British Journal of Haematology, 2001, 113, 479–482
SHORT REPORT

Selective *ex vivo* expansion of cytomegalovirus-specific CD4$^+$ and CD8$^+$ T lymphocytes using dendritic cells pulsed with a human leucocyte antigen A*0201-restricted peptide

ALESSANDRO M. VANNUCCHI, STEPHANIE GLINZ, ALBERTO BOSI, ROBERTO CAPORALE AND PIERLUIGI ROSSI-FERRINI
The Bone Marrow Transplant Unit, Division of Haematology, Azienda Ospedaliera Careggi, University of Florence, Florence, Italy

Received 21 November 2000; accepted for publication 22 January 2001

Summary. Adoptive transfer of *ex vivo*-generated cytomegalovirus (CMV)-specific T lymphocytes may be effective in preventing CMV disease in allogeneic haematopoietic stem cell transplantation (HSCT) recipients. We developed a procedure for expansion of CMV-specific T lymphocytes based on the antigen-presenting function of donor dendritic cells (DCs), pulsed with a human leucocyte antigen A*0201-restricted pp65 nonamer peptide. CMV-specific T lymphocytes were identified following induction of interferon γ (IFN-γ) secretion prompted by peptide exposure. Both CD8$^+$ and CD4$^+$ CMV-specific T lymphocytes were selectively produced in these cultures and showed CMV-restricted cytotoxicity. The simultaneous and selective expansion of CD4$^+$ and CD8$^+$ CMV-specific lymphocytes might be instrumental for more efficient *in vivo* function of infused CMV-specific lymphocytes.

Keywords: dendritic cells, cytomegalovirus, adoptive immunity, peptide, T clones.

Human cytomegalovirus (CMV) is a member of the herpesvirus family that, while causing self-limited disease in the normal host, is responsible for significant morbidity and mortality when reactivated in immunocompromized subjects. Haematopoietic stem cell transplantation (HSCT) recipients are at particular risk because of the concurrent role of several factors, the most important of which is a delayed reconstitution of CMV-specific, donor-derived, cellular immunity. The incidence of CMV reactivation after HSCT has been reported to be up to 70% and mortality from CMV disease as high as 40% (Reusser *et al*, 1991). Pharmacological agents, used both pre-emptively and for treatment of CMV disease, have modified the course of the disease in recent years, but they are also associated with significant toxicity (Nichols & Boeckh, 2000).

Alternative approaches to restore CMV-specific cellular immunocompetence are of potential interest. The work of Riddell *et al* (1992) and Walter *et al* (1995), who demonstrated that *in vitro*-expanded, CMV-specific, T-cytotoxic lymphocyte (CTL) clones restored CMV cellular immunity in HSCT patients, marked the era of *ex vivo*-generated immunocompetent cells. CMV-specific CTL clones were obtained by co-culturing donor peripheral blood mononuclear cells (PBMNCs) with CMV-infected autologous fibroblasts, the only cell type allowing CMV replication without cell lysis (Riddell *et al*, 1992; Walter *et al*, 1995). However, techniques that do not require virus manipulation would be more appropriate for a broader application of cell therapy in humans.

As dendritic cells (DCs) are the most potent antigen-presenting cells and have a key role in host defence against infections, we exploited their use in this setting. A procedure for *ex vivo* expansion of CMV-specific T lymphocytes that involves DCs pulsed with a human leucocyte antigen (HLA) A*0201-restricted CMV nonamer peptide (Diamond *et al*, 1997) was developed.

MATERIALS AND METHODS

Dendritic cells were obtained from the peripheral blood of HLA A*0201 CMV-positive sibling donors, after informed consent was given. PBMNCs were plated at 2–4 × 10^6/ml in six-well culture dishes in Aim-V medium (Life Technologies, UK) supplemented with 1% autologous plasma, and allowed to adhere for 2 h. After removing non-adherent cells, which were cryopreserved as the lymphocyte fraction,

Correspondence: Dr Alessandro M. Vannucchi, Bone Marrow Transplant Unit, Division of Haematology, Azienda Ospedaliera Careggi, University of Florence, Florence, Italy. E-mail: a.vannucchi@dfc.unifi.it

