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Cross-linking of the CAMPATH-1 antigen (CD52) mediates growth inhibition in human B- and T-lymphoma cell lines, and subsequent emergence of CD52-deficient cells

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SUMMARY

The CAMPATH-1H (CD52) antigen is a 21 000–28 000 MW glycopeptide antigen that is highly expressed on T and B lymphocytes and is coupled to the membrane by a glycosylphosphatidylinositol (GPI) anchoring structure. The humanized CAMPATH-1H anti-CD52 antibody is extremely effective at mediating depletion of both normal and tumorigenic lymphocytes *in vivo* and has been used in clinical trials for lymphoid malignancy and rheumatoid arthritis. Cross-linking GPI-anchored molecules, including CD52, on the surface of T lymphocytes in the presence of phorbol 12-myristate 13-acetate or anti-CD3, results in cellular activation. In the present study we have investigated the functional effects of cross-linking CD52 on T and B tumour cell lines. Cross-linking CD52 on either a B-cell line, Wien 133, which expresses high levels of endogenous CD52 or Jurkat T cells transfected and selected to express high levels of CD52 resulted in growth inhibition. This effect showed slower kinetics and occurred in a lower percentage of cells than growth inhibition stimulated via T- or B-cell receptors. Growth inhibition of the Wien 133 line was followed by the induction of apoptosis, which appeared independent of the Fas/Fas L pathway. Wien 133 cells surviving anti-CD52 treatment were selected and cloned and found to have down-regulated CD52 expression, with a characteristic biphasic pattern of 10% CD52-positive, 90% negative by fluorescence-activated cell sorter analysis. Interestingly, surface expression of other GPI-linked molecules, such as CD59 and CD55, was also down-regulated, but other transmembrane molecules such as surface IgM, CD19, CD20, HLA-DR were unaffected. The present study and previous work show that this is due to a defect in the synthesis of mature GPI precursors. Separation of CD52-positive and negative populations *in vitro* resulted in a rapid redistribution to the mixed population. Injection of CD52-negative cells into nude mice to form a subcutaneous tumour resulted in a substantial increase in expression of CD52. These results suggest that the defect in the Wien 133 cells is reversible, although the molecular mechanism is not clear. These observations have relevance to the clinical situation as a similar GPI-negative phenotype has been reported to occur in lymphocytes following CAMPATH-1H treatment *in vivo*.

INTRODUCTION

The CAMPATH-1H antigen is a 21 000–28 000 mw glycopeptide which is highly expressed on normal T and B lymphocytes.[1] The antigen comprises a short peptide sequence of 12 amino acids and a large complex N-linked oligosaccharide and is attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor.[2,3] The antigen is also expressed on a large proportion of lymphoid cell malignancies.[1] Antibodies directed against the CD52 antigen have been used as therapeutic agents in a number of lymphoproliferative diseases including non-Hodgkins lymphoma (NHL).[4] A panel of rat monoclonal anti-CD52-specific antibodies, including both IgM and IgG isotypes, has been shown to be very efficient at cell lysis in the presence of human complement.[5] *In vivo*, however, only the rat IgG2b antibody (CAMPATH-1G) and the humanized version (CAMPATH-1H)[6] proved to be effective in the depletion of normal and transformed lymphoid cells.[4,7] The reason for this has been attributed to the fact that both of these antibodies bind to Fcγ receptors on effector cells and thereby bring about cell killing via antibody-dependent cell-mediated cytotoxicity (ADCC).[4,8]

Whether the depletion of lymphocytes by CAMPATH-1G and CAMPATH-1H *in vivo* is entirely due to ADCC and complement-mediated lysis is unknown. An additional mechanism by which antibodies could have a direct effect on lymphocyte depletion is by inducing programmed cell death

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or apoptosis.[9,10] The response of normal T and B lymphocytes to ligation of antigen receptors can vary according to both the state of cellular maturation and the way antigen is presented to cells resulting in either clonal expansion, differentiation or anergy, or in clonal deletion by apoptosis. Many transformed B-[11] and T-[10] cell lines have been shown to undergo growth inhibition or apoptosis following ligation of either the antigen-specific receptor complex or certain other cell surface receptors.[12] We have recently shown that cross-linking CD52 on normal human T lymphocytes, as observed for other GPI-linked molecules, triggers clonal expansion of normal human T lymphocytes.[13] It was therefore of interest to determine the direct effects of cross-linking CD52 on transformed lymphocytes as they are an important clinical target of the antibody. The majority of *in vitro* maintained lymphoid-derived tumour cells only express CD52 at very low levels.[1] An exception to this was a B-cell lymphoma line, Wien 133, derived from a child with non-endemic Burkitt-type acute lymphoblastic leukaemia, which expressed high levels of CD52 continuously in culture.[14] Phenotypically this line appears to be a relatively mature B-cell line expressing high levels of surface IgM (sIgM), low levels of sIgD and also human leucocyte antigen (HLA) DR, CD19 and CD20. In addition, Jurkat cells were stably transfected with CD52 under the β-actin promoter and clones expressing high levels of CD52 were selected.[15] The results showed that cross-linking CD52 on both the B and T tumour cell lines resulted in profound growth inhibition. More detailed examination of the Wien 133 cells revealed that this was followed by induction of apoptosis, which appeared to be independent of the Fas/Fas ligand pathway. Cells surviving anti-CD52 treatment were found to have down-regulated CD52 expression as well as other GPI-linked proteins.

MATERIALS AND METHODS

Antibodies and reagents

The CD52 rat-specific monoclonal antibody (mAb) CAMPATH-1G (IgG2b) is a variant derived by loss of the Y3 myeloma light chain and class switching from the hybridoma clone YTH 34·5 (IgG2a).[16] The reshaped humanized version of this antibody (CAMPATH-1H) was joined to the human IgG1 constant region and expressed in Chinese hamster ovary (CHO) cells as previously described.[6] The humanized anti-CD4 mAb was produced in NSO myeloma cells as previously described.[8] $F(ab)_2$ fragments of CAMPATH-1H were prepared by pepsin digestion as previously described, and complete digestion confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).[17] Rabbit polyclonal or mouse mAb against human μ or γ Fc regions or anti-$F(ab)_2$ were obtained from Sigma (Poole, Dorset). Fluorescein isothiocyanate (FITC)-labelled CAMPATH-1H $F(ab)_2$ was kindly provided by Fiona Crisp (Wellcome, Beckenham, UK), and FITC-labelled anti-IgG, anti-IgD and anti-IgM antibodies were all obtained from Sigma. FITC-labelled anti-DR, CD16, CD19 and CD20 were obtained from Becton-Dickinson (Oxford, UK).

