Effect of Campath-1H Antibody on Human Hematopoietic Progenitors In Vitro

By M.H. Gilleece and T.M. Dexter

The humanized antibody CAMPATH-1H has been shown in pilot studies to be beneficial in the treatment of lymphoid malignancy and other lymphoproliferative diseases. The antigen recognized by this antibody is not confined to lymphoid cells, and work with rat antibodies of similar specificity has not eliminated the possibility of damage to human hematopoietic progenitors, particularly those capable of repopulating bone marrow and sustaining hematopoiesis. This study aimed to discover if hematopoietic progenitor cells were affected by treatment with CAMPATH-1H, with or without human complement. Bone marrow mononuclear cells from healthy volunteers were treated with saturating concentrations of CAMPATH-1H, human complement, or CAMPATH-1H plus human complement. The CD34-positive fraction of the mononuclear cells was treated similarly. Residual progenitor activity was measured in the colony-forming unit-granulocyte, erythroid, monocyte, megakaryocyte assay and compared with untreated controls. There was no significant difference (at the 5% level) between treated and control cells. Mononuclear cells were divided into CAMPATH-1H-positive and CAMPATH-1H-negative fractions by fluorescein isothiocyanate-CAMPATH-1H labeling and fluorescence-activated cell sorter separation. Hematopoietic progenitors were predominantly found in the CAMPATH-1H-negative fraction. Furthermore, mononuclear cells treated with CAMPATH-1H and complement were equivalent to controls in experiments that investigated the capacity of these cells to form hematopoietic foci in long-term cultures.

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MATERIALS AND METHODS

CAMPATH-1H antibody. Humanized CAMPATH-1H, a genetically engineered human IgG1k monoclonal antibody (MoAb)

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containing the six complementarity determining regions from an IgG2b rat MoAb (CAMPATH-1G) specific to CDw52, was supplied by Wellcome Foundation, Ltd. (Beckenham, Kent, UK).

Bone marrow samples. The study was approved by the local ethics committee. Iliac crest aspirates were obtained under local anaesthesia, after informed consent, from healthy volunteers for use in cytotoxicity studies with CAMPATH-1H and subsequent assays for hematopoietic progenitors in semisolid cultures or on preformed stromal layers derived from long-term cultures. Functional long-term bone marrow cultures were initiated using marrow from sternal scrapes from patients undergoing coronary artery bypass surgery. The samples were suspended immediately after collection in sterile 300 mOsm/kg Iscove’s modified Dulbecco’s medium (IMDM, Gibco, Grand Island, NY) containing 40 U/mL preservative free heparin (CP Pharmaceuticals, Ltd, Wrexham, UK) and 2% fetal calf serum (FCS, Flow Laboratories, Irvine, CA).

Preparation of long-term cultures. The cultures were established using a modified version of the technique of Gartner and Kaplan,13 as described in Coutinho et al.18 Briefly, red blood cells were depleted by mixing the bone marrow aspirate cell suspension with methylcellulose, final concentration 0.1%, for 30 to 40 minutes at room temperature. The nucleated cells remaining in suspension were recovered and washed twice before being resuspended at 2 × 10^6 cells/mL in long-term culture medium (LTCM) containing 340 mOsm/kg IMDM, 10% FCS, 1% horse serum (HS) and 5 × 10^{-7} mol/L hydrocortisone sodium succinate (Sigma, St Louis, MO). The suspension was incubated at 33°C in tissue culture flask, gassed with 5% CO2 in air. The cultures were fed weekly by replenishing half of the spent medium with fresh LTCM and were observed for the appearance of hematopoietic foci on the stromal layer.

Enrichment of bone marrow mononuclear cells. Bone marrow mononuclear cells (BMMNC) were obtained by separation of fresh marrow aspirates over J-PREP (1.077 g/mL density, TechGen International, London, UK).

CD34 antigen-positive cell enrichment. BMMNC expressing CD34 antigen were selectively apaned by a panning procedure using Cellector SBA and CD34 cell culture flasks (Applied Immune Sciences, Menlo Park, CA). This technique first depletes T cells, B cells, monocytes, fibroblasts and red blood cells by virtue of their adherence to immobilized soy bean agglutinin. The nonadherent cells are then incubated with immobilized CD34 antibody. Using this technique, we achieved a consistent 20-fold enrichment of CFUs as judged by performance of varying concentrations of enriched cells in CFU-GEMM assay conditions (data not shown).

