Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Different mechanisms of Campath-1H-mediated depletion for CD4$^+$ and CD8$^+$ T cells in peripheral blood

Hayden Lowenstein, Akeesha Shah, Alan Chant and Abrar Khan

Department of Surgery, Division of Transplantation Surgery and Immunology, University of Vermont, Burlington, VT, USA

Keywords
Campath-1H, CD52, immunosuppression, T cells.

Correspondence
Abrar Khan, MD, MPhil, FACS, University of Vermont, 89 Beaumont Ave, D-317 Given, Burlington, VT 05405-0068, USA. Tel.: +1 802 656 9695; fax: +1 802 656 0680; e-mail: abrar.khan@med.uvm.edu

Received: 20 April 2006
Revision requested: 12 May 2006
Accepted: 20 July 2006

doi:10.1111/j.1432-2277.2006.00382.x

Abstract

It is assumed that complement and noncomplement-mediated mechanisms are similarly responsible for Campath-1H-mediated killing of all T-cell subtypes *in vivo*. However, the differing surface expression of CD52 on T-cell subtypes suggests that may not be the case. The purpose of this study is to determine the extent and mechanism of Campath-1H-mediated elimination of different T-cell subtypes in peripheral blood. Whole blood or lymphocytes isolated from peripheral blood of healthy volunteers by Ficoll density centrifugation were incubated with Campath-1H, with or without complement and/or serum, and the resultant T-cell elimination mechanisms studied. For CD4$^+$ T lymphocytes, 60% and 40% cell death and for CD8$^+$ T lymphocytes 23% and 77% cell death, in peripheral blood, was mediated by complement and noncomplement mediated mechanisms, respectively. CD4$^+$ T cells demonstrated approximately twice the amount of surface CD52 compared with CD8$^+$ T cells, consistent with primarily complement-mediated killing for CD4$^+$ T cells. Thus, peripheral blood supports differential and partial elimination of T-cell subtypes, suggesting that the complete T-cell elimination seen in transplant recipients is most likely due to contribution from other lymphoid organs.

Introduction

Campath-1H (Alemtuzumab) is a humanized monoclonal antibody against the CD52 antigen, a 12 amino acid, heavily glycosylated glycosylphosphatidylinositol-linked cell surface protein. Campath-1H has been used therapeutically for various illnesses [1,2], and has also been used in solid organ transplantation [2–4].

The mechanism of Campath-1H-mediated killing *in vitro* is a combination of complement-mediated lysis and antibody-dependent cell-mediated cytotoxicity (ADCC) [5–8]. It is assumed that similar mechanisms are responsible for Campath-1H-mediated killing of all T-cell subtypes. In this study, we focus on determining whether peripheral blood can support equivalent elimination of all T-cell subtypes seen in transplant recipients. For example, it may be possible that Campath-1H-mediated killing is enhanced by other immunosuppressants given *in vivo*, thus confounding its individual mechanism of T-cell killing [9–11]. Additionally, it is possible, given the large variation in surface expression of CD52, that either complement or noncomplement-mediated killing may predominate for any T-cell subtype. If complement-mediated killing is in fact important, then it is reasonable to hypothesize that the degree of surface expression of CD52 on various T-cell subtypes should play an important role, as more CD52 implies more antibody binding, and hence more killing. In this study, we will establish the main mechanism of Campath-1H-mediated killing of CD4$^+$ and CD8$^+$ T cells in peripheral blood and also establish the importance of differential CD52 expression on T-cell subtypes.

Materials and methods

Immune cell killing assay

Fresh blood from healthy volunteers was used. The indicated concentration of Campath-1H (Ilex Inc,

Montville, NJ, USA) was added to 1 ml of fresh blood, and incubated in 50 ml centrifuge tubes (37 °C) for the indicated time. The blood was kept in motion continuously to simulate blood flow. Control tubes were treated similarly, but without Campath-1H. The blood was subjected to ammonium chloride (AC) lysis to isolate peripheral blood mononuclear cells (PBMNCs). Alternatively, Ficoll density centrifugation was used to isolate the lymphocytes. Live cells were counted using Trypan Blue (Mediatech Inc, Cellgro, Herndon, VA, USA) exclusion. The *absolute number* of cells remaining in 1 ml of blood in each tube was then determined. Experiments were done in triplicate unless otherwise indicated. Blood from transplant recipients was treated similarly, but without Campath-1H.

Fluorescence activated cell sorting (FACS)

All FACS staining was done at 4 °C. 5×10^5 peripheral blood mononuclear cells (PBMNCs), as isolated above, were placed in FACS tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and washed twice with FACS solution (1% BSA/PBS). Cells were then incubated with Campath-1H for 30 min, washed twice with FACS solution, and then secondarily stained for 30 min with a goat antihuman IgG1-FITC (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Alternatively, the cells were stained directly with a mouse antihuman CD52-FITC. When necessary, the surface expression of CD52 after treatment with Campath-1H could also be determined because mouse antihuman CD52-FITC-binding was not blocked by Campath-1H. Cells were washed twice with FACS solution and then stained for 40 min with a cocktail of various combinations of the following mouse antihuman antibodies (Caltag Laboratories, Burlingame, CA, USA): anti-CD4 APC-AlexaFluor 750, anti-CD8 PE-Cy 5.5, anti-CD3 PE, anti-CD25 APC, and anti-CD52-FITC. Necessary single color and fluorescence minus one controls for up to 5-color flow cytometry were performed with every experiment. These controls also verified that, among T cells, there was no cross reactivity with the goat antihuman IgG1–FITC (anti-CD52 secondary antibody) and other mouse antihuman fluorochromes used (data not shown).

Statistical analysis

GRAPHPAD PRISM V4.01 (GraphPad Software, San Diego, CA, USA) was used to construct graphs, calculate medians, and display SD bars. All experiments were done in triplicate and each experiment done at least three times. When indicated in the figure legend, a 2-way ANOVA analysis with Bonferroni post-test was performed to determine the statistical significance of the graph.

Ammonium chloride (AC) lysis: isolation of peripheral blood mononuclear cells

A 500-ml, 10× stock concentration of ammonium chloride lyzing solution (ACLS) was prepared as follows: 40.1 g NH_4Cl, 4.2 g $NaHCO_3$, 1.85 g disodium EDTA was added to 450 ml of deionized water. The pH of the solution was calibrated to 7.4 using an Accumet Basic pH meter (Fisher Scientific, Pittsburgh, PA, USA) and the final volume was adjusted to 500 ml. The solution was then stored at 4 °C.