Cell lines

The Wien 133 B-cell lymphoma cell line maintained in Iscove's medium containing 10% heat-inactivated fetal calf serum (FCS), supplemented with penicillin, streptomycin and L-glutamine (Flow Laboratories, High Wycombe, UK). The cell line was cloned by limiting dilution to ensure a homogeneous population for these experiments. A representative clone C1 was chosen. The Jurkat T-cell lymphoma cell line clone E6-1 was obtained from the European Collection of Animal Cell Cultures (ECAC; Salisbury, UK), and transfected with pRDN-1 vector containing the CD52-coding region expressed from the β-actin promoter and G-418-resistant cells were selected and cloned as described previously.[15] Several clones of Jurkat cells expressing different levels of CD52 were selected for further study. Transfected cells were cultured in RPMI-1640 plus usual supplements and also G-418 at 500 μg/ml.

Cell growth inhibition assay

Tumour cells (2×10^4/well) were incubated in flat-bottomed 96-well microtitre plates (Costar) in the presence or absence of various antibodies at indicated concentrations in appropriate media for 5 days at 37°/5% CO_2. Alternatively in some experiments, cells were preincubated with saturating concentrations of primary antibody (10 μg/ml) for 1 hr at 4°, followed by washing and addition of secondary cross-linking reagent. Cell growth was monitored by pulsing cultures with 1 μCi/ml [methyl-^{3}H]thymidine (Amersham International, Amersham, UK) for 4–6 hr, and the amount of DNA synthesis was measured using a beta-plate scintillation counter (LKB, Pharmacia). Results are expressed as mean ± SEM c.p.m. of four to six replicates or alternatively as percentage of growth (c.p.m.) of cells which were untreated.

Selection of CD52 low variants of Wien 133 cell line

Wien 133 cells were pretreated with CAMPATH-1H and anti-human IgG, excess antibody washed off and cultured with Iscoves's medium with usual supplements for 7 days, after which the majority of cells had died via apoptosis. Fresh medium was added every 7–10 days, and live cells expanded in cultures over the next 2–3 weeks. The cells were analysed for expression of different cell surface markers by fluorescence-activated cell sorter (FACS) analysis and cloned by limiting dilution at 3, 1 and 0·3 cells per well. At least 10 individual clones, which were designated CV1, -2, -3 etc. were phenotyped by FACS analysis.

Flow cytometry

Expression of different cell surface markers was performed by FACS analysis. Cells were incubated with directly conjugated FITC or phycoerythin (PE)-labelled (CD32, CD19, CD20), HLA-DR antibodies, obtained from Becton-Dickinson. Anti-human anti-μ, and anti-γ and anti-δ antibodies FITC-conjugated directly were obtained from Sigma. For recognition of other GPI-linked molecules, rat anti-CD59 (YTH 53.1) was kindly provided by Dr Geoff Hale (University of Oxford, UK) and detected by FITC-conjugated anti-rat IgG and mouse anti-CD55 (Serotech, Oxford, UK) was detected with FITC goat anti-mouse IgG. Isotype-matched controls were used in all experiments. All flow cytometry was performed using a FASCcan flow cytometer (Becton-Dickinson), using Lysis II software.

Detection of apoptosis

At various times after antibody treatment the Wien 133 cell lines were analysed for evidence of apoptosis. The morphology of the cells was analysed using cytospin preparations which were stained in Giemsa May–Grunwald stain. The percentage of apoptotic bodies in each preparation was determined by counting at least 500 cells by light microscopy. Alternatively apoptosis was monitored by propidium iodide staining of cells, followed by analysis on FACScan as previously described.[18]

CD52 mRNA levels

Messenger RNA was prepared from parental and CD52 low cell lines using the guanidine thiocyanate method and mRNA was isolated by magnetic separation on a Dynal column (Gibco BRL, Paisley, UK). Relative levels of CD52 mRNA in the different cell types were determined using RNA dot blots with doubling dilutions of RNA. The filters were probed with a ^{32}P-labelled cDNA of the whole CD52 antigen as previously described.[15] The filter was reprobed with a ^{32}P-labelled β-actin probe to check for equal loading.

Quantification and Western blot analysis of CD52 and CD59 antigen in Wien 133 cells

An enriched glycolipid fraction, enriched for GPI-linked proteins, was obtained from identical cell equivalents of the parental or different clones expressing low levels of CD52, as described previously.[3] This fraction was used to coat enzyme-linked immunosorbent assay (ELISA) plates at a range of dilutions, followed by washing thoroughly and addition of CAMPATH-1H antibody, and detection with alkaline phosphatase-conjugated goat anti-human IgG. The amount of antigen in cell equivalents was calculated using a standard curve with affinity-purified CD52 antigen kindly provided by Dr R. Lifely (Glaxo-Wellcome, Stevenage, UK). To confirm that the signal measured in the ELISA was indeed specific for the presence of CD52 antigen, the chloroform/methanol extracts were also subjected to SDS–PAGE and Western blot analysis. The CD52 antigen was further characterized by pretreatment with *N*-glycanase, which reduces the molecular weight from 21 000–28 000 to a core of 6000, as previously described.[2,3]

Relative total cellular CD59 levels between parental and low CD52 cells lines were determined by lysing cells followed by SDS–PAGE and Western blot analysis using a rat CD59-specific antibody (YTH 53.1.), followed by detection with goat anti-rat IgG conjugated to horseradish peroxidase, and developed using ECL.

In vivo *growth of Wien 133 cells*

Different numbers of parental or CD52 low variant cell lines [CV or monocycle-resistant (MR)] were injected subcutaneously into the flank of nude mice and tumours were allowed to establish for varying periods of time. Tumours were then dispersed and subjected to FACS analysis for expression of different cell surface molecules, including CD52, CD59, surface IgM, CD20, CD19 and HLA-DR, immediately after isolation from the animal and after different times following *in vitro* culture.