**CFU-GEMM assay.** This was performed as described previously.18 Briefly, BMMNC or CD34 antigen-positive enriched cells were resuspended at 10^7 or 5 × 10^5 cells/mL, respectively, in 1.2% methylcellulose, 10% 5637 bladder-carcinoma cell line-conditioned medium (as a source of colony-stimulating factors),19 2 U of recombinant erythropoietin (Terry Fox Lab, Vancouver, Canada), 1% bovine serum albumin (Sigma) and 30% FCS, plated at 0.25 mL/well in triplicate, incubated in humidified 5% CO2, 5% O2, and 80% N2, at 37°C and scored at 9 and 14 days. Colonies of more than 50 cells were scored as CFU-E, BFU-E, GM-CFC, or CFU-MIX and we included single lineage granulocyte or macrophage colonies among the GM-CFC.

**Incubation of BMMNC with CAMPATH-1H and complement.** Optimum saturating concentrations of CAMPATH-1H and recombinated human complement (Sigma) were determined by chromium 5 mmol/L 51Cr 185MB (DuPont [UK] Ltd, Stevenage, UK) release studies as described by Hale et al.18 Briefly, peripheral blood mononuclear cells were labeled with chromium 5 mmol/L 51Cr 185MB before sequential incubations with antibody and a comple-ment source; each assay was performed in triplicate and 51Cr release by lysed cells was measured by a gamma counter. At a final concentration of 25%, reconstituted human complement was as effective as autologous or blood group AB donor serum in lysing peripheral blood mononuclear cells previously incubated with 200 μg/mL CAMPATH-1H (Wellcome). CAMPATH-1H was titrated in a similar assay using 25% autologous serum as a source of complement; maximum lysis of peripheral blood mononuclear cells was achieved at final concentrations of 1 to 100 μg/mL with little cell lysis at concentrations of 0.1 μg/mL or less.

Aliquots of 10^6 BMMNC in 100 μL medium (300 mOsm IMDM, 1% RSA, and 1% FCS, heat inactivated) were incubated with 100 μL CAMPATH-1H 400 μg/mL or medium for 10 minutes at 26°C before adding reconstituted human complement, final concentration 25%, or medium and incubating at 37°C for 45 minutes. Surviving cells were then washed and plated in CFU-GEMM assay conditions to assess residual progenitor activity. One aliquot of untreated cells was plated directly in the CFU-GEMM assay.

**Progenitor activity of FITC-CAMPATH-1H-labeled BMMNC.** Aliquots of 10^6 cells were incubated with fluorescein isothiocyanate (FITC)-labeled CAMPATH-1H 400 μg/mL and separated into positive and negative fractions by a Becton Dickinson fluorescence activated cell sorter IV (FACS, Becton Dickinson, Oxford, UK). FITC-Tetanus Toxin 46 human IgG, MoAb (Wellcome) was used to eliminate false positives caused by nonspecific binding. Positive and negative fractions, together with controls consisting of sorted and unsorted unlabeled cells, were then plated at 10^5 cells/mL in CFU-GEMM assay conditions.

**CD34 antigen-positive BMMNC incubated with CAMPATH-1H and complement.** CD34 antigen positive-enriched BMMNC were sequentially incubated with CAMPATH-1H antibody or medium followed by complement or medium as described above before washing and plating in the CFU-GEMM assay at 5 × 10^5 cells/mL.

Long-term culture initiating cells incubated with CAMPATH-1H and complement. Further aliquots of CD34 antigen-positive, enriched BMMNC were incubated with CAMPATH-1H and complement or medium alone, washed, resuspended in LTCM, and seeded at 8 × 10^5/mL onto performed irradiated (15 Gy; caesium 137 gamma rays dose rate 4 Gy/min) stromal layers gassed with 5% CO2 in air and incubated at 33°C (the stromal layers were previously shown to be functional by their ability to support hematopoietic foci before irradiation.) The hematopoietic potential of the seeded cells was determined by their ability to form hematopoietic foci on these stromal layers, as well as production of hematopoietic progenitor cells (assessed in the CFU-GEMM assay in the ensuing weeks). The freshly inoculated cultures were fed weekly by replenishing half of the spent medium with fresh medium. Nonadherent cells removed at the same time were counted and aliquots were plated in CFU-GEMM assay conditions.

**Statistical analysis.** The effects of CAMPATH-1H plus complement on day 14 GM-CFC were evaluated using analysis of covariance, the covariate being day 9 GM-CFC. Day 14 BFU-E and CFU-MIX and day 9 CFU-E colonies were evaluated by Friedman nonparametric analysis of variance. The effects of CAMPATH-1H plus complement on the production of nonadherent cells and colony forming cells in long-term bone marrow cultures were assessed by repeated measures analysis of covariance. All statistical operations were performed using the statistical computer package Biomedical Data Processing (BMDP) (Statistical Software, Berkeley, CA).

