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# Glycosylphosphatidylinositol (GPI)-anchored surface antigens in the allogeneic activation of T cells*

J. SCHUBERT, A. STROEHMANN, C. SCHOLZ & R. E. SCHMIDT *Division of Clinical Immunology, Department of Medicine, Hannover Medical School, Hannover, Germany*

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## SUMMARY

GPI-linked surface molecules have recently been described as structures with an activation potential for human T lymphocytes. To study the role of these molecules in T cell activation we analysed GPI-deficient or normal T cells from patients with paroxysmal nocturnal haemoglobinuria (PNH). On activation with allogeneic Epstein–Barr virus (EBV)-transformed B cell lines GPI-deficient freshly separated T cells or continuously growing T cell lines exhibited a significantly lower proliferation or cytokine production compared with their normal counterparts. In contrast, stimulation via the T cell receptor-associated CD3 structure resulted in a comparable response. There was no difference in activation of normal T lymphocytes when GPI-deficient B cells were used as stimulators compared with normal B cells obtained from the same PNH patient. We conclude from these data that GPI deficiency in PNH leads to a functional deficiency of GPI-deficient T cells. In contrast, no difference in activation of T lymphocytes for GPI-deficient cells on the stimulator cell level was observed.

**Keywords** GPI-linked antigen T cell activation allogeneic stimulation paroxysmal nocturnal haemoglobinuria

## INTRODUCTION

GPI-linked surface proteins are now generally accepted as molecules able to mediate T cell activation [1–3]. Since these molecules are linked to the membrane via a glycolipid bridge which has access only to the outer leaflet of the membrane lipid doublelayer [4] the mechanisms of this activation remain unknown. Recently, it has been suggested that GPI-linked molecules take part in large non-covalent complexes which also contain protein tyrosine kinases [5–7]. In addition, a link of GPI-anchored proteins to the T cell receptor (TCR) in T lymphocytes has been suggested [8]. In order to investigate the role of surface expression of GPI-linked proteins in T cell activation, GPI-deficient lymphocytes from patients with the GPI deficiency disease paroxysmal nocturnal haemoglobinuria (PNH) were studied [9]. PNH is characterized by the emergence of GPI-deficient blood cells due to a somatic mutation within the PIG-A gene of an early haematopoietic precursor cell [10–12]. In recent studies we were able to establish that there is a functional deficiency of T lymphocytes, especially in response to lectins due to the GPI deficiency resulting from a mutation in the PIG-A gene [9]. In contrast, stimulation via the TCR-associated CD3 structure resulted in a comparable response. This shows that the basic ability of cells in PNH to proliferate and to produce cytokines is identical to that of normal cells. Here we extended these studies by an allogeneic activation system using Epstein–Barr virus (EBV)-transformed B cells which more closely resembles the *in vivo* situation in which activation is more complex and requires more cell-to-cell contact. In contrast to CD3-mediated activation, the response of GPI-deficient T cells to allogeneic B cell blasts is much weaker than that of normal cells obtained from the same patient.

A. S. current address: III Medizinische Klinik, Universitätskliniken der Charité, D-10098 Berlin, Germany.

Correspondence: Jörg Schubert MD, Abt. Klinische Immunologie, Medizinische Hochschule, D-30623 Hannover, Germany.

* This study is dedicated to Professor Fritz Hartmann on his 75th birthday.

## PATIENTS AND METHODS

*Reagents*

All MoAbs used in these studies have been characterized previously [9,13]. PE-labelled antibodies Leu-4 (CD3), Leu-3a (CD4) and Leu-2a (CD8) were obtained from Becton Dickinson (Heidelberg, Germany). CD48 MoAb J4.53 was purchased from Dianova (Hamburg, Germany). BRIC110 (CD55) and BRIC229 (CD59) were purchased from IBGRL (Bristol, UK). CD48 MoAb MEM-102 was kindly provided by Dr V. Horejsi (Czech Academy of Sciences, Prague, Czech Republic).