RESULTS

Growth inhibition of B-cell lymphoma by cross-linking CD52

The effects of cross-linking the CD52 antigen using the humanized anti-CD52-specific antibody, CAMPATH-1H on the growth of the B-cell line Wien 133 are shown in Fig. 1. CAMPATH-1H antibody cross-linked in solution with anti-human IgG resulted in growth inhibition of Wien 133 cells as measured by the ability to incorporate [^{3}H]thymidine. CAMPATH-1H antibody alone had no effect; anti-human IgG alone, had a slight effect, which was variable between experiments, whereas the combination of the two antibodies resulted in significant reduction in cell growth (Fig. 1a).

In order to confirm that the effects observed were a result of cross-linking CD52 and not due to formation of immune complexes and/or interaction with surface immunoglobulin molecules or Fc receptors, several control experiments were performed. Growth inhibition was also observed following cross-linking CD52 using the rat anti-CD52 antibody CAMPATH-1G followed by an anti-rat IgG that did not cross-react with human IgG (Fig. 1b). Inhibition of growth of the Wien 133 cells was also observed by cross-linking CD52 with $F(ab)_2$ fragments of both CAMPATH-1H and anti-huIgG, indicating that FcR cross-linking was not necessary to mediate the observed effects (Fig. 1c). Additional evidence that the growth inhibitory effects were not due to immune complex formation was that the addition of a humanized IgG1 anti-CD4 antibody, (of the same isotype as CAMPATH-1H) and anti-huIgG did not result in growth inhibition (Fig. 1a). Pre-incubation with anti-huIgG followed by washing and addition of CAMPATH-1H did not result in growth inhibition, whereas if the antibodies were added in reverse order or together, significant growth arrest was observed (data not shown). This shows that cross-linking of the CD52 antigen was required to induce growth inhibition.

Comparison of growth inhibition of Wien 133 cells induced by surface immunoglobulin and CD52

The Wien 133 cell line was shown by FACS analysis to express high levels of surface IgM. Addition of soluble anti-μ to the cells caused almost complete growth inhibition (99%). Although surface expression of CD52 was comparable with that of sIgM, growth inhibition did not exceed 80% and secondary cross-linking was obligatory. A comparison of the time–course of growth inhibition induced by anti-μ or anti-CD52 antibodies is shown in Fig. 2. The effects of anti-μ were extremely rapid, with maximal effects occurring by 18–24 hr. CAMPATH-1H alone had no effect on cell growth, whereas anti-IgG alone inhibited growth to some extent at 24 hr, after which the cells showed some recovery. The combination of CAMPATH-1H and anti-huIgG together induced significant growth inhibition, although this was much slower (48–96 hr) than observed for anti-μ.

Cross-linking CD52 induces apoptosis in Wien 133 cells

Growth inhibition of certain lymphoid-derived tumours induced by ligating various cell surface receptors is sometimes also accompanied by programmed cell death or apoptosis. Therefore a number of experiments were performed to

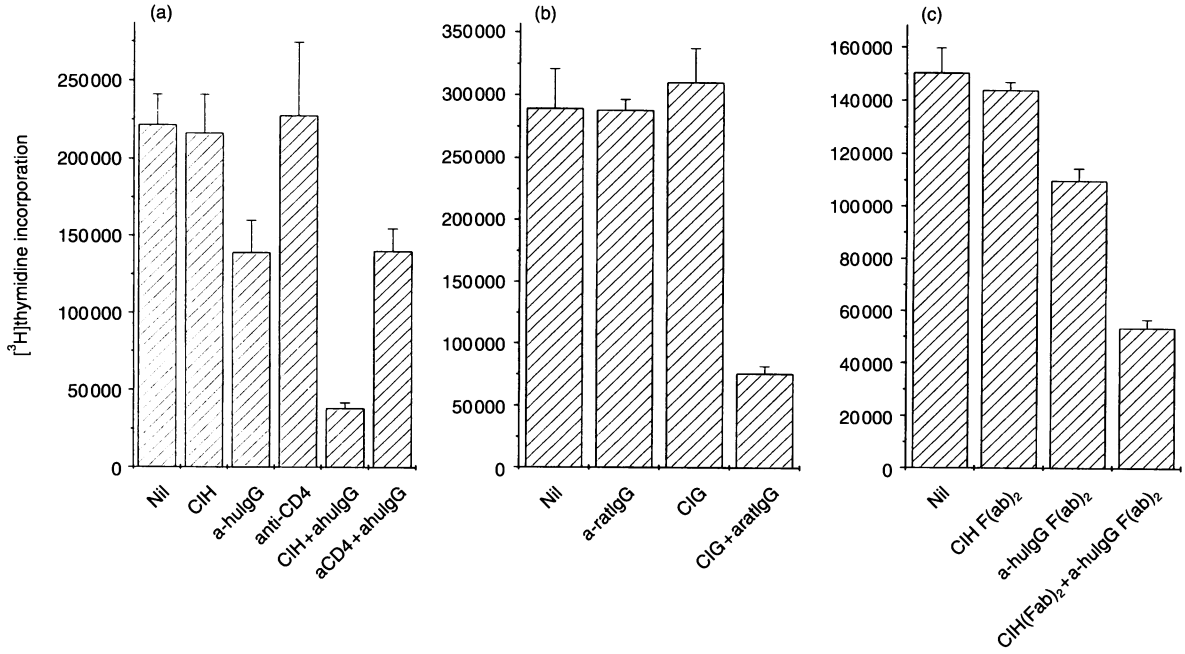

Figure 1. Inhibition of cell growth of the B-cell Wien 133 cell line by cross-linking the CD52 antigen. Wien 133 cells (2×10^4/well) were cultured for 4 days in the presence of (a) medium alone, CAMPATH-1H (CIH) alone, anti-huIgG, anti-CD4, CIH + anti-huIgG, anti-CD4 + anti-huIgG; (b) medium, anti-rat IgG, CAMPATH-1G (CIG), CIG + anti-huIgG (c) medium alone, CIH $F(ab)_2$, anti-huIgG $F(ab)_2$, CIH $F(ab)_2$ + anti-huIgG $F(ab)_2$. Antibodies were added in solution at a final concentration of 20 μg/ml. [^{3}H]thymidine was added for the last 6 hr of the culture. Results are expressed as mean ± SEM of quadruplicate cultures.