© 2001 Blackwell Science Ltd

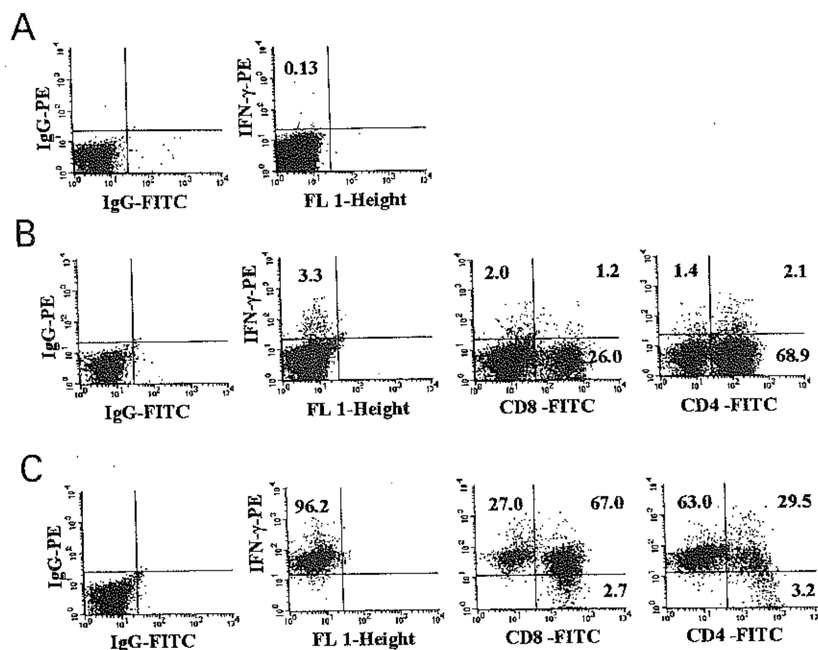

Fig 1. FACS analysis of *ex vivo*-expanded CMV-specific T lymphocytes, based on the IFN-γ secretion assay. See text for details of procedure. The figure depicts one typical experiment out of nine performed. (A) Fresh PBMCs, not stimulated with the peptide before assay (this represents background levels of spontaneously IFN-γ-secreting cells). (B) Cells after 3 week cultures with peptide-loaded DCs, not stimulated with peptide before assay (this represents background levels of spontaneously IFN-γ-secreting cells after culture). Co-expression of CD4 and CD8 is also shown. (C) Cells from 3 week cultures with peptide-loaded DCs, primed overnight with CMV-specific peptide before assay (this represents CMV-specific, IFN-γ-secreting lymphocytes, either CD4 or CD8). Control plots using PE- or FITC-labelled isotype antibodies are shown in the left column. Analysis was performed on gated live cells (propidium iodide).

medium containing recombinant human (rh) interleukin 4 (IL-4; 500 U/ml), rh tumour necrosis factor α (TNFα; 10 ng/ml) and rh granulocyte macrophage colony-stimulating factor (GM-CSF; 1000 U/ml) (all from Euroclone, UK) was added to the plates, and half-replenished every second day. The frequency of *ex vivo*-generated DCs was assessed by immunophenotyping with CD1a–fluorescein isothiocyanate (FITC) and CD86–phycoerythrin (PE) monoclonal antibodies (mAbs; Euroclone).

DCs ($3–5 \times 10^6$/ml) were incubated with CMV $pp65_{495-503}$ peptide [NLVPMVATV, purity >95% by reverse phase high pressure liquid chromatography (RP-HPLC); MWG Biotech, Germany] (Diamond *et al*, 1997) at 10 μg/ml for 2 h. Then, they were irradiated at 3000 rad and co-cultured at a ratio of 1:20 thawed donor lymphocytes in Aim-V medium. Three days later, rhIL-2 (20 U/ml) was added and the medium half-changed on an alternate-day basis. Cultures were restimulated weekly for 2 weeks with either freshly prepared or thawed, peptide-pulsed, DCs at the original 1:20 ratio according to the number of alive lymphocytes in culture. Following the third stimulation, cells were processed for quantification of CMV-specific lymphocytes and CMV-specific cytotoxity.

Quantification of CMV-specific lymphocytes was performed using the live IFN-γ secretion assay (Miltenyi GmbH, Germany). Briefly, cells were exposed overnight to CMV peptide (10 μg/ml) or to an irrelevant 9-mer Mart-1 peptide (AAGIGILTV; specific for melanoma and melanocytes) as a negative control, and then incubated with a bispecific reagent (anti-CD45–anti-IFNγ) that binds, respectively, to the lymphocyte membrane and cell-secreted IFN-γ molecules. IFN-γ secreting cells were revealed using fluorescence-activated cell sorter (FACS) analysis with anti-IFN-γ–PE mAbs and anti-CD4/CD8–FITC mAbs (Brosterhus *et al*, 1999).