*Patients and cell preparations*

All patients tested (patients 1, 2, 3, 4 and 5) had a long-standing history of chronic or sudden intravascular haemolysis. In all cases the clinical diagnosis of PNH was established by a positive Ham test or sucrose test. In addition, flow cytometric analysis revealed GPI-deficient cells in the granulocyte, monocyte, and erythrocyte fractions, as described previously. Lymphocytes were affected by the GPI-anchoring defect at a proportion of 5–35%. All five PNH patients tested in these studies were proven to be $EBV^-$ serologically.

Heparinized blood was collected from PNH patients and healthy donors by venous puncture and mononuclear cells (PBMC) were obtained by Ficoll–Hypaque density centrifugation (Biochrom, Berlin, Germany). Adherent cells were obtained by incubating PBMC twice on Petri dishes (Falcon, Heidelberg, Germany) at 37°C. After three washes with RPMI 1640 medium containing 10% fetal bovine serum (FBS) adherent cells were removed with ice-cold PBS.

*Flow cytometric analyses*

Indirect immunofluorescence was performed according to the standard methods. Two-colour analysis was carried out with indirect immunofluorescence using FITC-labelled goat anti-mouse antiserum (GM-FITC), followed by direct immunofluorescence in the presence of mouse serum as described previously [14]. Ten thousand cells in each sample were analysed using a FACScan (Becton Dickinson). Fluorescence intensity was depicted on a four-decade logarithmic scale, single-parameter analysis as histograms and two-colour analysis as contour plots, respectively.

*Cell separation*

After separation of adherent cells, peripheral blood lymphocytes (PBL) of the PNH patients (1, 2 and 3) were incubated with the biotinylated CD48 antibody MEM-102 for 30 min. Cells were then washed and incubated with avidin–FITC (Becton Dickinson) for 20 min. After washing, antibody binding was proven by flow cytometry. Cells were then incubated with biotin-coupled magnetic beads (Miltenyi, Bergisch-Gladbach, Germany). Separation was performed as described [9]. Purity of the cell preparations was > 95%.

*Generation of GPI-deficient T cell lines*

T cell lines were generated as described in detail previously [9]. Briefly, separated lymphocytes from PNH patients (2, 3, 4 and 5) were plated at 1000 cells/well onto a feeder cell layer consisting of allogeneic PBMC and EBV-transformed B cell lines (JoJo). Colonies were expanded in culture medium containing 10% lymphocyte conditioning medium (LCM). Contaminating natural killer (NK) lymphocytes were removed using CD16 MoAbs (3G8; Dianova) followed by magnetic separation according to the manufacturer's instructions (Dynal, Hamburg, Germany).

*Generation of EBV-transformed B cell lines*

Unseparated PBMC from one PNH patient were suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Eggenstein, Germany) containing 20% FBS and cyclosporin A (Sandoz, Basle, Switzerland) at a concentration of 2 $\mu$g/ml. EBV-containing supernatant from B95–8 marmorset cell line (kindly provided by Dr G. W. Bornkamm; GFL, Munich, Germany) was added to each well [15]. Transformed colonies were expanded and analysed for their expression of GPI-anchored surface molecules.

*Proliferation tests*

Cells were incubated at $5 \times 10^4$ cells/well. In the case of MACS-sorted cells various cell numbers/well were used. Depending on the number of cells obtained from the columns $2 \times 10^4$–$5 \times 10^4$ cells/well were seeded. Adherent cells were added at a density 10% of total cells. Cells were stimulated for 4 days, pulsed with $^3$H-thymidine, and then harvested 18 h later. Solid-phase bound CD3 MoAb was OKT3-bound to Sepharose used at 100 beads/well.

*Production and quantification of interferon-gamma*

Interferon-gamma (IFN-$\gamma$) was measured in the supernatants of T cell lines after 48 h culture at a density of 50 000 cells/well. Cells were stimulated with either solid-phase bound OKT3, phorbol myristate acetate (PMA; 5 ng/ml) or EBV-transformed B cell lines (JoJo) as an allogeneic stimulus. IFN-$\gamma$ in the supernatants was measured with a complete ELISA test kit (Biomar, Marburg, Germany) according to the manufacturer's instructions.

