Antibody selection against CD52 produces a paroxysmal nocturnal haemoglobinuria phenotype in human lymphocytes by a novel mechanism

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The CD52 antigen is a lymphocyte glycoprotein with an extremely short polypeptide backbone and a single N-linked glycan, and it is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. Treatment of rheumatoid arthritis patients with CAMPATH-1H, a humanized monoclonal antibody against CD52, resulted, in a small number of cases, in the appearance and persistence of CD52-negative T cells. Similarly, CD52-negative B cells emerged following *in vitro* treatment of a CD52-positive human B cell line with CAMPATH-1H. Both the B and T CD52-negative cells were also found to be defective in surface expression of other GPI-anchored proteins. Biochemical analysis revealed a severe defect in the synthesis of a mature GPI precursor in both the B and T cell lines. Therefore the phenotype of these CD52-negative B and T cells closely resembles that of lymphocytes from patients with paroxysmal nocturnal haemoglobinuria (PNH), in which the first step of the GPI-biosynthetic pathway, i.e. synthesis of GlcNAc-phosphatidylinositol, is blocked. In all cases studied to date, this defect maps to a mutation of the phosphatidylinositolglycan class A (PIG-A) structural gene. We therefore amplified the PIG-A gene from both the GPI-negative B and T cells by PCR and determined the nucleotide sequence. No differences from the wild-type sequence were detected; therefore a classical PNH mutation cannot be responsible for the GPI-biosynthesis defect in these cell lines. Significantly, the GPI-negative phenotype of the B cells was reversible upon separation of the positive and negative cells, resulting in a redistribution to a mixed population with either CD52-positive or -negative cells, whereas populations of 100% CD52-negative T cells were stably maintained during culture. Therefore, whereas the GPI-biosynthesis deficiency in the T cell lines may be due to a mutation in another gene required by the GPI-biosynthetic pathway, the reversible nature of this block in the B cell lines suggests a less direct cause, possibly an alteration in a regulatory factor. Overall, these data demonstrate that the PNH phenotype can be generated without a mutation in the PIG-A structural gene, and thereby identify a novel mechanism for the development of GPI deficiency.

INTRODUCTION

The CD52 antigen is an abundant cell surface glycosylphosphatidylinositol (GPI)-anchored lymphocyte glycoprotein that was discovered during the development of antibodies for T cell depletion from bone marrow for graft versus host disease therapy. The CD52 antigen was subsequently purified and its structure fully elucidated, but its function remains unknown. This unusual glycoprotein, with an apparent molecular mass of 21–28 kDa, has a peptide backbone comprising only 12 amino acids, a single N-linked oligosaccharide attached to Asn-3 and a GPI anchor attached to the C-terminus of the protein [1,2].

The CAMPATH-1 antibodies, which recognize CD52, have been used in a range of clinical studies. The humanized monoclonal antibody CAMPATH-1H, derived from the rodent antibody CAMPATH-1G (rat IgG2b), has been used in experimental therapy for rheumatoid arthritis [3] and refractory B-cell non-Hodgkin lymphoma [4]. CAMPATH-1H treatment resulted in complete depletion of CD52-positive cells, including T and B lymphocytes, monocytes and natural killer cells, from the peripheral blood, but CD52-negative lymphocytes emerged in 12% of patients (3 out of 25) after treatment [5]. At 1–2 months following treatment, most T cells and 40–80% of the B cells were CD52-negative [5]. The CD52-negative B cells did not persist beyond 3 months, but the CD52-negative T cells could still be detected after 20 months [5]. The percentage of CD52-negative T cells varied between patients, presumably as a result of different rates of re-emergence of the CD52-positive T cells, and included both CD4 and CD8 subsets [5].

CD52-positive and -negative T cell lines, and corresponding clones, were established from two of the rheumatoid arthritis patients, and designated BT and RP [5]. After 6 months in culture the original proportion of CD52-negative cells was maintained. The CD52-positive and -negative clones, the original line and CD52-negative selected lines were all found to proliferate normally in response to antibody to CD3, phytohaemagglutinin (PHA) and concanavalin A [5]. In a second experiment, a human B cell line was treated with CAMPATH-1H in the presence of a second cross-linking antibody. The surviving B cells were found to be negative for the expression of CD52, or to express low levels.

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired clonal stem cell disorder [7], characterized by an increased susceptibility of erythrocytes to autologous complement [8,9]. In patients suffering from PNH, cells of both haematopoietic and lymphoid lineages are deficient in the surface expression of a number of GPI-anchored proteins. This absence of expression of GPI-anchored proteins is due to a defect in the transfer of *N*-acetylglucosamine from UDP-GlcNAc to phosphatidylinositol, the first step in GPI anchor biosynthesis [10–12].