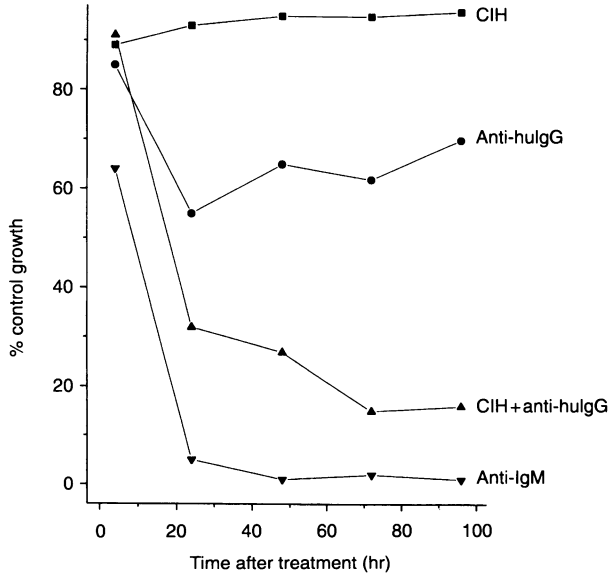

Figure 2. Comparison of time-course of growth inhibition induced by anti-CD52 and anti-Ig antibodies. Wien 133 cells were cultured for varying periods of time after treatment with medium alone, CAMPATH-1H (CIH), anti-huIgG, CIH + anti-huIgG at 20 μg/ml or anti-IgM at 4 μg/ml. [^{3}H]thymidine was added for the last 6 hr for each time-point. Results are expressed as % control growth of mean c.p.m. of cells in medium alone for each time-point.

determine whether cross-linking CD52 also induced apoptosis in Wien 133 cells. Light microscopy of cells treated with Giemsa/May–Grunwald stain allowed identification of apoptotic cells. Apoptotic cells were identified as having condensed nuclei, blebbed membranes and being smaller than normal cells due to shrinkage of the cell cytoplasm. Cells cultured with soluble anti-IgM, resulted in the appearance of a significant number of apoptotic bodies as early as 4 hr, reaching 20% by 24 hr and >98% by 72 hr. Cross-linking CD52 using CAMPATH-1H and anti-human IgG, did not result in appearance of apoptosis until 72 hr and the percentage of cells showing apoptosis at any one time was 18–20% (Fig. 3a). A small (<3%) percentage of apoptotic cells was observed in cells treated with anti-human IgG, in agreement with the degree of growth inhibition observed with this treatment. Only a few apoptotic cells were observed in cultures treated with either medium alone or CAMPATH-1H alone without further cross-linking. The presence of apoptotic cells detected following either anti-μ or CAMPATH-1H and anti-human IgG was confirmed by electron microscopy.

Changes in DNA composition due to cells undergoing apoptosis can also be demonstrated by staining DNA with propidium iodide and analysing the cells by FACS. FACS analysis of cells at 96 hr following treatment with CAMPATH-1H and anti-human IgG, showed that a much higher proportion of cells contained low molecular weight DNA than in samples treated with medium, or CAMPATH-1H and anti-human IgG antibodies alone. (Fig. 3b). Cells treated with anti-μ contained only small DNA fragments by this time.

Recent studies have indicated that induction of Fas and Fas ligand in certain T-cell tumour lines is an important pathway for apoptosis,[19] and therefore Fas expression following cross-linking of either surface IgM or CD52 on the Wien 133 cells was determined. The cells were negative for Fas expression prior to treatment and no increase in expression

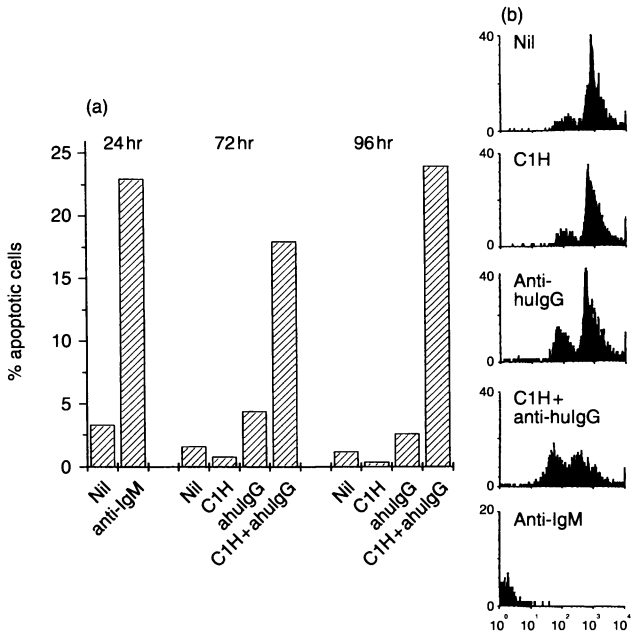

Figure 3. Evidence for apoptosis occurring in Wien 133 cells treated with anti-IgM or CD52 antibodies. Wien 133 cells were treated with medium alone, anti-IgM, CAMPATH-1H (CIH), anti-huIgG or CIH + anti-huIgG for different time periods. Cells were recovered and analysed. (a) Counting number of apoptotic cells as assessed by morphological appearance following Geimsa/May–Grunwald staining, with 500 cells counted per group. (b) Assessment of DNA damage by propidium iodide staining and FACS analysis.