## RESULTS

*Distribution of GPI-deficient T cells from PNH patients*

In normal donors the GPI-anchored CD48 antigen is homogeneously expressed on all lymphocytes. Therefore, CD48 MoAbs are ideal reagents for distinction and separation of GPI-deficient lymphocytes from PNH patients [14]. Two-colour immunofluorescence of T cells from a representative PNH patient (patient 2) is shown in Fig. 1. A proportion of 6·5% of $CD3^+$ T cells is affected by the GPI-anchoring defect. In addition, the number of GPI-deficient $CD4^+$ and $CD8^+$ T cells is comparable. $CD48^-$ cells represent 7% of total $CD4^+$ cells and 6·5% of total $CD8^+$ cells, respectively.

*Decreased activation of GPI-deficient lymphocytes in response to allogeneic EBV-transformed B cells*

Allogeneic EBV-transformed B cell lines were used as stimulators to investigate the role of GPI-anchored surface molecules

**Table 1.** Proliferation of magnetic column separation (MACS)-separated $CD48^+$ and $CD48^-$ PBL

| | Medium | | CD3 | | B cell blasts | |
|---|---|---|---|---|---|---|
| Patient | $CD48^+$ | $CD48^-$ | $CD48^+$ | $CD48^-$ | $CD48^+$ | $CD48^-$ |
| 1 | 70 | 65 | 2491 | 2418 | 7548 | 1428 |
| 2 | 1500 | 1000 | 27 663 | 29 794 | 61 149 | 8931 |
| 3 | 70 | 65 | ND | ND | 10 219 | 4478 |

Cells were tested in response to either solid-phase bound CD3 MoAb or allogeneic B cell blasts. Proliferation is given as ct/min measured by $^3$H-thymidine uptake. The interindividual variation of the maximal proliferation is due to the variable number of cells available (20 000/well, 50 000/well, and 25 000/well; for patients 1, 2 and 3, respectively) after cell separation in each experiment. Standard deviation was < 10% in each experiment.

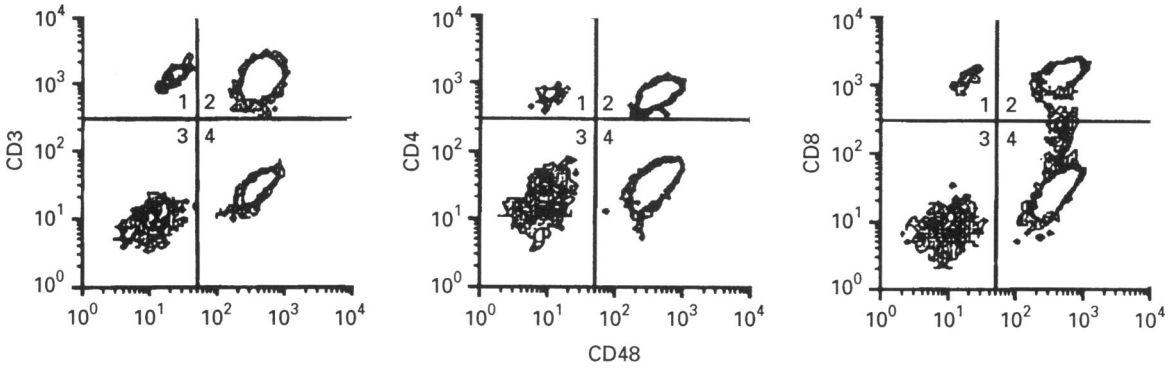


**Fig. 1.** Two-colour immunofluorescence of peripheral blood mononuclear cells (PBMC) of a representative paroxysmal nocturnal haemoglobinuria (PNH) patient. Cells were stained with PE-labelled CD3, CD4 or CD8 MoAbs (ordinate) and CD48 antibody labelled with FITC-labelled goat anti-mouse antiserum (GM-FITC) (abscissa). Statistics were performed by means of gating the contour plots.