The following describes the AC lysis procedure for 1 ml of blood: 15 ml of 1× ACLS was added to 1 ml of blood. The sample was incubated for 10 min at room temperature. Following incubation, the sample was centrifuged at 300 *g* for 5 min at 22 °C. The supernatant was discarded and cells washed twice in 10 ml of phosphate-buffered saline (PBS, 37 °C). The cells were resuspended in 1 ml of 10% fetal bovine serum in Isocove's Medium (10% FBS/IMDM) and counted using the Trypan Blue exclusion technique.

Ficoll gradient isolation of lymphocytes

Blood was diluted 1:1 with PBS (37 °C). Three milliliters of Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ, USA) were added to 15 ml conical tubes. Up to 4 ml of the blood solution per tube was layered over the Ficoll-Paque Plus and centrifuged at 300 *g* (18 °C) for 40 min. The upper plasma layer was removed using a Pasteur pipette, and a different Pasteur pipette was used to remove the lymphocyte layer, which was then placed in 20 ml of PBS (37 °C). Cells were spun at 400 ***g*** (7 min, 18 °C), washed with warm PBS, and resuspended in 10% FBS/IMDM. For Fig. 3, cells were resuspended in human serum or medium, keeping the total volume at 1 ml (the original volume of blood). This kept the cell density the same as it would be *in vivo*.

Determining the absolute number of cells

The cell suspension derived after AC lysis (or Ficoll density centrifugation) was thoroughly mixed. Ten microliters of this suspension were mixed with 30 µl of Trypan Blue. From this mixture, 10 µl was loaded onto a hemocytometer and viewed under a Micromaster Inverted Microscope (Fisher Scientific, Pittsburgh, PA, USA). The number of cells in opposite quadrants was counted and their average applied to the following equation to determine cell number per milliliter: cell

average × (dilution factor) × 10^4. The value obtained for cell number per milliliter of suspension is equivalent to the number of PBMNCs obtained from the original milliliter of blood. A total of 0.5×10^6 cells were then placed into the appropriately labeled 12 × 75 mm polystyrene FACS tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) for flow cytometry. The cells were then stained with the appropriate antibodies as described above.

Flow cytometry analysis: determining the absolute number of specific T-cell populations in control and Campath-1H-treated blood

After staining with antibodies, the cells were resuspended in 3.7% formaldehyde and immediately run on a BD LSRΠ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Postcytometry analysis was performed using the FLOWJO software V4.6.2 (Tree Star Inc, Ashland, OR, USA). The absolute number of cells for a given population was determined by multiplying the reported percentage on FLOWJO with the total number of cells counted after AC lysis. Percent cell survival for any given cell population was then determined using the absolute numbers of the given cell population and comparing it with the absolute number of the control for the same cell population.

Patient data

Human studies were performed in accordance with ethical standards (Institutional Review Board) laid down in an appropriate version of the 2000 Declaration of Helsinki. Patients received Campath-1H 30 mg, 20 mg Prednisone (tapered over several months), and Tacrolimus to keep levels around 10 ng/ml.

Results

Peripheral blood does not facilitate complete T-cell killing by Campath-1H

To determine the ability of peripheral blood to facilitate Campath-1H-mediated elimination of lymphocytes, fresh peripheral blood from healthy volunteers (PB-HV, *in vitro* in Fig. 1a) was incubated with 5 μg/ml of Campath-1H for 4 h at 37 °C. The blood was AC lysed, lymphocyte populations analysed by flow cytometry, and absolute numbers of cells surviving plotted as percent control (Fig. 1a). Peripheral blood from a kidney transplant patient (PB-TP, 'patient' in Fig. 1a) who received one dose of Campath-1H (30 mg) for induction was similarly analysed 15 h after Campath-1H administration. Lymphocytes, from PB-HV and PB-TP, were broken down into $CD8^+$, $CD4^+25^-$, and $CD4^+25^+$ subsets and the percent surviving plotted (Fig. 1a). There is a clear hierarchy in PB-HV where $CD8^+$ T cells are the least sensitive, $CD4^+25^-$ more sensitive, and $CD4^+25^+$ the most sensitive to Campath-1H-mediated elimination. PB-TP, on the other hand, shows complete and equivalent elimination of all three T-cell subtypes. This discrepancy of T-cell elimination can be due to the inherent nature of T cells in dialysis patients [12,13]. To eliminate this possibility, normal blood and blood from a dialysis patient (not transplanted) was exposed to Campath-1H *in vitro*. No difference in T-cell elimination was seen (Fig. 1b).

It is also possible that the discrepancy seen in Fig. 1a is because of insufficient complement available *in vitro* (PB-HV). To eliminate this possibility, the experiment in Fig. 1a was repeated with two aliquots. The first aliquot of peripheral blood (1 ml, 1× serum) and the second aliquot, containing twice the amount of human serum (2× serum), were incubated with Campath-1H (kept at 5 μg/ml), and analysed by FACS. No difference in Campath-1H-mediated killing of $CD4^+$ and $CD8^+$ T cells was observed (Fig. 1c). Thus, complement is not a limiting factor *in vitro*. Another possible reason for complete T-cell depletion *in vivo* is that steroids, given before Campath-1H administration, may enhance Campath-1H's ability to eliminate T cells. This possibility was also ruled out (Fig. 1d).

The discrepancy between PB-HV and PB-TP could also be explained by the fact that more time and more Campath-1H was available for killing *in vivo* (PB-TP) than *in vitro* (PB-HV). We exposed PB-HV to 0.25, 1.25, and 5 μg/ml of Campath-1H for 4 and 8 h. The blood incubated with Campath-1H for 8 h demonstrated the same degree of T-cell killing at each concentration of Campath-1H as did blood incubated for 4 h (Fig. 2a). Thus, elimination of T lymphocytes by Campath-1H is essentially complete by 4 h *in vitro*.

To ensure that sufficient Campath-1H was present *in vitro*, PB-HV was incubated with 0.25, 1.25, 5, and 20 μg/ml of Campath-1H for 4 h and analysed by FACS for percent survival of T-cell subtypes. There was only a small incremental increase in percent killing beyond 1–2 μg/ml of Campath-1H and no further killing beyond 5 μg/ml (Fig. 2b). Thus, 5 μg/ml of Campath-1H (as used in Fig. 1) represents an excess of Campath-1H (comparable to the estimated concentration found *in vivo* in PB-TP – a 75-kg person with 7% blood volume who receives 30 mg of Campath-1H has a blood concentration of approximately 6 μg/ml).

In summary, there is incomplete and differential elimination of T-cell subtypes by Campath-1H in peripheral blood.