GPI-deficient thymoma mutants belonging to complementation classes A, C and H are unable to transfer *N*-

Abbreviations used: GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; PIG-A, phosphatidylinositolglycan class A; PI-PLC, phosphatidylinositol-specific phospholipase C; PNH, paroxysmal nocturnal haemoglobinuria.

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acetylglucosamine to a phosphatidylinositol acceptor [13–15]. The human cDNA encoding PIG-A (phosphatidylinositolglycan class A), which repairs the biosynthetic defect in class A thymoma mutants, has been identified [16], and can restore the expression of GPI-anchored proteins in PNH lines [17]. In all PNH patients analysed to date, the defect in GPI anchor biosynthesis is due to a mutation in the PIG-A gene [17–21]. The PIG-A gene was localized to chromosome Xp22.1 [17], suggesting that a single acquired mutation within PIG-A would be sufficient to cause the deficiency in GPI anchor biosynthesis in both sexes.

We have analysed the CD52-negative B and T cells biochemically and genetically; all of the CD52-negative cells failed to express other GPI-anchored proteins on their cell surfaces and were devoid of the capacity to synthesize mature GPI precursors, consistent with the loss of all GPI-anchored proteins from the cell surface. Surprisingly, analysis of the PIG-A coding region detected no alterations from the wild-type sequence. These data demonstrate that a PNH phenotype may be generated by a novel mechanism not involving PIG-A inactivation.

MATERIALS AND METHODS

Cells

B cells were cultured in Iscove's modified Dulbecco's medium (Gibco–BRL) containing 10 % (v/v) foetal calf serum (Bioclear). T cells were maintained by stimulation every 7–14 days with irradiated peripheral blood mononuclear cells, 10 % Lymphocult T (Biotest) and either anti-CD3 (1 μg/ml; Serotec) or phytohaemagglutinin (5 μg/ml; Sigma) in RPMI (Gibco–BRL) containing 10 % foetal calf serum [5]. The CD52-positive B cell clone C1 was derived from the line WIEN133. C1 cells were cultured in the presence of CAMPATH-1H (20 μg/ml; Wellcome Foundation) and anti-(human IgG) (20 μg/ml; Becton Dickinson). After 2 weeks viable cells, cloned by limiting dilution, generated clones CV3 and CV5. CV5 *ex vivo* was derived by injecting CV5 cells subcutaneously into the flanks of nude mice; cells derived from tumours were designated CV5 *ex vivo* and had reverted to CD52-positive. CD52-positive and -negative T cell lines (BT and RP respectively) were established from two rheumatoid arthritis patients previously treated with CAMPATH-1H [5]. CD52-positive and -negative cells were separated by incubation with CAMPATH-1G and ovine anti-(rat IgG)-conjugated Dynalbeads (Dynal).

Flow cytometry

Cells were stained for CD52 with FITC-conjugated CAMPATH-1H Fab_2 fragment (Wellcome), for CD59 with YTH 53.1 (gift from G. Hale, University of Cambridge, U.K.) and FITC-conjugated anti-(rat IgG) (Becton Dickinson), and for CD55 with mouse anti-CD55 (Serotec) and FITC-conjugated anti-(mouse IgG) (Becton Dickinson). Analysis was performed using a FACScan flow cytometer (Becton Dickinson) and data were acquired using LYSIS II software.

Metabolic labelling and lipid extraction

Cells were incubated with 50 μCi/ml [1-^{3}H]ethanolamine hydrochloride (25 Ci/mmol) or D-[2,6-^{3}H]mannose (48 Ci/mmol) (Amersham) in RPMI/10 % foetal calf serum or glucose-free medium for 4 h at 37 °C [22]. T cell lines were collected 3 days after anti-CD3 or phytohaemagglutinin stimulation, when their metabolic activity was greatest [10]. Lipids were extracted with chloroform/methanol (2:1, v/v) followed by chloroform/methanol/water (10:10:3, by vol.) as described [22,23]. Extracts were dried in a Speedvac (Savant) and partitioned between butanol and water. Aliquots of the butanol phase were taken for scintillation counting. The butanol phases were analysed by TLC on Silica gel 60 plates (Merck) in chloroform/methanol/water (10:10:3, by vol.) (solvent A), chloroform/methanol/1 M NH_4OH (10:10:3, by vol.) (solvent B) or chloroform/methanol/1 M HCl (10:10:3, by vol.) (solvent C) in a saturated atmosphere. Radioactivity was located with an Automatic TLC Linear Analyser (Berthold).

Chemical modifications and enzymic digestions

Samples (20000 c.p.m.) were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) (Boehringer Mannheim), GPI-specific phospholipase D (GPI-PLD) (from rat serum), phospholipase A_2 from *Crotalus adamanteus* venom (Sigma) or nitrous acid, as described previously [22].

