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Heike H. Breitinger, Markus T. Rojewski, Hubert Schrezenmeier. Antibodies to glycosylphosphatidyl-inositol anchored proteins (GPI-AP) in antithymocyte and antilymphocyte globulin: possible role for the expansion of GPI-AP deficient cells in aplastic anemia. *Annals of Hematology*, 2009, 88 (9), pp.889-895. 10.1007/s00277-008-0688-0 . hal-00535024

HAL Id: hal-00535024

<https://hal.science/hal-00535024>

Submitted on 11 Nov 2010

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Antibodies to glycosylphosphatidyl-inositol anchored proteins (GPI-AP) in antithymocyte and antilymphocyte globulin: possible role for the expansion of GPI-AP deficient cells in aplastic anemia

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Received: 19 September 2008 / Accepted: 18 December 2008 / Published online: 13 January 2009
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Abstract Antithymocyte globulin (ATG) and antilymphocyte globulin (ALG) are currently used successfully for immunosuppressive treatment of aplastic anemia. In this study we have investigated whether commercial ATG/ALG preparations contain antibodies against glycosylphosphatidyl-inositol anchored proteins (GPI-AP), which could be responsible for emergence of GPI-deficient populations in aplastic anemia after ATG/ALG therapy. We analyzed four commercial ATG/ALG preparations by competitive binding assays using flow cytometry. Quantification was achieved by calculating the concentration of ATG/ALG required to give 50% inhibition of binding the specific fluorochrome-labeled monoclonal antibody (EC50). High concentrations of antibodies against the GPI-anchored protein CD52 were found in all preparations (Lymphoglobulin[®] Genzyme, Thymoglobulin[®] Genzyme, ATGAM[®] Pharmacia & Upjohn, and ATG-Fresenius S Fresenius). Antibodies against the GPI-anchored protein CD48 are present in significant concentrations except in the preparation ATGAM[®]. CD16 antibodies were found in lower concentrations. We could not detect significant concentrations of antibodies against the GPI-anchored proteins CD157 and CD14. Campath-1H, a monoclonal antibody against the GPI-anchored protein CD52, has been used as immunosuppressive tool for T-cell depletion. CD52 antibodies in ATG/ALG preparations might contribute in the same way to the

immunosuppressive effects in treatment of aplastic anemia. It is known that in a substantial proportion of patients with aplastic anemia GPI-deficient cells are present in a low level at diagnosis or emerge after immunosuppressive therapy. GPI-anchored antibodies in ATG/ALG preparations might lead to a relative advantage for pre-existing GPI-deficient cells caused by an escape from the antibody-mediated attack.

Keywords Glycosylphosphatidyl-inositol anchored proteins · Antithymocyte globulin · Antilymphocyte globulin · Aplastic anemia · Immunosuppression

Introduction

Antithymocyte globulin (ATG) and antilymphocyte globulin (ALG) are polyclonal immunoglobulins (IgG) prepared by immunizing horses or rabbits with human thymocytes, lymphocytes, or T-cell lines. We use ATG as abbreviation for all preparations. They contain a mixture of antibody specificities [1–3]. ATG has become the golden standard in immunosuppressive treatment of aplastic anemia (AA). Remission rates to combined immunosuppression with ATG, cyclosporin A, and corticosteroids reach up to 60–80% in aplastic anemia [4, 5]. However, the mechanism of action responsible for induction of remissions in AA is still unknown. There is evidence that several mechanisms are involved [6]. There is a close clinical interrelation between AA and paroxysmal nocturnal hemoglobinuria (PNH). In AA patients treated with ATG, there is a risk of 25% to develop secondary clinical PNH within about 15 years [7]. This clinical interrelation is based on a pathophysiological link: we and others could demonstrate that cells with a deficiency of glycosylphosphatidyl-inositol anchored pro-

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teins (GPI-AP) are present at diagnosis or emerge after immunosuppressive therapy in a substantial proportion of AA patients [8–12]. Like in primary, classical PNH, the GPI-AP deficient population in AA is characterized by mutations of the PIG-A gene [13]. These findings support the hypothesis that in many patients with AA PIG-A mutated, GPI-deficient cells are present at a low level. Failure of “normal”, non-GPI-deficient cells confer a relative advantage to the GPI-AP deficient population. An immune attack directed preferentially against non-GPI-deficient cells could explain both bone marrow failure and emergence of GPI-AP deficient hematopoiesis.

A transient emergence of GPI-deficient T-lymphocytes and monocytes could be observed after therapy with Campath-1H, a monoclonal antibody directed against the GPI-anchored protein CD52 [14–16].

These findings support the hypothesis that there is a relative survival advantage for GPI-AP-negative cells compared to GPI-AP-positive cells caused by an escape from an immune attack.

These observations prompted the question whether antibodies against GPI-anchored proteins are present in polyclonal ATG preparation. These antibodies might—similar to monoclonal CD52 antibodies—confer a selective advantage to GPI-deficient cells thus supporting outgrowth of pre-existing GPI-deficient cells after immunosuppressive treatment with ATG for AA or after ATG as part of the conditioning regimen for stem cell transplantation.

Therefore, we analyzed whether commercial ATG preparations contain antibodies against GPI-anchored proteins and determined their quantity and specificities of the antibodies. Using competitive binding assays we have investigated four commercial ATG products and could quantify the antibodies by calculation the 50% inhibition concentrations (EC50).