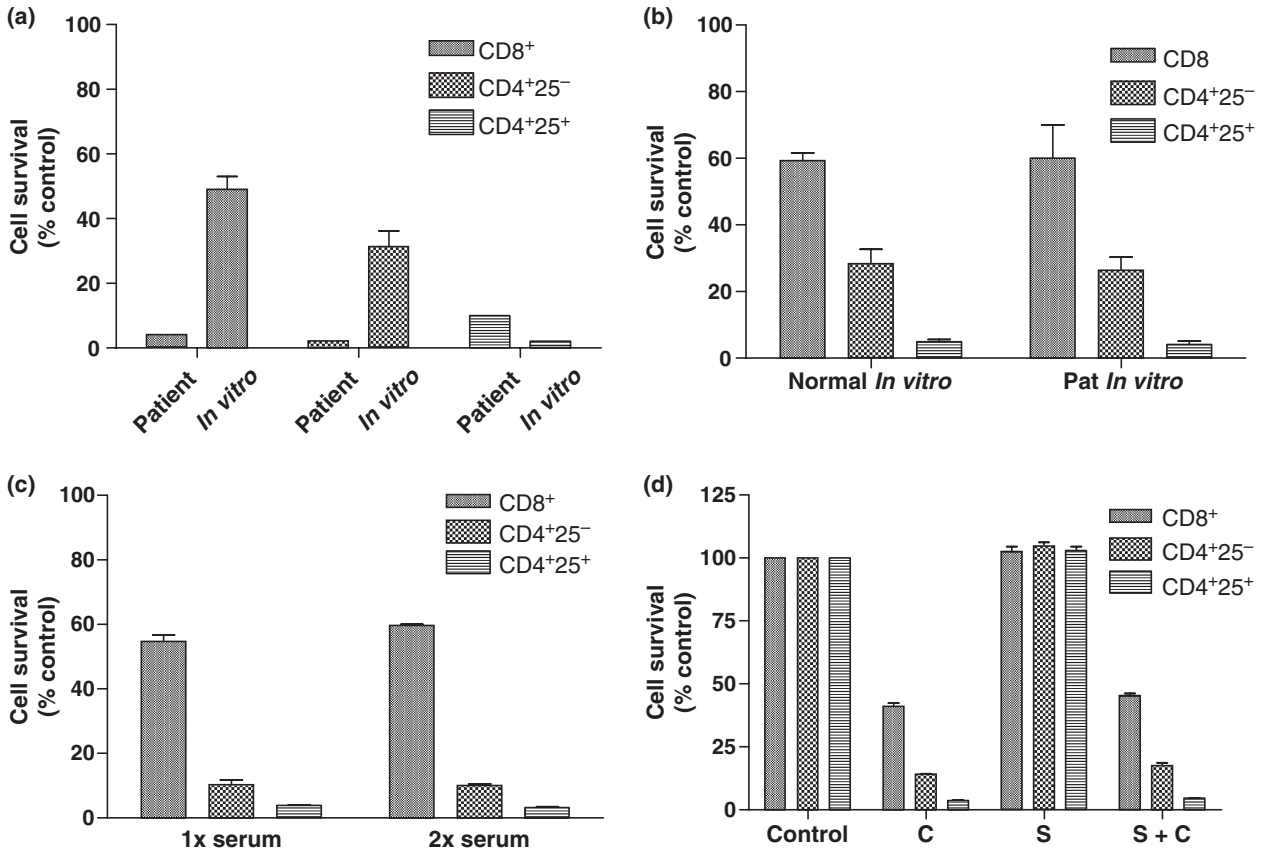

Figure 1 Campath-1H-mediated killing of T-cell subsets is incomplete in peripheral blood. (a) Fresh blood from a healthy volunteer, which was incubated at 37 °C for 4 h with 0 μg/ml (control) and 5 μg/ml of Campath-1H, and blood from a transplant recipient treated with 30 mg of Campath-1H 15 h before was subjected to AC lysis to isolate peripheral blood mononuclear cells. The cells were analysed using forward vs. side scatter and 5-color flow cytometry. Percent cell survival was then plotted for the T-cell subtypes indicated. There is incomplete killing of $CD8^+$ and $CD4^+25^-$ T-cell subtypes *in vitro*. Patient data are representative of at least three other patients and the control used, to determine the percent T-cell depletion, was the patient's own blood prior to Campath-1H administration. *In vitro* data represent one healthy volunteer's blood and the error bars represent the experiment done in triplicate (CD8 – 49% ± 4, $CD4^+25^-$ – 31.3% ± 5, $CD4^+25^+$ – 2% ± 0). Comparing patient and *in vitro* T-cell subset killing, *P*-values for CD8, $CD4^+25^-$ and $CD4^+25^+$ are <0.0001, <0.004, and <0.0001, respectively. (b) Fresh blood from a normal volunteer and from a dialysis patient, not transplanted, was exposed to Campath-1H *in vitro* as in A. No difference in killing of T-cell subsets was noted (*P*-values for CD8, $CD4^+25^-$ and $CD4^+25^+$ are <0.9, <0.7, and <0.5, respectively). (c) Blood (1 ml) from a healthy volunteer (containing normal amount of serum, 1× serum) was exposed to Campath-1H as in A. Additionally, serum from 1 ml of blood was isolated by centrifugation, and added to a separate 1-ml aliquot of normal blood (2× serum). Campath-1H concentration was kept at 5 μg/ml, and both, 1× and 2× serum, were treated and analysed as in A, and percent cell survival plotted for $CD8^+$, $CD4^+25^-$, and $CD4^+25^+$ T cells. Data represent one healthy volunteer's blood done in triplicate (1× serum: CD8 – 54.7% ± 2, $CD4^+25^-$ – 10.3% ± 1, $CD4^+25^+$ – 3.9% ± 0.1, 2× serum: CD8 – 59.7% ± 0.3, $CD4^+25^-$ – 10% ± 0.6, $CD4^+25^+$ – 3.2% ± 0.1. Error bars are not visible when triplicate values are very close to each other. (d) Four aliquots of fresh human blood were incubated with nothing, Campath-1H (5 μg/ml), Solumedrol (1 mg/ml), and Solumedrol + Campath-1H, and percent cell survival calculated. Solumedrol did not enhance the Campath-1H-mediated T-cell elimination (C = Campath-1H, S = Solumedrol).