PCR amplification of the PIG-A gene

Genomic DNA was extracted as described previously [24]. Portions of exons 2–6 corresponding to the entire PIG-A coding region were amplified from genomic DNA using primer pairs containing *Bam*HI and *Xho*I restriction sites (bold). Exon 2, (a) CCAGT**GGATCC**CTGAGCTGAGATCCTGTTTTACTC and (b) CTGGC**CTCGAG**CTATGGGAAAAAAGTCTACAATGC; exon 3, (c) AGAT**GGATCC**AAGTGGATTCTCAGTCGTTCTGGTG and (d) CACA**CTCGAG**CCTAAGGTACGCATGCAGTTAAAACC; exon 4, (e) CGGA**GGATCC**GTAGCATGAGTTTTCACTCCTTTC and (f) TTCG**CTCGAG**CAGAAATCCCAACCATGAATGCCC; exon 5, (g) GGTT**GGATCC**GATCTTCCTGAGGTATGATTATGG and (h) GCTG**CTCGAG**CAAGTAAGAGTTCAGACACAATC; exon 6 coding region, (i) TCGA**GGATCC**GTTTATCATGGGACAGGTGATGG and (j) CAGG**CTCGAG**GCAAGGTTATTTTCCATAGTC. Primers were designed within the introns so as to include intron boundaries and to preclude amplification of the chromosome 12 PIG-A pseudogene [25].

PCRs were performed in a reaction volume of 100 μl in buffer containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % (w/v) gelatin (Perkin–Elmer), 200 μM of each dNTP (Perkin–Elmer) and 5 % DMSO. Reactions were performed in a Biometra TRIO-Thermoblock, using 1.25 μg of each primer and approx. 1 μg of genomic DNA with 2.5 units of *AmpliTaq* DNA polymerase (Perkin–Elmer). Exons 3–6 were amplified by an initial denaturing step at 97 °C for 1.5 min, followed by 30 cycles of 55 °C for 1 min, 72 °C for 2 min and 93 °C for 1 min, with a final 2 min at 55 °C and 10 min at 72 °C. Exon 2 was amplified under the same conditions, except that annealing was performed at 50 °C. Products were analysed by electrophoresis in 1 % agarose gels stained with ethidium bromide (0.25 μg/ml), and then cloned into pBluescript (Stratagene) and used to transform DH5α competent cells (Gibco–BRL). Plasmids were purified using Wizard Minipreps (Promega) and amplification products were purified with a QIAEX II Gel Extraction Kit (QIAGEN). Both plasmid inserts and products were sequenced by dideoxy chain termination using an Applied Biosystems Model 373 DNA Sequencer (Perkin–Elmer).

RESULTS

Characteristics of the CD52-negative B cell clones and the T cell line

FACScan analyses for CD52 of the WIEN 133-derived parental cell line, C1, and a representative CAMPATH-1H selected clone,

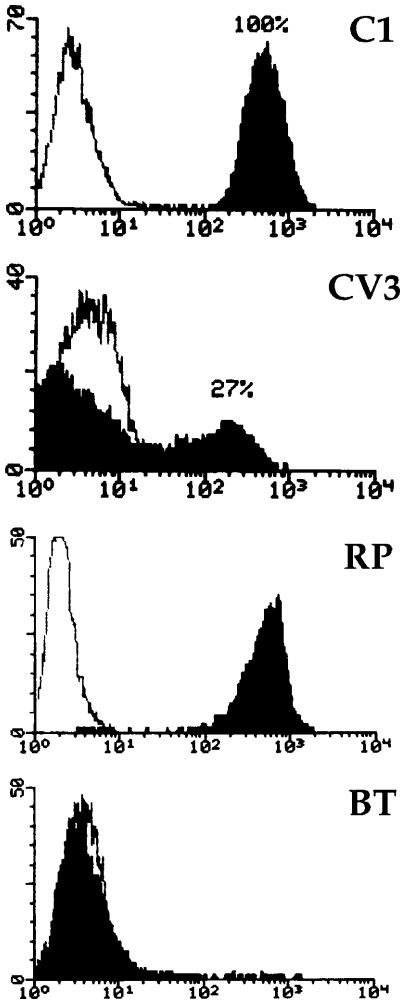

Figure 1 FACScan analysis of B and T cell lines

Expression of CD52 by the B cell clones C1 and CV3 and the T cell lines RP and BT is shown. The shaded histograms represent cells stained with FITC-conjugated CAMPATH-1H Fab_2, while the open histograms are controls of the same cells stained with FITC-conjugated anti-(rat IgG) only.