CTL activity was evaluated using the $DiO_{18}(3)$ cytotoxicity assay (Mattis *et al*, 1997). Effector cells were recovered from DC–lymphocyte co-cultures. As target cells, autologous, CMV-infected fibroblasts propagated *in vitro* from donors' skin explants (4 out of 10 experiments) or T2 cells (all experiments; CRL-1992; ATCC, USA) loaded with either CMV peptide or Mart-1 peptide were used. Fibroblasts had been infected *in vitro* with AD-169 CMV strain (VR-538; ATCC). Target cells (1×10^7) were labelled with 15 μg/ml of green fluorescent dye $DiO_{18}(3)$ (a dialkylcarbacyanine perchlorate amphiphilic probe that inserts into the cell membrane exposing the charged fluorophore; Molecular Probes, USA), and then plated at different effector/target (E/T) ratios for 4 h. Propidium iodide (10 μg/ml) and RNase A (100 μg/ml) were added for counterstaining. The activity of cytotoxic T lymphocytes was measured by analysing at least 20 000 target cells/sample using a FACScan flow cytometer. Live target cells showed only green fluorescence, whereas dead target cells were indicated by both green and red fluorescence; red fluorescence alone indicated dead effector cells. The percentage of dead target cells and of specific lysis was calculated according to Mattis *et al* (1997).

RESULTS AND DISCUSSION

DCs were generated from PBMNCs cultured with IL-4, GM-CSF and TNFα; the frequency of $CD1a^+/CD86^+$ cells was 78 ± 12 ($n = 12$; range 63–92%). In cases where high numbers of DCs developed, aliquots were cryopreserved after being pulsed with peptide and irradiated; thawed cells maintained stimulatory properties in lymphocyte co-cultures, as we observed in parallel experiments with freshly generated DCs.

Identification of CMV-specific T lymphocytes generated in culture was based on the detection of IFN-γ, a marker of