$CD4^+$ T-cell depletion by Campath-1H in peripheral blood is primarily complement-mediated: $CD8^+$ T-cell depletion is not

To determine the mechanism of this differential killing of T cell subsets, whole blood (PB-HV) was exposed to Campath-1H, and the survival of absolute numbers of $CD4^+$ and $CD8^+$ T cells calculated (positive control, Fig. 3b, bars on left). Additionally, lymphocytes isolated by Ficoll density centrifugation were incubated with Campath-1H with human serum (representing complement-mediated killing, Fig. 3b, second bar from left) and no serum (representing noncomplement-mediated killing, Fig. 3b, second bar from right). The degree of $CD4^+$ and $CD8^+$ T-cell survival for these three groups is compared with lymphocytes with neither serum nor Campath-1H

Journal compilation © 2006 European Society for Organ Transplantation **19** (2006) 927–936

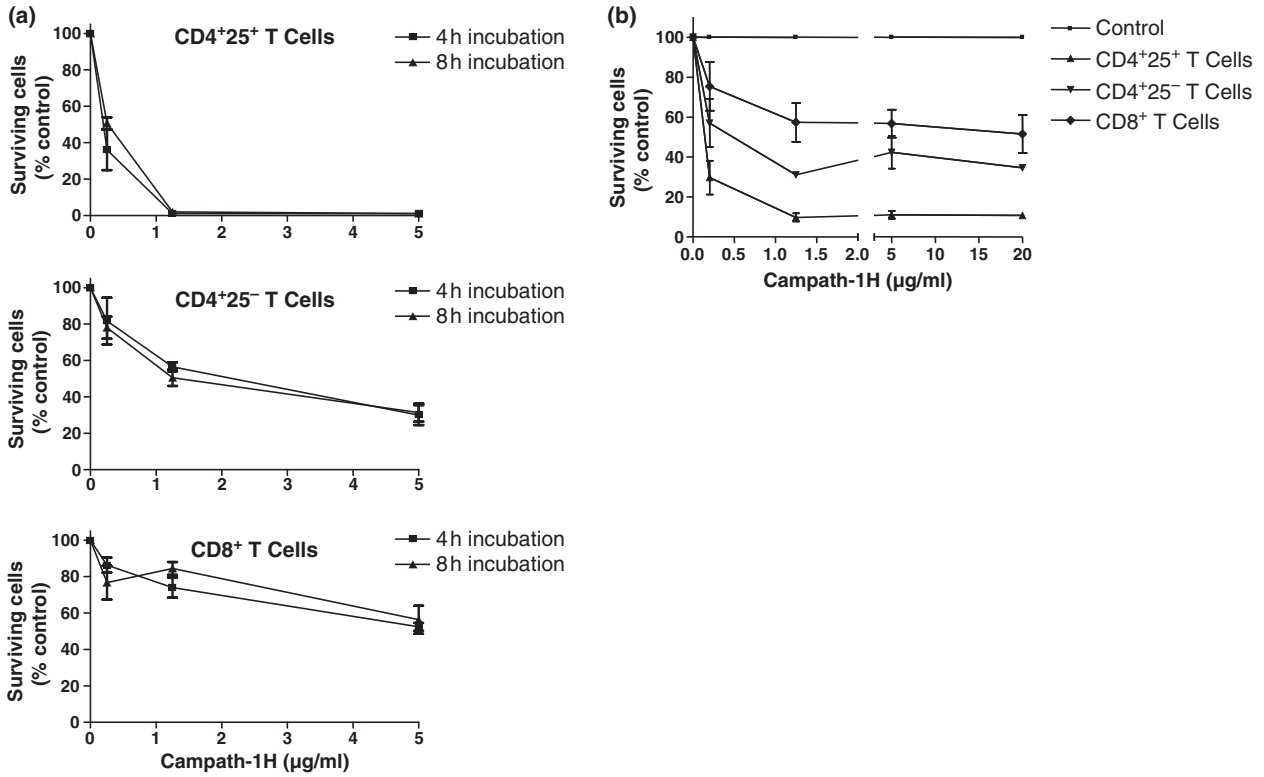

Figure 2 Campath-1H-mediated killing is complete after 4 h. Fresh human blood from one healthy donor was incubated, in triplicate, at 37 °C for 4 and 8 h with the indicated concentrations of Campath-1H, AC lysed, and analysed by 4-color flow cytometry. (a) Percent cell survival for the indicated T-cell subsets was calculated as in Fig. 1. All T-cell subsets ($CD4^+25^+$, $CD4^+25^-$, $CD8^+$) demonstrated the same degree of killing after 4 and 8 h of incubation with the indicated concentrations of Campath-1H. (b) Fresh human whole blood, from one healthy donor, was incubated, in triplicate at 37 °C for 4 h with the indicated concentrations of Campath-1H. The samples were then subjected to AC lysis to isolate the peripheral blood mononuclear cells, and the cells analysed with 4-color flow cytometry. Absolute numbers of various T-cell subsets were then calculated (see Materials and methods) and compared with the absolute numbers of control cells, and the percent surviving cells then plotted as a function of Campath-1H concentration for each T-cell subset analysed. Campath-1H concentration of approximately 1.25 μg/mL results in essentially maximal killing, with small incremental killing between 1.25 and 5 μg/ml of Campath-1H. A 2-way ANOVA with a Bonferroni post-test for each curve gave a *P*-value of 0.0001. Error bars when not visible are obscured by data points because variation was very small.

(negative control, Fig. 3b, defined as 100% survival, bars on right). Figure 3a shows the experimental protocol. Of the $CD4^+$ T cells eliminated (Fig. 3b, lower graph), 40% *cell death* was due to noncomplement-mediated killing (100−67)/(100−67) + (67−18) (numbers are percentages shown in Fig. 3b) whereas 60% was due to complement-mediated killing (67−18)/(100−67) + (67−18). On the other hand, for $CD8^+$ T cells (Fig. 3b, upper graph), 77% *cell death* was due to noncomplement-mediated killing whereas only 23% was due to complement-mediated killing. Thus, complement-mediated killing plays a major role in Campath-1H-facilitated elimination of $CD4^+$ T cells in peripheral blood, whereas noncomplement-mediated killing plays a major role in $CD8^+$ T-cell elimination. It is interesting to note that noncomplement-dependent killing (ADCC) is similar for $CD4^+$ and $CD8^+$ T cells (Fig. 3b, second bar from right). To verify that noncomplement-dependent T-cell killing is in fact mediated by ADCC, the experiment in Fig. 3b was repeated, but in the presence of increasing concentrations of human IgG1 (Fig. 3c). There was a clear IgG1 dose-dependent inhibition of Campath-1H-mediated cell death.