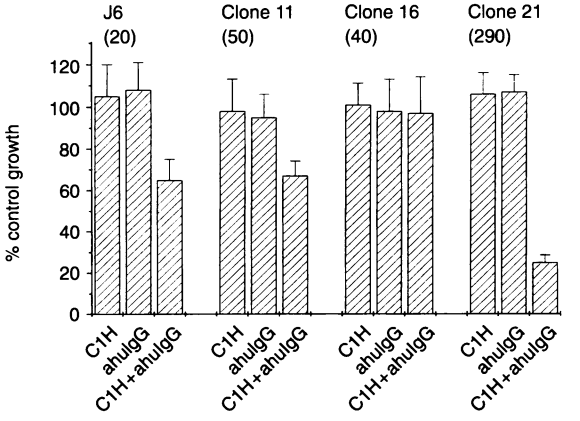

Figure 4. Jurkat cells transfected and selected to express high levels of CD52 are growth inhibited by cross-linking CD52. Parental or CD52-transfected Jurkat cells (2×10^4/well) were incubated with medium alone, CAMPATH-1H (CIH), anti-huIgG or CIH + anti-huIgG all at 20 μg/ml for 4 days. [^{3}H]thymidine was added for the last 6 hr of culture, and results are expressed as percentage inhibition of c.p.m. of medium-alone group. The mean fluorescence intensity (MFI) of CD52 expression for each clone is shown in brackets.

was observed following either antibody treatment. In addition anti-Fas antibody did not induce apoptosis in the Wien 133 cells and growth inhibition by cross-linked anti-CD52 was not blocked by anti-fas antibody (data not shown). These results suggest that apoptosis in these cells was not occurring via the Fas pathway.

As the kinetics of induction of growth arrest and apoptosis were relatively slow following treatment with CAMPATH-1H and anti-human IgG, it was thought possible that the effects observed could be due to induction of a cytokine which then subsequently induced apoptosis. Tumour necrosis factor-α (TNF-α) would be a potential candidate as the cells were extremely sensitive to this cytokine. However, addition of supernatants from cells treated with CAMPATH-1H and anti-human IgG for 96 hr to fresh cells did not induce any growth inhibition. Also the growth inhibitory effects of cross-linking CD52 were not blocked by the addition of a neutralizing anti-TNF-α antibody and secreted TNF-α was not detected in supernatants from cells treated in this way as determined by a sensitive ELISA (data not shown).

Attempts to block the induction of growth inhibition by the addition of several different cytokines over a range of physiological concentrations was unsuccessful. Interleukin-1 (IL-1), IL-2, IL-5, IL-6, IL-7 and interferon-γ (IFN-γ) did not change the extent of growth inhibition observed following addition of CAMPATH-1H and anti-human IgG. Addition of IL-4 slightly increased the degree of inhibition of cell growth from 70% to 90%. No effect on growth inhibition was observed following addition of anti-CD23 and IL-1 or anti-CD40 to cultures (data not shown).

Cross-linking CD52 on Jurkat T cells also induces growth inhibition

Although the results on the B-cell lymphoma Wien 133 strongly suggest that cross-linking CD52 induces growth inhibition, because of the presence of surface immunoglobulin and Fc receptors it is difficult to determine the relative contributions of all these molecules. We therefore examined the effects of cross-linking the CAMPATH-1H antigen on Jurkat cells which do not express surface immunoglobulin or FcR. The level of CD52 on these cells only induced modest growth inhibition, and therefore Jurkat cells were transfected with DNA encoding the CD52 gene. Transfection of CD52 into these cells followed by limiting dilution cloning produced clones that varied in expression of CD52. Only clones which expressed a high level of CD52 antigen (MFI > 290) were significantly inhibited in their growth following treatment with CAMPATH-1H, indicating a minimum threshold of antigen density to achieve this effect (Fig. 4). Both the parental line and the CD52 transfectants were inhibited in their growth to a similar extent, when treated with cross-linked anti-CD3 antibodies.

Selection of CD52-deficient Wien 133 cells

As discussed above, cross-linking CD52 on Wien 133 cells resulted in growth inhibition followed by cell death by apoptosis, such that 10 days post-treatment very few cells were surviving. However, after continuous culture and media replenishment the surviving cells expanded over the next 2–4 weeks. These cells were phenotyped by FACS and were found to be low for CD52 expression. A similar population of CD52 low expressing cells was also obtained by placing the parental population under selection of monocyte-mediated cytotoxicity in the presence of CAMPATH-1H antibody. Both of these low CD52 populations were cloned by limiting dilution and each line showed a similar pattern with 80–95% negative, 5–20% positive for CD52 expression. (Fig. 5). This pattern remained stable in *in vitro* culture over several months.

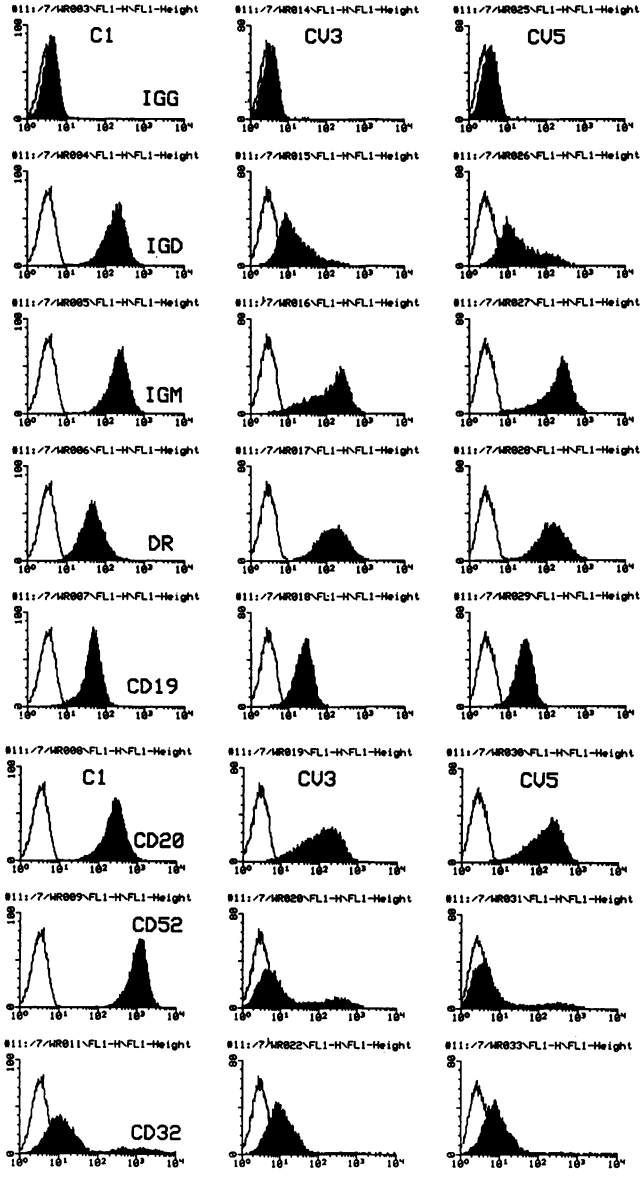

Figure 5. Phenotypic analysis of Wien 133 cells following *in vitro* selection by cross-linking with anti-CD52 antibodies. FACS analysis of parental (C1) and two clones derived from anti-CD52-mediated selection (CV3 and CV5). Cells were stained with FITC-labelled isotype controls and antibodies against various cell surface markers and analysed using FACScan.