**RESULTS**

Effect of CAMPATH-1H with or without complement on unseparated bone marrow mononuclear cells. Figure 1
CAMPATH-1H AND HUMAN HEMATOPOIETIC PROGENITORS

Mean colony numbers, derived from nine experiments performed in triplicate, for day 9 CFU-E and for day 14 GM-CFC, BFU-E, and CFU-Mix are shown. The results show that there was no significant difference at the 5% level in the number of colonies produced from cells previously incubated with CAMPATH-1H with or without complement compared with untreated cells (n = 8; day 14 GM-CFC = .6332, BFU-E = .6823, CFU-MIX = .3974; day 9 CFU-E P = .3691).

FITC-CAMPATH-IH cells assayed for hematopoietic progenitor activity. BMMNC were labeled with FITC-CAMPATH-IH and positive and negative cells were retrieved by FACS and assayed for the presence of hematopoietic progenitors as described in previous sections. Unlabeled control cells were either assayed without further manipulation or after passage through the FACS. The mean percentage of BMMNC-binding FITC-CAMPATH-1H was 15.8% (SD 5.44, n = 8). Results of progenitor assays in four experiments are shown in Table 1. The fraction of cells that bind to CAMPATH-1H contains less progenitor cells than the negative fraction.

The in vitro repopulating capacity of early hematopoietic progenitors following treatment with CAMPATH-IH. Data indicate that the progenitor cells that initiate hematopoiesis in long-term bone marrow cultures are equivalent (or closely related) to the hematopoietic stem cell. We assessed the response of these cells to CAMPATH-1H plus complement treatment. The results of observations of CAMPATH-1H and complement treated or untreated CD34 antigen-positive, enriched cells seeded onto functional stromal layers in four experiments are shown in Figs 3 and 4. Foci were observed in all cultures and appeared within 2 weeks of seeding, while hematopoietic progenitors persisted for 3 to 6 weeks of culture in standard conditions. Treatment with CAMPATH-IH plus human complement did not affect subsequent production of hematopoietic foci or nonadherent progenitors (P = .6060) or colony forming cells (P = .7533).

DISCUSSION

Studies with conventional rat CAMPATH-I MoAbs have assessed the use of the antibodies in a variety of clinical situations. For example, CAMPATH-I-M has been used to deplete bone marrow of T cells before allogeneic transplan-

Table 1. Colony Forming Capacity of FITC-CAMPATH-1H Positive/Negative Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>GM-CFC</th>
<th>BFU-E</th>
<th>CFU-E</th>
<th>CFU-Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 (100)</td>
<td>100 (44)</td>
<td>100 (32)</td>
<td>100 (4)</td>
</tr>
<tr>
<td>FACS-sorted unlabeled</td>
<td>162</td>
<td>100</td>
<td>138</td>
<td>100</td>
</tr>
<tr>
<td>FITC-CAMPATH-1H positive</td>
<td>24</td>
<td>45</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>FITC-CAMPATH-IH negative</td>
<td>84</td>
<td>145</td>
<td>175</td>
<td>100</td>
</tr>
</tbody>
</table>

The colony forming capacity of FITC-CAMPATH-1H positive cells compared with FITC-CAMPATH-1H negative cells, unsorted cells, and unlabelled sorted cells in four separate experiments. CFCs are expressed as a percentage of control where absolute values are given in brackets.
tation resulting in a reduction in GVHD. However, as with other methods of T-cell depletion, there has been no significant rise in overall survival because of the increased incidence of graft rejection. There is also an increased incidence of leukemic relapse in CAMPATH-1 antibody-treated marrow suggesting a coincident depletion of antitumor effector mechanisms.

The expression of CAMPATH-1 by a variety of normal and abnormal lymphoid cells has prompted investigation of in vivo therapy using CAMPATH-1 antibodies. In vivo therapy of allogeneic bone marrow transplant recipients with CAMPATH-1 antibody may avoid early graft rejection by removing the residual host immunocompetent lymphocytes that persist despite marrow ablative conditioning regimes and may also contribute to elimination of residual leukemia. In fact, CAMPATH-1M has been shown to have an immunosuppressive effect in vivo when used therapeutically or prophylactically in organ transplantation. CAMPATH-1 antibodies may also have a role in the treatment of autoimmune diseases such as rheumatoid arthritis where T cells are implicated in pathophysiology.

CAMPATH-1M, administered intravenously, has been shown to reduce the peripheral lymphocyte count in lymphoid malignancies, accompanied by consumption of complement, but without any effect on malignant cells in bone marrow and solid tumor masses. CAMPATH-1G, used similarly, depleted lymphocytes from marrow and spleen as well as peripheral lymphocytes. Furthermore, the fall in peripheral lymphocytes with CAMPATH-1M was transient and succeeded by a rebound rise suggesting that sequestration had occurred; by comparison, the fall induced by CAMPATH-1G was sustained and not associated with appreciable consumption of complement. Cell-mediated cytotoxicity is more effective with CAMPATH-1G than with CAMPATH-1M and these results suggest that Fc receptor-mediated killing may be crucial.