Campath-1H-induced killing correlates with surface expression of CD52

If complement-mediated killing is a general mechanism of Campath-1H-mediated killing of T cells, then such killing should be proportional to surface CD52 expression. More specifically, as complement-mediated killing is the principal mechanism for $CD4^+$ T-cell killing and not for $CD8^+$ T-cell killing (in peripheral blood), then it is reasonable to hypothesize that there should be significantly more CD52 expression on $CD4^+$ than $CD8^+$ T cells. Would this logic also apply to other immune cells? We generalized our query to families of immune cells such as

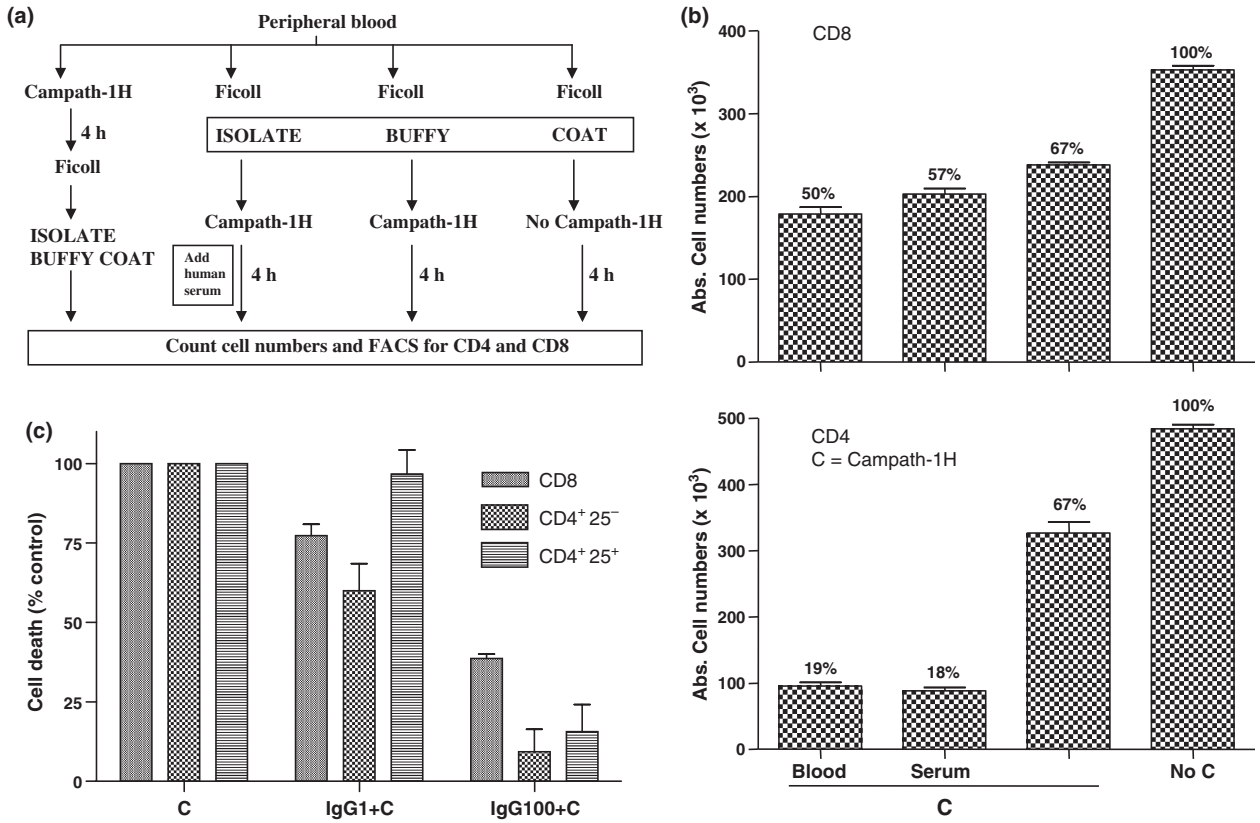

Figure 3 Campath-1H-mediated killing of CD4 T cells in peripheral blood is mostly dependent on complement. Blood from one healthy volunteer was divided into four aliquots of 1 ml each. The first was retained as whole blood. The second, third, and fourth aliquots were treated with Ficoll density gradient centrifugation to isolate the buffy coat and eliminate the serum and complement. To the second aliquot, serum from 1 ml of whole blood was reintroduced (represents complement mediated killing), and to the third (represents noncomplement killing) and fourth (negative control) nothing was added. The first three aliquots were exposed to 5 μg/ml of Campath-1H and the fourth remained as negative control. The entire experiment was done in triplicate. After 4 h of incubation with Campath-1H, viable cells were counted and analysed by multicolor flow cytometry and absolute numbers of remaining $CD4^+$ and $CD8^+$ T cells calculated. (a) Schematic of experimental protocol. (b) Absolute numbers of surviving CD4 and CD8 T cells are plotted for all four aliquots described above. Percent cell survival is indicated above the bars and is relative to the far right bars (negative controls, 100% survival). T cells isolated as in A remained similarly viable regardless of whether they were suspended in human serum or in medium for 4 h (data not shown). *P*-values for bar graphs from left to right (Campath-1H + blood, Campath-1H + serum, Campath-1H alone) for CD4 vs. CD8 are <0.0002, <0.0001, and <0.93, respectively. (c) Lymphocytes isolated as in A were incubated with 5 μg/ml of Campath-1H only (control) or in conjunction with human IgG1 (1 and 100 μg/ml). Cell death with Campath-1H only was used as 100% cell death (control, bars on left). There is a significant dose-dependent inhibition of death in all T-cell subsets when human IgG1 is added. Human IgG alone (1 or 100 μg/ml) had no effect on T-cell subset viability (data not shown). *P*-values (IgG1 + C vs. IgG100 + C) for CD8, $CD4^+25^-$ and $CD4^+25^+$ are <0.01, <0.0005, and <0.0002, respectively. C = Campath-1H, IgG1 = human IgG1 at 1 μg/ml, IgG100 = human IgG1 at 100 μg/ml.

granulocytes, monocytes, and lymphocytes to determine whether killing of immune cells in general correlates with surface expression of CD52. Whole blood (PB-HV) was treated with different concentrations of Campath-1H for 4 h, AC lysed, subjected to 5-color flow cytometry, and the results plotted in Fig. 4a. Lymphocytes were the most sensitive, monocytes less sensitive, and granulocytes the least sensitive to Campath-1H-mediated killing. CD52 expression on normal untreated lymphocytes, monocytes, and granulocytes is shown in Fig. 4b, which demonstrates that surface CD52 expression is highest in lymphocytes [mean fluorescence intensity (MFI) = 11 628], less in monocytes (MFI = 8198), and least in granulocytes (MFI = 161). Thus, killing correlates with CD52 expression in lymphocytes, monocytes, and granulocytes as a whole. The same principle is upheld even if the lymphocytes are broken down into $CD4^+CD25^+$, $CD4^+25^-$, and $CD8^+$ T-cell subtypes and compared with granulocytes and monocytes (Fig. 5a). A plot of CD52 expression (in normal untreated cells) on granulocytes, monocytes, and the T-lymphocyte subsets, juxtaposed to a plot of percent cell survival of the same cells after Campath-1H incubation (Fig. 5b,c) reveals a clear hierarchy. From least to most surface expression of CD52 are granulocytes, monocytes, $CD8^+$, $CD4^+CD25^-$, and $CD4^+CD25^+$ T cells, where the MFI of CD52 is 161, 8198, 8339, 14867, and 15629,