CV3, are shown in Figure 1. Most significantly, the CD52-deficient clone was also found to be negative for CD55 and CD59, two additional GPI-anchored antigens, suggesting a general defect in GPI-anchor metabolism (Table 1). Unexpectedly, staining of all negative clones, i.e. CV3 and CV5, produced two peaks in the FACScan profile, clearly indicating the presence of both GPI-negative and GPI-positive populations (Figure 1 and Table 1). These CD52-negative B cell clones, generated by limited-dilution cloning at 0.3 cell/well, were subsequently recloned, and all five clones obtained showed similar FACS profiles, i.e. mostly CD52-negative cells with a minority (10–40 %) of positive cells. This behaviour is unusual, as simple reversion would be unlikely to account for the maintenance of subpopulations of CD52-positive cells at a discrete level. We chose to study this phenomenon further by isolating highly purified CD52-positive and -negative populations using CAMPATH-1G and sheep anti-(rat IgG)-conjugated magnetic beads, followed by a return to culture. Over a period of several days the cells in both cultures returned to the original proportions of CD52-positive and -negative cells (Table 2). The apparent

Table 1 FACScan analysis of CD52-negative cells for surface expression of other GPI-anchored proteins

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

		Positive cells (%)		
Cell type	Cell line	CD52	CD55	CD59
B cells	C1	100	100	100
	CV3	27	24	26
	CV5	16	14	16
T cells	RP	100	100	100
	BT	0	0	0

Table 2 Expression of CD52 by the B cell line CV3 during culture

CD52-positive and -negative cells were separated from a CV3 culture with magnetic beads (see the Materials and methods section), and the individual populations were returned to culture. At periodic intervals samples were withdrawn from the cultures for analysis of the level of CD52 surface expression. The percentage of CD52-positive cells immediately after separation was not determined, as the cells were still attached to the beads, but was assumed to be 100 %. NA, not analysed.

	CD52-positive cells (%)						
No. of days ...	0	Separation	3	6	7	10	14
CD52-negative	23	0	NA	NA	17	NA	21
CD52-positive	17	100	55	28	NA	15	NA

conservation of CD52 expression on ~25 % of the total cell population did not correlate with the cell cycle (J. Tite, unpublished work).

We also analysed two T cell lines derived from individuals treated with the CAMPATH-1H antibody, 19 and 16 months after treatment (RP and BT respectively). The former line, RP, was 100 % CD52-positive whereas the latter, BT, was completely negative for CD52 surface expression. The BT cell line was also negative for CD55 and CD59 (Figure 1 and Table 1), suggesting that it too, in common with the CV3 and CV5 lines, had a general defect in GPI-anchor metabolism. In contrast with the CV3 and CV5 cells, the 100 % CD52-negative BT population did not revert to CD52 expression during extended culture [5].

Identification of the mature GPI-anchor precursor in B cells

The failure of the CD52-negative cells to express other GPI-anchored proteins on their cell surface clearly suggested that a general defect, either in GPI-precursor lipid biosynthesis or in attachment, was present in these cells. We therefore chose to determine whether the CD52-negative cells synthesized mature GPI-anchor precursors by metabolic labelling with ethanolamine. We used the well-characterized murine T cell line EL4 throughout as a positive control for our studies [15], and initially investigated the CD52-positive B cell line C1.

Ethanolamine metabolic labelling identified one peak, designated mGPI, which migrated in solvent system A with an R_F of 0.4, consistent with a polar glycolipid of the GPI class putatively similar to the species designated as 'core' [15,26,27]. Considerable

Table 3 Correlation between mGPI synthesis and the percentage of CD52-positive cells in different cell lines

The percentage of the total ethanolamine incorporation into lipid represented by the mature B cell GPI-lipid mGPI was calculated following resolution by TLC and integration of the mGPI peak. The amount of mGPI in the C1 cell line was set at 100%. The percentage of CD52-positive cells was determined by FACScan analysis.

Cell line	CD52-positive cells (%)	mGPI content (%)
C1	100	100
CV3	27	42
CV5	16	14
CV5 *ex vivo*	45	64