in the activation requiring cell-to-cell contact. GPI-deficient and normal lymphocytes were separated using the CD48 MoAb MEM-102 followed by magnetic column separation (MACS). Both GPI-deficient and normal lymphocyte fractions exhibited a purity of > 95%. GPI-deficient lymphocytes exhibited a weaker response to allogeneic B cells compared with their normal counterparts obtained from the same PNH patient. In contrast, activation via the TCR-associated CD3 structure resulted in a comparable response of the two fractions. This was consistent comparing three different PNH patients (Table 1). The ct/min difference comparing various PNH patients was due to different cell numbers available after magnetic column separation. To exclude that the activation differences of $CD48^+$ and $CD48^-$ cells were due to an unspecific activation mediated by the separating antibodies used, lymphocytes of a normal donor were treated the same way and compared with untreated lymphocytes. No unspecific activation could be detected in these experiments (data not shown).

*Production of IFN-γ by GPI-deficient T cell lines*

To examine further the effects of the lack of GPI-anchored surface structures on response to allogeneic EBV-transformed B cell blasts, polyclonal T cell lines, either GPI-deficient or normal, were established using allogeneic PBL and EBV-transformed B cells as feeders. These T cell lines obtained from the same PNH patient were tested for their ability to produce IFN-γ. GPI-deficient T cell lines from four different PNH patients released about half the amount of the cytokine upon stimulation with allogeneic B cells compared with their normal counterparts (Fig. 2). In contrast, stimulation with immobilized CD3 MoAb resulted in a comparable response.

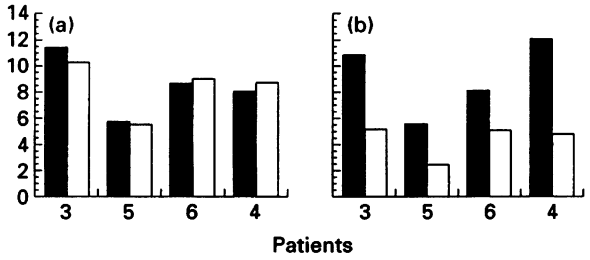


**Fig. 2.** Production of IFN-γ by GPI-deficient (□) *versus* GPI-positive (■) polyclonal T cell lines. Cells were either stimulated with solid-phase bound CD3 MoAb (a) or with allogeneic Epstein–Barr virus (EBV)-transformed B cell blasts (b). The amount of IFN-γ after 48 h production given in ng/ml on the ordinate was measured by an IFN-γ-specific ELISA.

*Stimulation of normal PBMC with GPI-deficient allogeneic B cells*

The role of GPI-anchored proteins was further investigated by addressing the question of whether their expression is required not only on the responder but also on the stimulator cell level. GPI-deficient EBV-transformed B cell blasts from one patient (patient 3) were established and used for stimulation of normal PBMC in comparison with normal B cell blasts obtained from the same patient. There was no difference in the proliferation of allogeneic PBMC when either GPI-deficient or normal B cell lines were used as stimulators (data not shown). The same results could be obtained by using the GPI-deficient mutant cell line JY-5 in comparison with the normal parental cell line JY as stimulators.

## DISCUSSION

From previous studies it has been suggested that the absence of GPI-linked surface molecules leads to a functional deficiency of lymphocytes and haematopoietic cells [9,16]. In this context, normal T cells were compared with GPI-deficient T cells from PNH patients which can be distinguished by their lack of GPI-linked molecules due to a mutation within the PIG-A gene and a disrupted assembly of GPI-anchor molecules. In previous studies, GPI-deficient T cells exhibited a much weaker response to lectins than their normal counterparts [9]. In contrast, stimulation via the TCR-associated CD3 structure did not show any difference.