Journal compilation © 2006 European Society for Organ Transplantation **19** (2006) 927–936

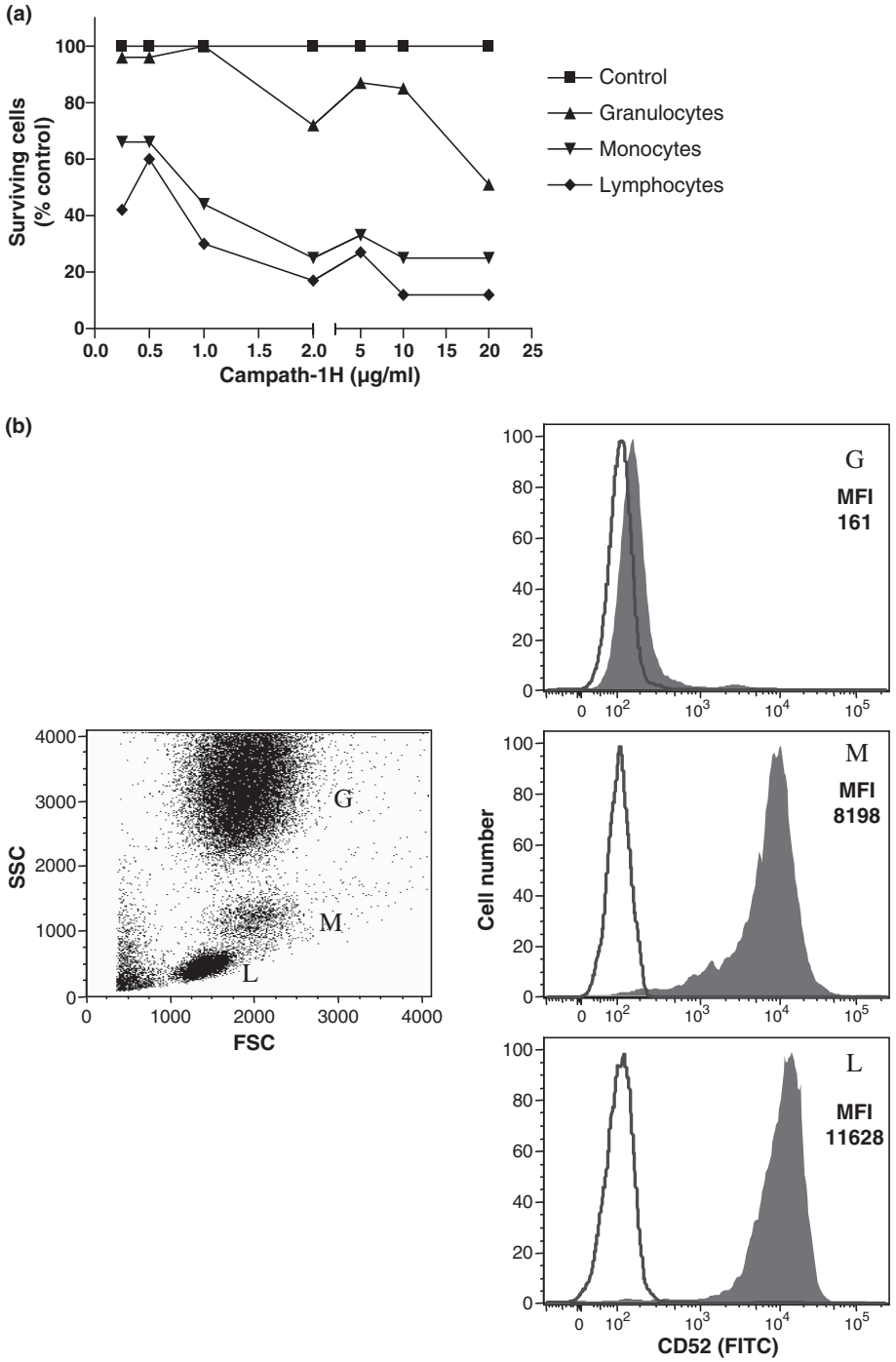

Figure 4 Campath-1H-mediated killing of lymphocytes, monocytes, and granulocytes is proportional to cell surface expression of CD52. (a) Campath-1H incubation protocol was as in Fig. 1. Lymphocytes, monocytes, and granulocytes were analyzed as whole populations, using forward vs. side scatter and cell survival as percent control is plotted as a function of Campath-1H concentration. Granulocytes showed the least amount of killing, monocytes more killing, and lymphocytes the most killing at all concentrations of Campath-1H (not done in triplicate, but see Fig. 5a). (b) Control cells (no Campath-1H) were lyzed by AC lysis solution and analyzed by flow cytometry. Lymphocytes (L), monocytes (M), and granulocytes (G) are plotted as forward vs. side scatter (left). Their respective CD52 MFI is shown in the accompanying histograms (right). Lymphocytes express the most amount of CD52 (MFI = 11,628), monocytes somewhat less (MFI = 8198), and granulocytes the least (MFI = 161). Combining the information in A and B demonstrates that killing in lymphocytes, monocytes, and granulocytes correlates with surface CD52 expression.