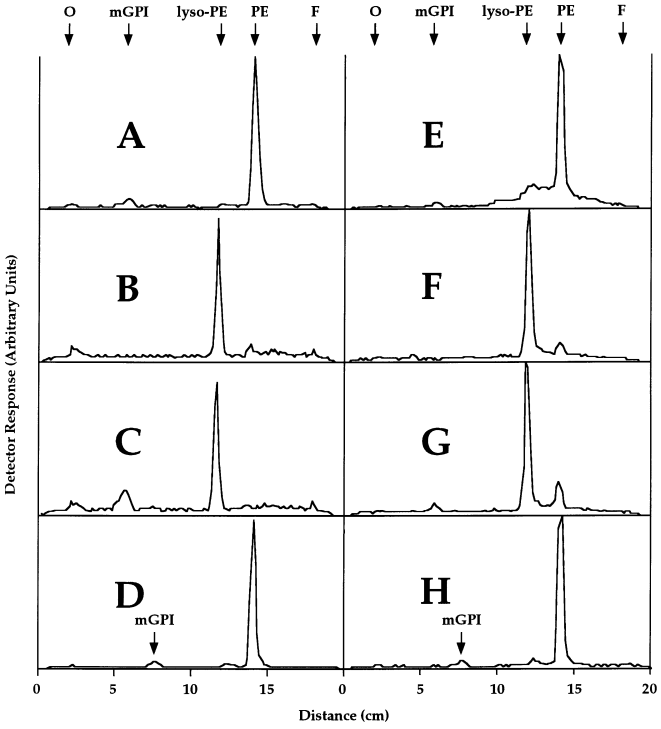

Figure 2 Identification of mGPI as a mature GPI precursor

In (**A**) and (**E**), mouse EL4 T cells and human C1 B cells were metabolically labelled with [^{3}H]ethanolamine, and extracted lipids were analysed by TLC in solvent A. EL4 and C1 cell ethanolamine-labelled lipid extracts were treated with GPI-PLD (**B** and **F**), GPI-PLD in the presence of 1,10-phenanthroline (**C** and **G**) or PI-PLC (**D** and **H**). Vertical scales are adjusted so that the dominant peak phosphatidylethanolamine (PE) or lyso-PE is set to 100%. The positions of mGPI, PE, lyso-PE, the solvent front (F) and the origin (O) are indicated.

quantities were also recovered of a labelled phospholipid which co-migrated with a phosphatidylethanolamine standard, but the identity of this species was not investigated further. A species with the same R_F was also labelled by all three partially GPI-positive cell lines (results not shown and Table 3). Additionally, a species was also detected that had [^{3}H]GlcNAc and [^{3}H]mannose labelling, migrating identically to [^{3}H]-ethanolamine-labelled mGPI, consistent with mGPI being a GPI species (results not shown). To confirm that mGPI is indeed a GPI-anchor precursor, we subjected the ethanolamine-labelled EL4 and C1 extracts to enzymic and chemical treatments. Both mGPI from C1 and 'core' from EL4 were sensitive to GPI-PLD, marked by a shift of the peak towards the origin (Figure 2). A

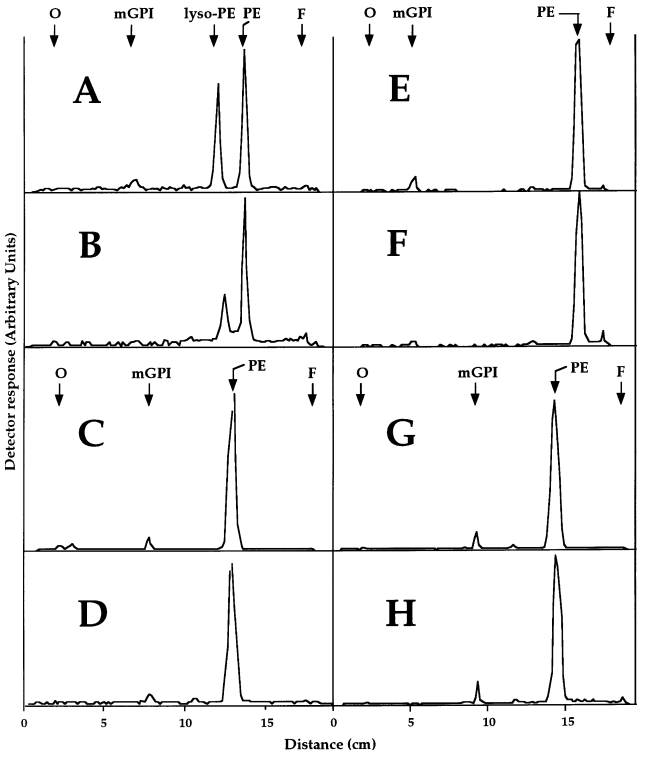

Figure 3 mGPI is highly similar to the mature GPI precursor from EL4 cells

Mouse EL4 T cells and human C1 B cells were metabolically labelled with [^{3}H]ethanolamine and lipids were extracted. In (**A**) and (**B**) the ethanolamine-labelled EL4 extract was either mock-treated (**A**) or treated with nitrous acid (**B**) and analysed by TLC in solvent A. In (**C**)–(**H**), ethanolamine-labelled lipids extracted from both EL4 cells and human B cells were then analysed by TLC in three different solvent systems (solvent A, **C** and **D**; solvent B, **E** and **F**; solvent C, **G** and **H**). The positions of mGPI, phosphatidylethanolamine (PE), lyso-PE, the solvent front (F) and the origin (O) are indicated.