Here, we extended these studies by choosing an allogeneic stimulus resembling the *in vivo* situation, thus requiring more cell-to-cell contact. We were able to demonstrate that GPI-deficient T cells are less activated by allogeneic EBV-transformed B cells compared with normal cells. In contrast, stimulation via CD3 resulted in a comparable response, showing that the basic ability of the responder cells to proliferate and to produce cytokines is almost the same as in their normal

counterparts. The deficient allogeneic stimulation could be demonstrated by an impaired proliferation of freshly isolated T lymphocytes and impaired production of the cytokine IFN-$\gamma$. In contrast to the GPI deficiency on the responder cell level, deficiency on the stimulator cell level did not significantly affect the activation of allogeneic T cells as measured by the proliferative response to GPI-deficient B cell blasts.

It has been suggested that GPI-anchored molecules are not only important on the responder but also on the stimulator cell level, since GPI-linked CD58 and CD59 molecules interact with CD2 on the T cell level [17,18]. However, we could not detect any difference of T cell proliferation in response to allogeneic GPI-deficient B cell blasts. This could be confirmed by using the GPI-defective mutant JY-5 in comparison with the normal parental EBV-transformed B cell line JY. These findings do not really exclude such a mechanism, since other stimulatory molecules might not exhibit the same activating potential as allogeneic HLA determinants. However, our data suggest that expression of GPI-linked molecules on the stimulator cell site might not be so important for supporting a sufficient T cell response. In contrast, the deficiency of GPI-anchored molecules on the responder cell level leads to a significant functional impairment of responding cells.

It is still a mystery how GPI-anchored surface molecules can mediate activation signals into a cell. Recently, large noncovalent molecular complexes have been described as containing protein tyrosine kinase activity and thus mediating activation signals. Obviously, GPI-anchored molecules are residing in microdomain-like structures containing glycolipids and cholesterol [19,20]. In addition, they may even contain transmembranous proteins as signal transducers [5]. However, such mediating molecules have not been described so far [6]. In T cells a link between the signal transduction via GPI-anchored molecules and the zeta-chain of the TCR has been suggested [8]. GPI-anchored surface molecules have also been described to play a role in adhesion; e.g. CD48, CD58 and CD59 [17,18,21,22]. Especially in the context of activation of the protein kinase C (PKC) they have been found to mediate T cell activation [23]. These studies suggest that such molecules might play a role as important amplifying signals supporting the specific signal by the TCR. Therefore, their absence on the surface of GPI-deficient T cells could explain the functional deficiency.

Functional alterations due to the absence of GPI-anchored proteins might also influence thymocyte maturation. Apart from a lacking surface expression of the CD45RO antigen which characterizes previously activated T cells, GPI-deficient T lymphocytes exhibit the characteristics of mature T cells such as normal expression of the TCR or costimulatory molecules such as CD4 and CD8 [9]. However, one might argue that the observed alterations could be due to selected subpopulations of T cells, e.g. with a lack of CD28 molecules which is responsible for the altered response to allogeneic stimulation. However, the finding that the polyclonal T cell lines do not exhibit any difference in the expression of such molecules such as CD28, CD11a, etc., would rather favour the explanation of a functional deficiency due to the absence of GPI-anchored surface molecules.

The finding that the lack of GPI-linked surface molecules leads to a functional deficiency further elucidates the hypothesis of clonal expansion of GPI-deficient progenitor cells within the bone marrow. The functional deficiency of bone marrow progenitor cells from PNH patients was established in recent investigations [16,24]. According to the hypothesis of clonal expansion, GPI-deficient cells might also occur in normals, but the normal marrow overgrows the deficient cells because of their functional deficiency. This hypothesis has been supported by the recent observation of the sustained emergence of GPI-deficient lymphocytes which also contain a mutation within their PIG-A gene after treatment of patients with high-dose CD52 antibody Campath-1 [25]. In the case of PNH, there is an injury only of normal marrow cells and the GPI-deficient clone can expand, which leads to the clinical symptoms found in PNH [7].

In conclusion, our data suggest that the allogeneic response to EBV-transformed B cells is impaired in GPI-deficient cells, whereas the response to GPI-deficient B cells is not significantly affected. Whether GPI-deficient cells also respond differently to soluble antigens presented by autologous antigen-presenting cells is currently under investigation.

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