Expression of other B-cell markers, namely CD19, CD20, HLA-DR, CD32 and sIgM, was similar to that found on the parental line (C1), whereas sIgD appeared considerably reduced on the CD52 low cells. However, it was striking that cell surface expression of other GPI-linked molecules, such as CD59 and CD55, was also reduced (Table 1).

The variant cells were not inhibited in their growth by cross-linking CD52, as would be expected by the low density of CD52 on their cells, however they were fully sensitive to growth inhibitory effects induced by anti-μ (Fig. 6). This suggests that a major change in the sensitivity of cells to growth inhibition or apoptosis had not occurred as a consequence of the CD52 selection.

Table 1. Analysis of CD52-negative cells for surface expression of other GPI-anchored proteins; % positive cells

Cell line	CD52	CD55	CD59
C1	100	100	100
CV3	27	24	28
CV5	16	14	16

Cells were analysed for surface expression of CD52, CD55 and CD59 by FACScan using antibodies described in the text (see the Materials and Methods). There is close correlation between percentages of cells expressing all three markers, indicative of a common block in surface expression.

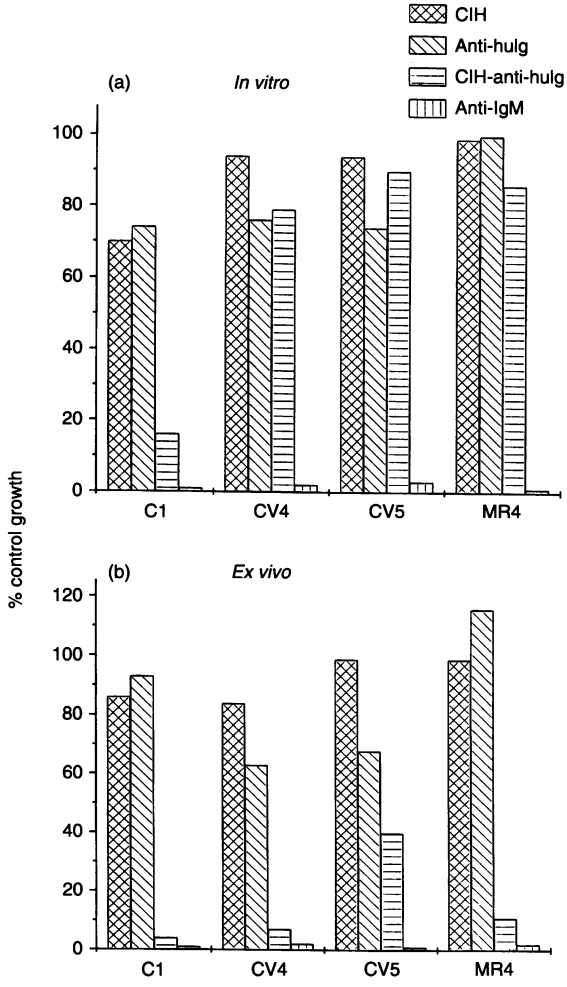

Figure 6. Variant CD52 low Wien 133 cells are no longer susceptible to growth inhibition induced by cross-linking CD52. Parental (C1) and selected clones (CV4, CV5, MR4) (2×10^4/well) were treated with medium, anti-huIgG, CAMPATH-1H (CIH), CIH + anti-huIgG (20 μg/ml) or anti-IgM (4 μg/ml) for 4 days and [^{3}H]thymidine added for last 6 hr of culture. Results are expressed as percentage control growth of medium alone. (a) *In vitro* selected cells; (b) *in vitro* selected cells after growth in nude mice.

Analysis of defect in low CD52 variant clones

To investigate whether the low level of CD52 expression was associated with transcriptional regulation the levels of mRNA

in parental and variant cells CV4 and MR4 were compared using RNA dot blots. MR4 had similar levels of CD52 cell surface expression as that of CV3 and CV5. The results shown in Fig. 7(a) indicate that there was no marked difference in the levels of CD52 mRNA in the parental and variant lines. Similar loading for mRNA was confirmed using the β-actin probe.

To investigate whether there was a block in cell surface expression of CD52 and other GPI-linked molecules, total chloroform/methanol cellular extracts were made. The relative amounts of CD52 that were obtained from the parental versus the variant cell lines, closely matched the level of cell surface expression, whether monitored by Western blotting for CD52 antigen or a more quantitative ELISA (Fig. 7b). That the antigen monitored was CD52 was confirmed by the identical migration pattern of affinity-purified CD52 and the susceptibility to *N*-glycanase treatment. The amounts of CD52 antigen present in chloroform/methanol extracts of whole cell extracts determined by ELISA correlated with the density of protein on Western blots as well as the level of cell surface expression. C1 cells had 86 nmol, CV3 had <4·6 nmol and CV4 had 12·6 nmol. The results therefore indicate that there is not a block in translation or transport of CD52. As expression of other GPI-linked molecules was also affected, it was possible that the defect may lie in generation or attachment of the GPI anchor itself. Unfortunately, because CAMPATH antibodies recognize an epitope which is partially composed of the GPI anchor and the peptide chain of CD52, the antibodies do not recognize the non-GPI anchor precursor form of the molecule.[20] However, the anti-CD59 antibody YTH 53.1, recognizes the extracellular domain of the molecule, independently of the GPI anchor. This antibody was therefore used to probe for the non-GPI-linked form of CD59 in the parental and variant lines. As shown by Western blotting similar levels of total CD59 protein were found in both parental and variant lines, although the variant lines only showed low cell surface CD59 expression (Fig. 7c). Taken together the data suggest that the most likely defect in the variant cells is associated with the synthesis, or attachment of the GPI anchor to various cell surface molecules.