GPI-PLD activity in rat serum, and not a phospholipase A_2, was responsible for the degradation of mGPI, as cleavage was inhibited by 1,10-phenanthroline (Figures 2C and 2G). mGPI and 'core' were resistant to PI-PLC, as expected if an acyl chain is attached to the inositol ring (Figures 2D and 2H; [27]). Furthermore, the mouse T cell species 'core' was sensitive to nitrous acid treatment, confirming the identification of 'core' in our murine extracts (Figures 3A and 3B). We were unable to determine the effect of nitrous acid on mGPI in the B cell lipid extract because of distortion of the control profiles, probably due to detergent in the incubation buffer. However, the murine EL4 precursor 'core' and the human B cell mGPI co-migrated in three different solvent systems (Figures 3C–3H), confirming that mGPI is a mature GPI precursor of B cells, with a very similar or identical structure to 'core' [15,26,27].

Correlation of the percentage of CD52-positive cells with the amount of mGPI in lipid extracts

We next labelled the various B cell clones with ethanolamine and analysed the extracted lipids by TLC in solvent A. The amount of mGPI present in the CD52-negative clones was clearly decreased compared with the CD52-positive cell line C1; moreover, mGPI amount correlated well with the percentage of CD52-positive cells present in each cell population (Table 3). These data clearly suggest the possibility that only the CD52-positive cells are capable of synthesizing mGPI, raising the

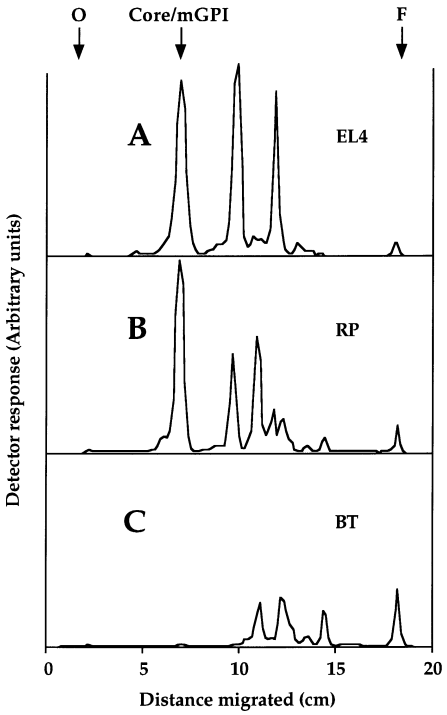

Figure 4 The T cell line BT is defective in the synthesis of mature GPI precursors

Mouse EL4 T cells and human RP and BT T cells were metabolically labelled with [^{3}H]mannose, and lipids were extracted and analysed by TLC. (**A**) EL4 cells synthesize a polar lipid at R_F 0.4 previously described as 'core'. In (**B**) and (**C**), the CD52-positive T cell line RP synthesizes a mature GPI precursor (mGPI), whereas extracts from the CD52-negative BT cell line do not contain this lipid. The positions of mGPI, the solvent front (F) and the origin (O) are indicated.

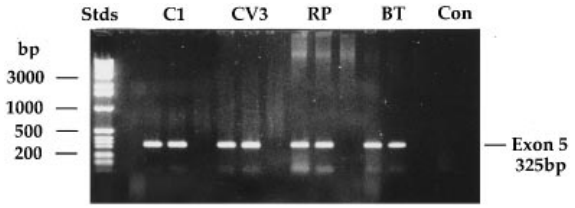

Figure 6 Amplification of exon 5 from GPI-deficient cells and their GPI-positive counterparts

The PIG-A exon 2–6 fragments were amplified by PCR from C1, CV3, RP and BT cells and the products were electrophoresed on a 1% agarose gel. In each case distinct strong bands were obtained for each fragment amplified from each cell type. The exon 5 products are shown as a representative example. For each set of lanes, the products of two independent reactions are shown, followed by a control reaction where primers were omitted. The final lane (Con) is a negative control containing no DNA template but otherwise identical to the other reactions. A 1 kb DNA ladder is shown on the left.

possibility that the defect is due to a PIG-A mutation of the type observed in patients with PNH.

We were also unable to detect a mature GPI precursor in the BT cell line. We did not investigate the structure of this lipid as thoroughly as for the B cells, as the identification of a mature GPI lipid in human T cells has already been made [15]. However, we did observe that in [^{3}H]mannose-labelled extracts of both EL4 cells and the RP cell line a major peak was observed at $R_F \sim 0.4$ (Figures 4A and 4B). This peak was sensitive to nitrous acid in both cell lines (results not shown). By contrast, the BT cell line failed to synthesize significant quantities of mGPI (Figure 4C).

Analysis of the PIG-A gene of GPI-deficient B and T cells

We analysed the PIG-A structural gene by PCR amplification of the individual exons from genomic DNA isolated from several of the cells in the study. Exons 2–5 and the 5′ portion of exon 6, corresponding to the C-terminus of the protein, were amplified by PCR from CV3 and BT cells and their GPI-positive counterparts C1 and RP respectively (Figures 5 and 6). The regions amplified covered the entire coding region as well as the intron splice sites. To minimize the possibility of PCR artifacts, each exon was amplified in two independent PCRs and the products cloned into pBluescript (Figure 6). One clone derived from each PCR was sequenced and compared with the published PIG-A genomic sequence (NCBI accession numbers D28788–D28791 [28]).