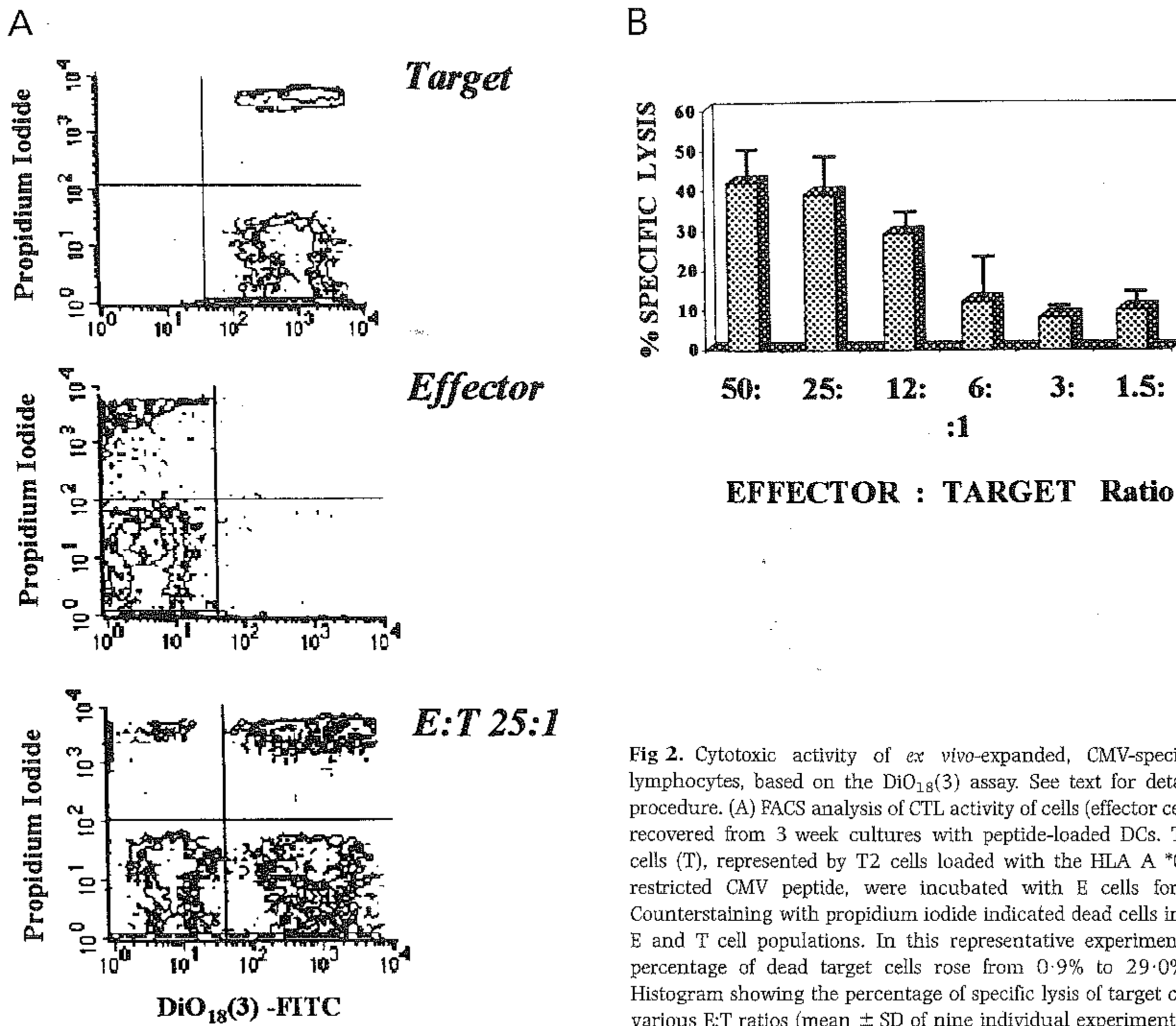

Fig 2. Cytotoxic activity of *ex vivo*-expanded, CMV-specific T lymphocytes, based on the $DiO_{18}(3)$ assay. See text for details of procedure. (A) FACS analysis of CTL activity of cells (effector cells, E) recovered from 3 week cultures with peptide-loaded DCs. Target cells (T), represented by T2 cells loaded with the HLA A *0201-restricted CMV peptide, were incubated with E cells for 4 h. Counterstaining with propidium iodide indicated dead cells in both E and T cell populations. In this representative experiment, the percentage of dead target cells rose from 0·9% to 29·0%. (B) Histogram showing the percentage of specific lysis of target cells at various E:T ratios (mean ± SD of nine individual experiments).

T-cell activation, at the single cell level using flow cytometry (Kern *et al*, 1998). In preliminary experiments ($n = 4$), we evaluated the effects of multiple weekly stimulation with peptide-pulsed DCs on the generation of CMV-specific T cells. Paralleling a progressive decrease of total cellularity, the frequency of cells releasing IFN-γ in response to peptide increased from 9 ± 5% at week 1 to 26 ± 15% at week 2, to 88 ± 10% at week 3. Thereafter, significant cell mortality ensued and all further experiments were performed using 3 week cultures.

Most cells (88 ± 10%, range 73–98%; data from nine donors) that remained alive in 3 week cultures produced IFN-γ in response to pp65 peptide (Fig 1); the ratio of $CD8^+$/$CD4^+$ cells ranged from 2:1 to 2:7 in different experiments. Specificity against CMV peptide was demonstrated by challenging cells with a Mart-1 peptide which failed to increase IFN-γ production above control values (data not shown). Starting from a theoretical input number of lymphocytes in a culture of 1×10^6, we recovered a mean of $3{\cdot}5 \times 10^5$ lymphocytes at the end of the 3 week culture; as the frequency of CMV-specific, INF-γ-secreting cells in response to peptide rose from <0·1% at the beginning of culture to >85% at week 3, the actual expansion factor of CMV-specific ($CD4^+$ and $CD8^+$) cells was > 230-fold.

To determine whether CMV-specific, IFN-γ-producing lymphocytes generated in culture displayed CMV-specific and restricted cytotoxicity (CTL), we used a sensitive flow cytometry assay (one typical experiment out of nine performed with individual donors is reported in Fig 2A). There was a correlation between the percentage of specific lysis and E:T ratio that reached a plateau above the level of 12:1 (Fig 2B) using bulk cultures. Preliminary experiments ($n = 3$;) using immunomagnetic selected $CD8^+$ or $CD4^+$ cells suggested that cytotoxic activity was mainly confined to the $CD8^+$ cell fraction.