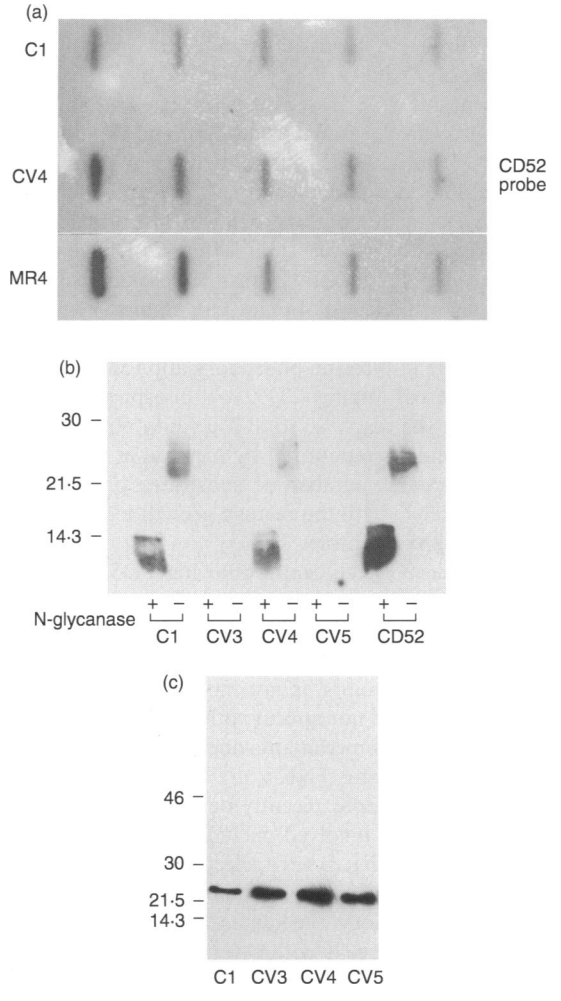

Figure 7. Analysis of defect in Wien 133 CD52 low cells. (a) Messenger RNA levels for CD52 in C1, CV4 and MR4 cells. (b) CD52 levels in C1, CV3, CV4 and CV5 determined by Western blotting, samples were treated (+) or not (−) with N-glycanase. (c) Western blot probed with anti-CD59 (YTH 53.1) of total cell extracts of C1, CV3, CV4 and CV5 cells.

Restoration of CD52 expression on Wien 133 variants cells following *in vivo* growth in nude mice

The CD52 antigen is expressed at high levels on normal T and B lymphocytes *in vivo*, however, the majority of T and B tumour cell lines cultured *in vitro*, express very low levels of CD52, with the main exception being the Wien 133 cells. To investigate the effect of the *in vivo* environment on CD52 expression, parental cells or variant clones of Wien 133 were injected into nude mice subcutaneously. After several weeks both parental and variant cell lines developed tumours, although parental C1 Wien 133 cells grew faster than the selected cells. At day 38 the incidence of tumours was higher and tumour size was bigger in parental compared with selected cells. Flow cytometric analysis of tumours growing in nude mice indicated that cells in tumours derived from the selected cells were now expressing higher levels of CD52 and CD59 than their *in vitro*-maintained counterparts (Fig. 8). This increased expression once established appeared quite stable for several months of *in vitro* culture.

To determine whether the enhanced CD52 expression following *in vivo* growth was associated with selection of a few pre-existing GPI/CD52 high cells or up-regulation of enzymes controlling GPI anchor production, the CV5 cells were treated with complement or sorted using dynal beads or by FACS to remove any CD52-positive cells. These were then injected into mice as described above or returned to *in vitro* culture. Again, cells from mice which had received sorted CD52-negative cells which were recovered after the tumours had established, had high levels of CD52 expression, indicating perhaps that the molecules are up-regulated in the *in vivo* environment. The CD52-negative population maintained *in vitro*, re-developed the same pattern of 10% CD52 low cells, as was seen in the original unsorted population within a few days and the CD52 low population also reverted to the same pattern.

Cells which increased expression of CD52 after *in vivo* growth were again sensitive to growth inhibition by cross-linking CD52 antigen with CAMPATH-1H and anti-huIgG (Fig. 6b).

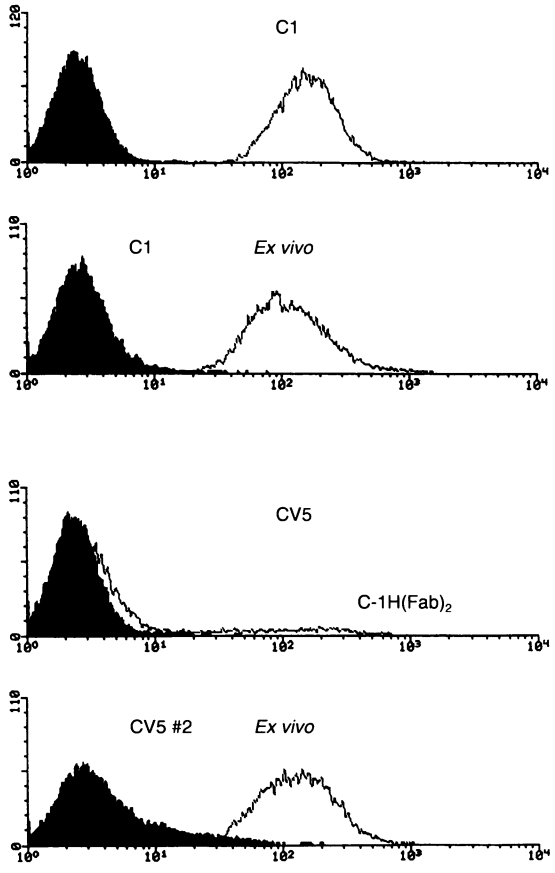

Figure 8. Expression of CD52 following *in vivo* growth in nude mice of low CD52-selected cells. CD52 expression levels on *in vitro* maintained CV3 cells and CV3 cells injected subcutaneously into nude mice to form tumours, as monitored by FACS analysis using FITC-conjugated Campath-1H $F(ab)_2$ fragment.