Two single base changes were detected in each of the exon 2 DNA sequences derived from both of the wild-type cell lines C1 and RP, and a single base change was also detected in one of the exon 3 sequences derived from the C1 B cell line. That these mutations were PCR or sequencing artifacts was demonstrated by sequencing the products of a third PCR directly. The sequence

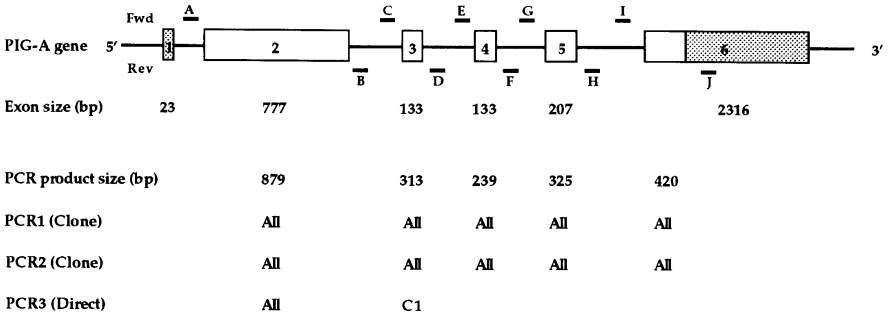

Figure 5 Strategy employed for amplification of the PIG-A gene and sequencing of the PCR products

Each exon is shown as an open box (not to scale) and the true sizes are indicated. Exon 1 and part of exon 6 are shaded to indicate that they are non-coding. Positions of primer pairs are represented by bars and letters above (forward primers) and below (reverse primers) the relevant exons, and the expected size of each product is given. Exon 1 and the 3′ end of exon 6 were not amplified. For each exon, a single clone was sequenced from two independent reactions (PCR1 and PCR2). To resolve ambiguities (see the text) a third amplification was performed where necessary (PCR3) and products were sequenced directly. 'All' indicates that products from C1, CV3, RP and BT cells were sequenced; 'C1' indicates that data were obtained for C1 cells alone.

obtained for each PIG-A exon from the GPI-positive cells C1 and RP was in this case identical to the published PIG-A sequence. Therefore no changes from the published sequence were present in the GPI-positive cells.

For the GPI-deficient cells two single base changes were detected in the exon 2 sequence of one PCR product derived from CV3 cells, and a single base change was detected in this exon of one product from BT cells. Again, these single base changes were suspected PCR artifacts, and additionally were silent changes. We again sequenced the products of a third PCR reaction directly to resolve these errors, and no mutations were detected. Furthermore, no mutations were identified in any of the intron splicing sites, eliminating the possibility of a mutation in the PIG-A gene as a result of incorrect intron splicing. Therefore there is no defect in the structural gene of PIG-A to explain the observed GPI deficiency.

DISCUSSION

We have shown that CD52-negative T cells produced by CAMPATH-1H treatment *in vivo*, and B cells generated by *in vitro* CAMPATH-1H treatment, are deficient in surface expression of both CD59 and CD55. As CD52, CD55 and CD59 are all anchored to the cell surface by a GPI anchor, these observations suggest that a generalized problem in GPI-anchor synthesis, addition or trafficking has arisen in these cell lines. We therefore investigated the capacity of the CD52-negative B and T cell lines for the biosynthesis of GPI lipids and compared these with the parental CD52-positive lines. As a B cell mature GPI lipid has not been identified as yet, we initially looked for a candidate precursor lipid in the GPI-positive B cell line C1.

Structural analysis of ethanolamine-labelled extracts from C1 cells allowed identification of a labelled polar lipid species, which we designated mGPI. This species had the expected properties of a mature mammalian GPI precursor. mGPI has an R_F of 0.4 in solvent system A, identical with that of the EL4 cell GPI precursor 'core' [15,26,27]. Additionally, mGPI was sensitive to GPI-PLD but resistant to PI-PLC, consistent with an acyl-inositol structure. Finally, mGPI and 'core' co-migrated in three different TLC solvent systems, confirming close structural similarity between the two lipids. In additional experiments using [^{3}H]mannose or glucosamine we observed a species that co-migrated with [^{3}H]ethanolamine-labelled mGPI from lipid extracts (results not shown), consistent with the assignment of mGPI as a mature GPI precursor in human B cells.