The procedure reported here for *ex vivo* generation of CMV-specific T lymphocytes deserves some comment. The use of peptide-loaded DCs has been exploited for generating Epstein-Barr virus (EBV)-specific CTLs (Subklewe *et al*, 1999); simultaneous expansion of CMV- and EBV-specific CTLs has also been obtained with EBV-transformed B-lymphoblastoid cell lines engineered to express pp65 (Sun *et al*, 1999). While the latter methodology does not have the

disadvantage of being HLA-restricted (such as the one described herein; however, HLA A*0201individuals represent about 40% of the European population), it requires exposure of clinical samples to lymphoblastoid cells which might not be worthwhile. Neverthless, it remains to be assessed in future studies whether CMV-specific lymphocytes generated with the peptide-based procedure will show full *in vivo* activity against naturally infected cells.

Finally, the generation of $CD4^+$ CMV-specific T cells in this culture system is of interest in light of the observation that helper T cells were required for persistence of *in vivo*-transferred $CD8^+$ CMV-specific T cells (Walter *et al*, 1995). Indeed, depletion and/or dysfunction of $CD4^+$ T cells resulted in reactivation and persistence of viruses including CMV and EBV (Polic *et al*, 1998). As efficient priming of $CD4^+$ cells by antigen-specific dendritic cells has recently been reported (Schlienger *et al*, 2000), it is probable that our system could be also employed for the induction of a specific CMV response by T lymphocytes obtained from CMV-negative HLA A*0201 donors; we are currently exploiting this possibility.

ACKNOWLEDGMENTS

This study was supported by grants from Foundation 'Istituto di Ricerca Virologica Oretta Bartolomei Corsi' and by private funds from Rotary Club, Serravalle Pistoiese.

The authors would like to thank Dr M. P. Piccini for helpful discussions and suggestions.

REFERENCES

Brosterhus, H., Brings, S., Leyendeckers, H., Manz, R.A., Miltenyi, S., Radbruch, A., Assenmacher, M. & Schmitz, J. (1999) Enrichment and detection of live antigen-specific CD4+ and CD8+ T cells based on cytokine secretion. *European Journal of Immunology*, 29, 4053–4059.

Diamond, D.J., York, J., Sun, J.-Y., Wright, C.L. & Forman, S.J. (1997) Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. *Blood*, 90, 1751–1767.

Kern, F., Surel, I.P., Brock, C., Freistedt, B., Radtke, H., Scheffold, A., Blasczyk, R., Reinke, P., Schneider-Mergener, J., Radbruch, A., Walden, P. & Volk, H.D. (1998) T-cell epitope mapping by flow cytometry. *Nature Medicine*, 4, 975–978.

Mattis, E.A., Bernhardt, G., Lipp, M. & Förster, R. (1997) Analyzing cytotoxic T lymphocyte activity: a simple and reliable flow cytometry-based assay. *Journal of Immunological Methods*, 204, 135–142.

Nichols, W.G. & Boeckh, M. (2000) Recent advances in the therapy and prevention of CMV infections. *Journal of Clinical Virology*, 16, 25–40.

Polic, B., Hengel, H. & Krmpotic, A. (1998) Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. *Journal of Experimental Medicine*, 188, 1047–1054.

Reusser, P., Riddell, S.R., Meyers, J.D. & Greenberg, P.D. (1991) Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood*, 78, 1373–1380.

Riddell, S.R., Watanabe, K.S., Goodrich, J.M., Li, C.R., Agha, M.E. & Greenberg, P.D. (1992) Restoration of cellular immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science*, 257, 238–241.

Schlienger, K., Craighead, N., Lee, K.P., Levine, B.L. & June, C.H. (2000) Efficient priming of protein antigen-specific human CD4+ T cells by monocyte-derived dendritic cells. *Blood*, 96, 3490–3498.

Subklewe, M., Chahroudi, A., Schmaljohn, A., Kurilla, M.G., Bhardwaj, N. & Steinman, R.M. (1999) Induction of Epstein-Barr virus-specific cytotoxic T-lymphocyte responses using dendritic cells pulsed with EBNA-3A peptides or UV-inactivated, recombinant EBNA-3A vaccinia virus. *Blood*, 94, 1372–1381.

Sun, Q., Pollok, K.E., Burton, R.L., Dai, L.J., Britt, J.W., Emanuel, D.J. & Lucas, K.G. (1999) Simultaneous ex vivo expansion of cytomegalovirus and Epstein-Barr virus-specific cytotoxic T lymphocytes using B-lymphoblastoid cell lines expressing cytomegalovirus pp65. *Blood*, 94, 3242–3250.

Walter, E.A., Greenberg, P.D., Gilbert, M.J., Finch, R.J., Watanade, K.S., Thomas, D.E. & Riddell, S.R. (1995) Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *New England Journal of Medicine*, 333, 1038–1044.