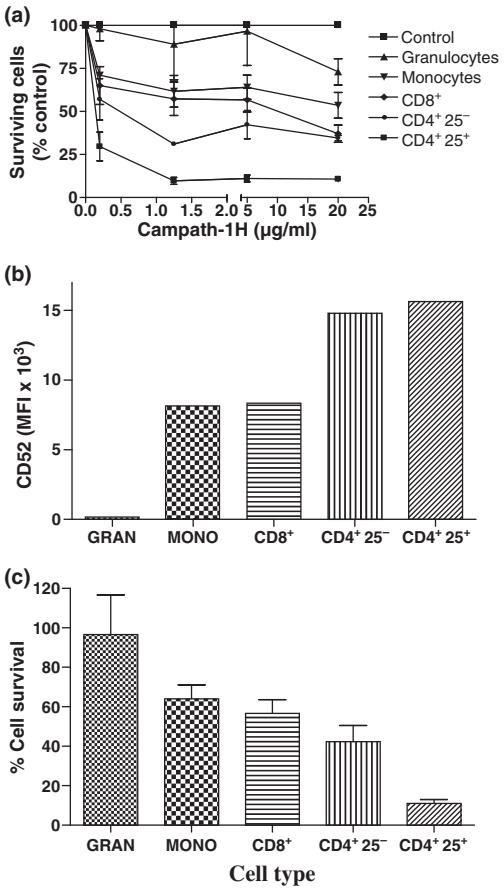

Figure 5 Campath-1H-mediated killing of lymphocyte subsets is proportional to CD52 expression. $CD4^+$ T cells express twice the amount of CD52 as do $CD8^+$ T cells. Campath-1H incubation protocol was as described in Fig. 1. Peripheral blood mononuclear cells (PBMNC) were then isolated using AC lysis solution and cells analyzed using 5-color flow cytometry. Percent surviving cells were calculated as in Fig. 1. (a) Lymphocytes were broken down into the T-cell subsets indicated, and the percent cell survival after Campath-1H treatment plotted as a function of Campath-1H concentration. Also included in the analysis is percent cell survival of granulocytes and monocytes. Error bars when not visible are obscured by data points because variation is very small. (b) Whole blood (not incubated with Campath-1H) was AC lyzed, and the indicated cell type analyzed by flow cytometry. Baseline CD52 MFI as a function of cell type is shown. (c) Whole blood was incubated with 5 μg/ml of Campath-1H, and PBMNCs isolated and analyzed in triplicate, as in A, and percent cell survival plotted as a function of cell type. Both (b) and (c) demonstrate that Campath-1H-mediated killing of T-cell subsets, and immune cells in general, is proportional to surface expression of CD52. Whereas a general trend of killing proportional to CD52 surface expression is seen, the difference in killing between monocytes and $CD8^+$ T cells ($P < 0.5$) and $CD8^+$ T cells and $CD4^+25^-$ T cells ($P < 0.26$) was not statistically significant.

respectively. Fig. 5c directly demonstrates that the sensitivity of these cell types to killing by Campath-1H in peripheral blood increases as CD52 surface expression

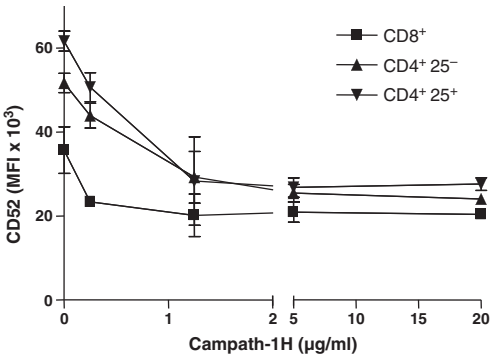

Figure 6 Cells surviving Campath-1H-mediated killing all express similarly low levels of surface CD52. Fresh human blood was incubated with 0 μg/ml and the indicated concentrations of Campath-1H for 4 h, and following AC lysis, CD52 expression of the surviving cells was determined using a 5-color flow cytometry. All cells expressing high levels of CD52 are eliminated as Campath-1H concentration increases, and all surviving T cells, regardless of subtype, demonstrate a similar low level of CD52 surface expression.

increases. Thus, complement-mediated killing predominates in $CD4^+$ T cells because they express approximately twice the amount of surface CD52 compared with $CD8^+$ T cells.

Levels of CD52 expression on cells surviving Campath -1H treatment are similar and low

As complement-mediated T-cell subset killing is proportional to surface expression of CD52, it follows that a certain minimum level of CD52 expression is required for this killing to occur. If this is true, then cells that survive Campath-1H exposure should have a low and somewhat similar level of CD52 expression. PBMNCs were treated with Campath-1H and CD52 expression determined on the remaining T cells. The FITC-antiCD52 used for this analysis is not blocked by Campath-1H (data not shown). As expected, concentrations of Campath-1H lower than 1–2 μg/ml (a level not sufficient for maximal killing, Fig. 2b) resulted in remaining T cells with relatively higher CD52 expression (Fig. 6). However, doses between 1 and 2 μg/ml of Campath-1H and higher (concentrations sufficient to induce maximal killing) resulted in remaining T cells, which showed a similar and low level of surface CD52 expression (Fig. 6). Thus, a certain amount of threshold CD52 surface expression is necessary to induce the complement-mediated killing in T-lymphocyte subsets. However, it is true that this experiment does not rule out the technical possibility that surviving T cells, upon exposure to Campath-1H, down-regulated their CD52 rapidly enough to avoid killing.

Discussion

The mechanism of T-cell depletion by Campath-1H mAb has been shown, *in vitro*, to be either complement-mediated or ADCC [5–8]. The components necessary for both processes – a target cell, an antibody bound to the target cell that fixes complement or binds the FcγIII receptor, and effector cells such as macrophages and natural killer (NK) cells – are all present in peripheral blood. Despite all components being available in peripheral blood, our results suggest that peripheral blood alone cannot facilitate *complete* T-cell killing by Campath-1H. Whereas it may be technically possible that the difference in T-cell killing between PB-HV and PB-TP is merely an artifact because T-cell killing in PB-HV is occurring *in vitro*, we believe that is not the case for a variety of reasons. First, it is clear from the literature that if all components necessary for killing by complement or ADCC are brought together *in vitro* that killing is in fact seen [5–8]. Second, when killing *in vitro* has been shown to be complement or ADCC-mediated, even when the necessary cellular components are in higher density than in peripheral blood [5–8], the best amount of killing seen is about 60–70%. However, these studies did not break down the killing into T-cell subsets as we have done, but 60–70% is a good approximation for overall killing in our studies as well. Third, there is in fact significant T-cell killing occurring in PB-HV, and almost complete killing occurring with the T-cell subtype $CD4^+25^+$ (Fig. 2), indicating that PB-HV is very capable of killing, and that any decrease in killing, like that seen in $CD8^+$ T cells, is not a result of *in vitro* artifact. Fourth, peripheral blood used in the PB-HV experiments was drawn minutes before the experiment was initiated and was kept at 37 °C throughout, thus making it unlikely that its properties and components would have been compromised in any significant way. Fifth, the rapidity with which T-cell killing is completed – after 4 h incubation with Campath-1H (Fig. 2a) – indicates again that the degree of T-cell killing observed is what peripheral blood is intrinsically capable of supporting, and that the process of T-cell killing is not compromised in any way. Sixth, if T-cell killing were in fact impaired in PB-HV, it would be unlikely to demonstrate such a compelling correlation with surface expression of CD52 (Fig. 5). Lastly, we have conclusively shown that lack of sufficient time (Fig. 2a), lack of sufficient Campath-1H (Fig. 2b), or lack of sufficient complement (Fig. 1c) were not confounding factors in our experiments.

What then is the reason for the difference in CD4 and CD8 T-cell killing seen *in vivo* and *in vitro*? One simple explanation could be that use of other immunosuppressants, as is usually the case in clinical practice [9,10,14,15], and in the patient described in Fig. 1a, may enhance Campath-1H-mediated killing. A form of steroid, for example, is usually given to patients prior to, during, or after Campath-1H administration, in addition to other immunosuppressants. Figure 3c, however, shows that steroids do not enhance the Campath-1H-mediated killing.