When we analysed lipid extracts from C1, CV3, CV5 and CV5 *ex vivo* cells we observed that mGPI levels were correlated with CD52 cell surface expression in mixed cell populations, and additionally that mGPI was completely absent from pure CD52-negative cell preparations. These data clearly suggest that the inability to express CD52, and other GPI-anchored surface proteins, is indeed due to defective GPI biosynthesis by these lymphocytes. Additionally the CD52-negative T cell line BT similarly failed to synthesize mature GPI precursors, in contrast with the CD52-positive RP cell line.

Therefore the phenotype of the GPI-deficient B and T cells strongly resembles that of PNH lymphocytes. In all PNH cells analysed to date the biosynthetic defect has been mapped to the first step of the pathway, i.e. transfer of *N*-acetylglucosamine from UDP-GlcNAc to phosphatidylinositol [10–12]. The gene encoding PIG-A, which is essential for the synthesis of GlcNAc-phosphatidylinositol [16], contains a variety of mutations in cells from PNH patients [17–21], and these are proposed to be the cause of deficient GPI-anchor biosynthesis in PNH patients. Therefore we analysed the PIG-A locus in our CD52-negative B and T cell lines. Most unexpectedly, we found that the PIG-A structural gene, including the intron splice sites, was identical to the wild-type sequence in all cases, ruling out a simple PIG-A mutation as an explanation for the GPI-deficient phenotype of these cells.

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. For example, the CV3 B cell population remained only $\sim 25\%$ CD52-positive, despite recloning. Most surprisingly, when the CD52-positive and -negative cells were separated and returned to culture, both populations reverted to the original distribution, again $\sim 25\%$ CD52-positive. This phenomenon was not observed for the BT cell line, where the CD52-negative cells, initially separated in the same manner as for CV3, did not revert to a CD52-positive phenotype even after 6 months in culture. Thus it would appear that, although both cell types are defective in the biosynthesis of GPI anchors, this defect is reversible in the B cells but permanent in the T cells, suggesting that the alterations in the B and T cells producing the GPI-negative phenotype are non-identical.

Re-expression of GPI-anchored proteins has been observed with sodium butyrate treatment of class H Thy-1-negative thymomas, and this presumably is the result of a stimulation of transcription rather than an alteration to a structural gene [29–31]. Such a mechanism may underlie the apparent ability of the GPI-deficient B cells to regain the capacity to synthesize GPI anchors. Separation of the cells into an all-GPI-negative population may provide a signal for re-activation of the affected gene. Furthermore, the persistence within a GPI-negative population of GPI-positive cells, which reappear in culture after removal, also suggests a signalling phenomenon between the cells. Conversely, the deficiency in GPI-anchor biosynthesis in the T cells is irreversible. As there is no defect in the PIG-A gene of the BT cell line, candidates include other genes involved in GPI biosynthesis. An additional possibility is disruption of the promoter, or other control element, of the PIG-A gene itself. Presumably such defects are rare as, unlike point mutations in the coding region which can result in truncation, nonsense or mis-splicing, point mutations within DNA control elements rarely have effects of sufficient intensity to account for a complete shutdown of GPI biosynthesis, and this would therefore necessitate consideration of more extensive alterations to the promoter.

Interestingly, in a separate trial, CD52-negative T cells with deficient surface expression of other GPI-anchored proteins emerged in a patient with refractory B cell non-Hodgkin lymphoma after treatment with CAMPATH-1H [4]. Reverse transcriptase-PCR analysis suggested that a deletion of exon 4 of the PIG-A gene could account for the GPI deficiency, a classical PNH defect [4]. As small numbers of PNH cells are most probably present in asymptomatic individuals, it is probable that, under appropriate selection, these cells can selectively proliferate [32,33]. Thus CAMPATH-1H treatment of the rheumatoid arthritis patients may have led to the expansion of pre-existing GPI-deficient cells. From the data presented here, it is apparent that such a deficiency can arise by more than one distinct mechanism.

Several direct conclusions can be drawn from the present study. A mature GPI anchor precursor, mGPI, has been identified in a B cell line, and a similar species has also been identified in a human T cell line. mGPI is highly similar to, and probably identical with, a GPI-anchor precursor characterized from the murine EL4 line [27]. A correlation was found between the percentage of CD52-positive B cells and the level of mGPI detected in the lipid extracts from these cells. While the phenotype

of these CD52-negative cells is very similar to that found in PNH cells, no mutations were identified in the PIG-A gene, indicating a novel mechanism underlying the GPI deficiency. The clinical importance of these findings has yet to be determined, but our data imply that a spectrum of defects may give rise to PNH-like lymphocytes. Our data also suggest that the use of monoclonal antibodies in therapy can lead to the expansion of abnormal or mutated cells, and therefore careful monitoring is essential during the follow-up period. Interestingly, in the present study CD52-negative cells were detected in only 12% of patients monitored, and all of these patients were in the group showing the longest remission.

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