However, treatment in vivo using CAMPATH-1G was associated with fever, rigors, rises in liver transaminases and, in a minority of patients, a significant rise in anti-IgG levels and bronchospasm. This is typical of rat MoAbs whose antigenicity in humans limits the duration of therapy to little more than 10 days in nonimmunosuppressed patients. Humanized antibodies combine rodent complementarity determining regions (plus adjacent amino acids) with human framework regions and human constant regions. The reduction in nonhuman components is expected to reduce the immunogenicity of the antibody. Human IgG1 has high activity in complement and cell-mediated lysis and was, therefore, selected as the basic structure of the humanized CAMPATH-1 antibody, CAMPATH-1H. CAMPATH-1H, showing identical specificity to the original rat antibody, was constructed using hypervariable loops of YTH,34.5 IgG2a. It has been administered to two patients with lymphoid malignancy as a daily intravenous dose for up to 43 days resulting in prolonged remission without any evidence of immunogenicity and with at least partial restoration of normal hematopoiesis. CAMPATH-1H has also been administered to eight patients with refractory rheumatoid arthritis as a daily intravenous dose for up to 10 days in a phase 1 open study and significant clinical benefit was reported in seven patients, although three of four patients who received 10 days of treatment did develop antiglobulins that were capable of blocking binding of CAMPATH-1H to its antigen. The introduction of the humanized CAMPATH-1H antibody for clinical use requires assessment of possible cross-reactivity or effects on proliferative potential of early hematopoietic progenitor cells. We have investigated this, using a variety of procedures that enrich for hematopoietic progenitor cells and, thus, reduce the possibility of effects of CAMPATH-1H be-
ing mediated by the presence of accessory cells. We have also tried to define the distribution of the CAMPATH-1 antigen in relation to proliferative capacity in CFU-GEMM assay conditions.

The mononuclear fraction of bone marrow cells is known to include most hematopoietic progenitors and is a convenient population for study. Incubation of BMMNC with CAMPATH-1H, either in the presence or absence of complement, resulted in no significant depletion of GM-CFC, BFU-E, CFU-E, and CFU-MIX. When BMMNC were further enriched for progenitor cells by selection with an antibody that recognized CD34, incubation with CAMPATH-1H (in the presence or absence of complement) did not significantly reduce the number of progenitor cells. These results suggest that the depletion of putative accessory cells by CAMPATH-1H does not influence subsequent proliferation and development of hematopoietic progenitor cells and that CAMPATH-1H has no direct toxic effect on hematopoietic progenitor cells.

The earliest hematopoietic cells that are capable of repopulating bone marrow can only be unequivocally shown by in vivo transplantation. However, the presence of these stem cells is also inferred from their ability to establish hematopoiesis when cultured in association with normal stromal cells in vitro. To examine if treatment of bone marrow cells with CAMPATH-1H had an effect on the in vitro repopulating cells, CD34-positive cells previously incubated with CAMPATH-1H plus complement were cocultured with marrow stroma. The results show that, in these conditions, there is no abrogation of the ability of the CD34-positive cells to establish hematopoiesis and to generate hematopoietic progenitor cells. The relatively low numbers of erythroid and mixed-lineage progenitors produced in these cultures is a reflection of the bias of this system toward myeloid differentiation and is not a result of CAMPATH-1H treatment.

Finally, corroborative evidence of the relative exclusion of hematopoietic progenitors from CAMPATH-1H binding is supplied by the results of progenitor assays of cells separated by FACS into CAMPATH-1H positive and negative fractions. The majority of progenitor activity was shown to be in the CAMPATH-1H-negative fraction. However, it is evident that there are some progenitors in the positive fraction and, whereas this may be attributable to FACS sensitivity, it may also support the possibility that some progenitors bind CAMPATH-1H but do not then fix complement to undergo lysis. Incubation of the CAMPATH-1H-positive cells with complement and subsequent CFU-GEMM assay might resolve this question. However, even if this hypothesis were true, our data do not indicate any toxicity to the progenitor cells resulting from CAMPATH-1H binding.

This study supports the view that humanized CAMPATH-1 antibody, CAMPATH-1H, is unlikely to impair early hematopoietic progenitor development when administered clinically. It does not provide information regarding possible effects of CAMPATH-1H on the more mature hematopoietic cells nor on the long-term effect of repeated administration on the most primitive hematopoietic progenitors.

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Effect of Campath-1H antibody on human hematopoietic progenitors in vitro

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