Another possibility is that T-cell killing, *in vivo*, is occurring more efficiently in other organs, such as the spleen, liver, or lymph nodes. As lymphocytes, NK cells, and macrophages are clustered together in secondary lymphoid tissues, it is quite possible that ADCC, mediated by macrophages or NK cells (e.g. in the spleen), is the main mechanism by which T cells that have survived elimination in peripheral blood are killed. Certainly, all lymphoid organs are not similar in their ability to mediate ADCC and some (e.g. the spleen) may be more efficient at ADCC than others [16]. Lastly, as Campath-1H also eliminates macrophages (Figs 4 and 5), and macrophages mediate ADCC, it is possible that the efficiency of ADCC in peripheral blood is reduced.

The finding that Campath-1H-mediated killing of T-cell subsets in peripheral blood is proportional to CD52 surface expression of the T cells suggests a possible mechanism to explain this difference in susceptibility to killing. There is very good correlation between the degree of killing and surface expression of CD52 (Fig. 5b,c). This would explain why $CD4^+$ T cells are eliminated more efficiently, by a complement-dependent mechanism, than $CD8^+$ T cells in peripheral blood. In fact, there is very little complement-mediated elimination of $CD8^+$ T cells, most likely because they express about half the amount of surface CD52 as do $CD4^+$ T cells. This, of course, leads to less Campath-1H binding and less fixing of complement.

The degree of noncomplement-mediated killing, or ADCC, is essentially the same for $CD4^+$ and $CD8^+$ T cells (about 33% of total number of cells, Fig. 3b). Clearly, the minimal amount of CD52 surface expression necessary to elicit ADCC is present on both $CD4^+$ and $CD8^+$ T cells, consistent with the fact that ADCC is more sensitive than complement-mediated lysis [8].

An interesting observation is that not only is the sensitivity of immune cells to killing correlated with surface CD52 expression, but the rate of re-emergence of T cells, and other immune cells in patients, treated with Campath-1H also correlates with CD52 expression (inversely proportional). For example, in the literature, it is well documented that macrophages return to approximately 25% and 100% of normal levels within 2 and 4 weeks, respectively, $CD8^+$ T cells return to about 25% of normal levels after 24 weeks, whereas $CD4^+$ T cells (not differentiated as CD25 negative or positive) return to about 25% of normal levels after approximately 52 weeks [10,11].

We have also observed similar patterns of repopulation in our transplant recipients (data not shown). This rate of return correlates very well with CD52 surface expression, which is low in macrophages (return fast), higher in $CD8^+$ T cells (return slower), and highest in $CD4^+$ T cells (return slowest). This would imply that Campath-1H continues to have an 'effect' long after it has been eliminated from peripheral blood, thus further implicating other organs in the complete and persistent elimination of T cells.

In summary, we have demonstrated two points. First, that the mechanism of Campath-1H-facilitated $CD4^+$ T-cell elimination in peripheral blood is mainly complement-mediated whereas that of $CD8^+$ T-cell elimination is mainly noncomplement mediated (ADCC). Second, T-cell subsets are not equally sensitive to complement-mediated elimination by Campath-1H in peripheral blood, owing primarily to the difference in expression of surface CD52. Presently, we are studying why CD52 is expressed differentially on T-cell subsets, and more basically, what is the function of CD52.

Acknowledgement

This study was funded by the Department of Surgery, University of Vermont.

References

1. Confavreux C, Vukusi S. Non-specific immunosuppressants in the treatment of multiple sclerosis. *Clin Neurol Neurosurg* 2004; **106**: 263.
2. Bloom DD, Hu H, Fechner JH, Knechtle SJ. T-lymphocyte alloresponses of campath-1H-treated kidney transplant patients. *Transplantation* 2006; **81**: 81.
3. Kaufman DD, Leventhal JR, Gallon LG, Parker MA. Alemtuzumab induction and prednisone-free maintenance immunotherapy in simultaneous pancreas–kidney transplantation comparison with rabbit antithymocyte globulin induction – long-term results. *Am J Transplant* 2006; **6**: 331.
4. Flechner SM, Friend PJ, Brockmann J, *et al.* Alemtuzumab induction and sirolimus plus mycophenolate mofetil maintenance for CNI and steroid-free kidney transplant immunosuppression. *Am J Transplant* 2005; **5**: 3009.
5. Isaacs JD, Wing MG, Greenwood JD, Hazleman BL, Hale G, Waldmann H. IgG4 monoclonal antibody that depletes target cells in humans. *Clin Exp Immunol* 1996; **106**: 427.
6. Dyes MJ, Hale G, Hayhoe FG, Waldmann H. Effects of Campath-1 antibodies *in vivo* in patients with lymphoid malignancies: influence of antibody isotype. *Blood* 1989; **73**: 1431.
7. Hale G, Clark M, Waldmann H. Therapeutic potential of rat monoclonal antibodies: isotype specificity of antibody-dependent cell mediated cytotoxicity with human lymphocytes. *J Immunol* 1985; **134**: 3056.
8. Riechmann L, Clark M, Waldmann H, Winter G. Reshaping human antibodies for therapy. *Nature* 1988; **332**: 323.
9. Kirk AD, Hale DA, Mannor RB, *et al.* Results from a human renal allograft tolerance trial evaluating humanized CD52-specific monoclonal antibody almetuzumab. *Transplantation* 2003; **76**: 120.
10. Knechtle SJ, Pirsch JD, Fechner Jr JH, *et al.* Campath-1H induction plus rapamycin monotherapy for renal transplantation: results of a pilot study. *Am J Transplant* 2003; **3**: 722.
11. Brett S, Baxter G, Cooper HN, Johnston JM, Tite J, Rapson N. Repopulation of blood lymphocyte sub-populations in rheumatoid arthritis patients treated with the depleting humanized monoclonal antibody, CAMPATH-1H. *Immunology* 1996; **88**: 3.
12. Litjens NH, van Druningen CJ, Betjes MG. Progressive loss of renal function is associated with activation and depletion of naive T lymphocytes. *Clin Immunol* 2006; **118**: 83.
13. Griveas I, Visvardis G, Fleva A, *et al.* Comparative analysis of immunophenotypic abnormalities in cellular immunity of uremic patients undergoing either hemodialysis or continuous ambulatory peritoneal dialysis. *Ren Fail* 2005; **27**: 279.
14. Weinblatt ME, Maddison PJ, Bulpitt KJ, *et al.* Campath-1H, a humanized monoclonal antibody, in refractory rheumatoid arthritis. *Arthritis Rheum* 1995; **38**: 1589.
15. Rebello PRUB, Hale G, Friend PJ, Cobbold SP, Waldmann H. Anti-globulin responses to rat and humanized campath-1H monoclonal antibody used to treat transplant rejection. *Transplantation* 1999; **68**: 1417.
16. Tay CH, Welsh RM. Distinct organ dependent mechanisms for the control of murine cytomegalovirus infection by NK cells. *J Virol* 1997; **71**: 267.