DISCUSSION

The data in the present study clearly show that cross-linking the CD52 receptor with the humanized mAb CAMPATH-1H can induce a direct growth inhibitory and apoptotic effect on two different lymphocytic tumour cell lines. Clearly antigen density was important as only the Wien 133 B-cell lymphoma and a CD52-transfected Jurkat cell line were sensitive to the growth inhibitory effects of the antibody. Other tumour cell lines, such as Epstein–Barr virus cells or parental Jurkat cells, which only express low levels of CD52 were not affected. The growth inhibitory effects on both the Jurkat and the Wien 133 cells were obtained in the absence of effector cells, such as natural killer cells or macrophages or active serum complement. However, cross-linking the antibody was required and therefore *in vivo*, FcR-positive cells could mimic these effects. As many FcR-positive cells also express cytolytic activity resulting in ADCC, the relative importance of ADCC or direct growth inhibition in patients treated with CAMPATH-1H in reducing tumour load is unclear. It has been shown that CAMPATH-1H antibody induces a strong first-dose reaction, associated with the release of inflammatory cytokines including TNF-α, IL-6 and IL-1. This phenomenon involves CD16 cross-linking, which supports a role for ADCC in the tumour clearance, at least in peripheral blood.[21] In addition, the time-course for tumour cell depletion in peripheral blood following antibody therapy is extremely rapid and more closely follows the time course for ADCC of 4–6 hr than that seen for direct growth inhibition which occurs over several days. It is possible that in other local microenvironments within certain tissues of the body the growth inhibitory effect may also occur. However, as the cells are coated with an opsonizing antibody it is unlikely that they would be cleared by the non-inflammatory phagocytic route of clearance of an apoptotic cell.[22] However, it is apparent that certain lymphoid tumours are more effectively treated by CAMPATH-1H than others. It has been shown that T-cell pro-lymphocytic leukaemia is cleared by CAMPATH-1H, which may be due to a direct apoptotic effect on the cells.[23]

It is unclear how CD52 cross-linking mediates growth inhibition and apoptosis. Numerous studies with a variety of B- and T-cell tumour lines[10–12,24] have shown that signalling via the antigen-specific receptor results in growth arrest, resulting in abortive cell cycle entry often accompanied by apoptosis. Several studies have shown that cells are sensitive to growth inhibitory effects at the G0/G1 stage of cell cycle.[25,26] The signalling pathways appear to involve the same molecules as those utilized by normal lymphocytes, including $p56^{lck}$, Zap70 or $p55/57^{lyn}$ activation.[27,28] In the current experiments, signalling via anti-CD3 in Jurkat cells or anti-μ in Wien 133 resulted in a complete growth inhibition and rapid apoptosis. The effects of anti-CD52 antibodies were much slower and complete inhibition was not observed. This may be related to the particular signalling pathways to which the different receptors are linked. It has been shown that GPI-linked proteins are found in clusters or GEMS on the cell surface, and that antibodies against GPI molecules can immunoprecipitate a variety of signalling molecules, including $p56^{lck}$ and $p59^{fyn}$.[29] A weak increase in tyrosine phosphorylation was observed in Wien 133 by cross-linking CD52 on phosphotyrosine blots compared with the response to anti-μ (data not shown). The strength of signal, as monitored by increase in tyrosine phosphorylation and the number of substrates phosphorylated, appears to correlate with the relative growth inhibitory effects of CD52 and IgM receptors.

The mechanism of cell death following CD52 cross-linking in this system is unknown, but at least in the Wien 133 cells, does not appear to be Fas-mediated. This would agree with reports in the literature that apoptosis of many B-cell lines, in contrast to T-cell tumours, is not mediated by the Fas pathway.[10] The effect does not appear to be mediated by a soluble ligand, as transfer of supernatants does not mediate this effect. In addition, neutralizing TNF-α antibody did not block the response. However, more recently described members of the TNF family could be involved, including wsl and TRAIL.[30]

The results show that *in vitro* selective pressure of the Wien 133 by cross-linking with secondary antibody to induce apoptosis or via monocyte-mediated antibody-dependent cell cytotoxicity results in cells with a very interesting phenotype, which grow out of the cultures. The cells have very low levels of CD52, as well as other GPI-linked molecules. Most other cell surface receptors are expressed at the same levels as those found on the parental cells (e.g. surface IgM, HLA-DR, CD19, CD20). Western blotting confirmed the presence of intracellular CD59. Normal lymphocytes which are defective in cell surface expression of GPI-anchored proteins, including CD52,

CD55 and CD59 have been shown to develop *in vivo* in a proportion of rheumatoid arthritis[31] and NHL[32] patients treated with CAMPATH-1H. A detailed biochemical and molecular analysis of GPI-anchor-negative T lymphocytes and the Wien 133 cells used revealed a severe defect in the synthesis of a mature GPI precursor.[32,33] This closely resembles the phenotype of lymphocytes from patients with paroxysomal nocturnal haemoglobulinuria, which is caused by mutations in the phosphatidylinositolglycan A (PIG-A) gene, which acts at the first step of the GPI biosynthesis.[34] In the CD52-negative B and T lymphocytes, the PIG-A gene was analysed by polymerase chain reaction and found to have a wild-type sequence and therefore suggests that in these cells a mutation in another gene along the pathway may be responsible for the defect.

Whereas the overall phenotypes of the CD52-negative B and T cells were superficially the same, marked differences were observed in the apparent stability of the CD52 deficiency. Whereas the T lymphocytes were completely negative for GPI-anchored proteins, and remained so for long periods in continuous culture, different B-cell clones always expressed low levels of CD52 on 10–20% of the population. When the CD52 low and-negative cells were separated and returned to culture, both populations rapidly reverted to the original distribution. Further evidence that the defect in the B cells was reversible was obtained by the observation that injection of CD52-negative Wien 133 cells resulted in cells reverting to high expression after growth *in vivo*. It is difficult to exclude the possibility that there was strong selection of the minor population of GPI anchor-positive cells *in vivo*. For example GPI-negative cells may be more sensitive to mouse complement as they do not express the complement protective factors, CD55 and CD59.

Overall the present results and those of previous studies have shown that cross-linking the CD52 receptor on normal and transformed lymphocytes is not a null event and can result in either clonal expansion or apoptosis depending on the activation state of the particular cell. These observations may underlie some of the biological effects of the CAMPATH-1H antibody, and illustrate the importance of understanding the mode of action of antibodies to be used in clinical trials.

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