follows: BV1 + 5.1, BV2 + 12, BV3 + 13, BV4 + 5.3, BV7 + 8, BV9 + 14, BV10 + 20, BV11 + 19F, BV15 + 17, BV16 + 21, BV18 + 23, BV22 + 24, BV61, BV6, BV63, BV25 were used unpaired. The total PCR volume is 40 μL containing Genamp PCR buffer (Perkin Elmer, Cambridge, UK), 2.0 mmol/L MgCl2, 0.2 mmol/L each dNTP, 2.5 mmol/L of each primers, and 5 μL complementary DNA. For the hot start, 0.5 units of Ampliqaq DNA polymerase were added last. Optimal cycling conditions were 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, for 35 cycles followed by final extension at 72°C for 10 min. For T-cell spectratyping, 1 μL of PCR product was denatured in 12 μL formamide and electrophoresed through Performance Optimized Polymer 4 (Perkin Elmer) on an ABI 110 automated sequencer (Perkin Elmer) in the presence of Tamra 500 size standard (Perkin Elmer). Genescan software 2.1 (Perkin Elmer) was used to analyze the data. For the purpose of analyzing the T-cell repertoire, a normal spectra-type was defined as comprising at least six different size classes, at intervals of three nucleotides without any gaps.

**REFERENCES**