Materials and methods

The antibody composition of ATG was analyzed by competitive binding assays using flow cytometry. We stained target cells expressing the respective antigens by monoclonal fluorochrome-labeled specific antibodies. By co-incubation with ATG- preparations we analyzed whether ATG contains antibodies, which block the binding of the fluorochrome-labeled antibody. Median fluorescence was measured from each sample without ATG and after preincubation with increasing concentrations of ATG. Staining without ATG corresponds to the maximal binding in the absence of a competing antibody.

The ATG concentration, which was necessary to inhibit 50% of the binding of the fluorochrome-labeled specific monoclonal antibodies (EC50), was calculated by nonlinear

regression using one side competition with the following formula:

$$\begin{aligned} \text{binding} &= \text{binding of negative control} \\ &+ (\text{max. binding} - \text{binding of negative control}) \\ &\div (1 + 10^{X - \log EC50}) \end{aligned}$$

The calculations were done with the computer program GraphPad Prism 3.00, San Diego, CA, USA.

The following batches of ATG were analyzed: Lymphoglobulin® Genzyme (Ch-B.: K0477L, N0098-21, N0562-14) and Thymoglobulin® Genzyme (Ch-B.: L0963J, M1097) from Genzyme GmbH Neu-Isenburg, Germany, ATG-Fresenius S (Ch-B.: H02B-2) from Fresenius AG, Bad Homburg, Germany and ATGAM® (Ch-B.: 695YM and 405YY) from Pharmacia & Upjohn Company, Kalamazoo, MI, USA.

The optimal concentration of the antibodies for the blocking studies was determined to be in saturation levels (the squared brackets indicate the employed volume of the monoclonal antibody). We used the following antibodies against GPI-anchored proteins: CD52-FITC (Campath-1H), [4 µl] (Wellcome Foundation, Beckenham, England), CD48-FITC (J4-57) [4 µl] and CD157 (BST-1/CD38-like, RF3) [1 µl] (both from Beckman Coulter, Krefeld, Germany), CD16-FITC (anti-Leu-11a, NKP15) [8 µl] (Becton Dickinson Biosciences—Immunocytometry Systems, Heidelberg, Germany), CD90-FITC (Thy-1, 5E10) [2 µl] (Becton Dickinson Biosciences Pharmingen™, Heidelberg, Germany) and CD14-FITC (MY4, 322A-1) [5 µl] (Beckman Coulter, Krefeld, Germany). We used as secondary antibody the DTAF-conjugated goat-anti mouse immunoglobulin [200 µl from a dilution of 1:200] (Dianova, Hamburg, Germany) for the unlabeled antibody CD157.

Besides these GPI-anchored antibodies we tested whether the ATG products contain CD2-, CD3-, CD95-, and CD34-antibodies using following specific monoclonal antibodies: CD2-FITC (39C1.5) [2.5 µl] and CD95-FITC (FAS/APO-1, UB2) [10 µl] (both from Beckman Coulter, Krefeld, Germany) and CD3-FITC (anti-Leu-4, SK7) [2.5 µl] and CD34-PE (8G12) [5 µl] (both from Becton Dickinson Biosciences—Immunocytometry Systems, Heidelberg, Germany).

The following cells with strong expression of the antigen were used as target cells: peripheral blood mononuclear cells from healthy donors were isolated by centrifugation of heparinized blood on a layer of Ficoll Hypaque (Biochrom AG, Berlin, density 1.077). Lymphocytes were used for competitive binding assays for CD2, CD3, CD48, and CD52, granulocytes were used for CD16 and CD157. Monoblastic peripheral blood cells from a patient with acute monoblastic leukemia were prepared with Ficoll and employed for the CD14 antibody. The cell line HUT78 was used for staining of CD90 and CD95. CD34-positive cells

were gained by positive selection of healthy donors (CD34 purity 98%). Informed consent was obtained from all donors.

Cells (3×10^5) were incubated with increasing concentrations of ATG (from 0.01/0.3 to 15 mg/ml) for 20 min at 4°C followed by incubation with the specific monoclonal antibody on equal conditions. The total sample volume was 100 μ l. In case of indirect labeled antibody, the cells were washed and afterwards they were stained with the DTAF-conjugated goat anti-mouse antibody. Isotype identical negative controls were taken for all antibodies.

Finally, after two washes, cells were resuspended in PBS 0.1% serum albumin (BSA) and were fixed in 2% paraformaldehyde.

Results

Four ATG preparations (Lymphoglobulin® Genzyme, Thymoglobulin® Genzyme, ATGAM® Pharmacia & Upjohn

and ATG-Fresenius S Fresenius) were analyzed by competitive binding assays for their content of antibodies to six GPI-anchored and four non-GPI-anchored proteins.

In order to validate our assay, we investigated whether we could detect antibodies that are known components of ATG (CD2 and CD3). We could confirm that all analyzed ATG preparations contain the antibodies CD2 and CD3. Low ATG concentrations from only 0.053 mg/ml to 0.626 mg/ml were required to achieve 50% binding inhibition (Figs. 1 and 2(a and b), Table 1) of monoclonal antibodies to CD2 and CD3.

With competitive binding assays we could demonstrate that commercial ATG products contain the GPI-anchored antibodies CD52 and CD48. In this case the co-incubation of ATG and fluorochrome-labeled specific monoclonal antibodies resulted in a dose-dependent inhibition of the monoclonal antibody binding by ATG (Fig. 1). We calculated the concentration of ATG, which is necessary to achieve 50% inhibition of binding the monoclonal fluorochrome-labeled antibody (EC50). Typical inhibition

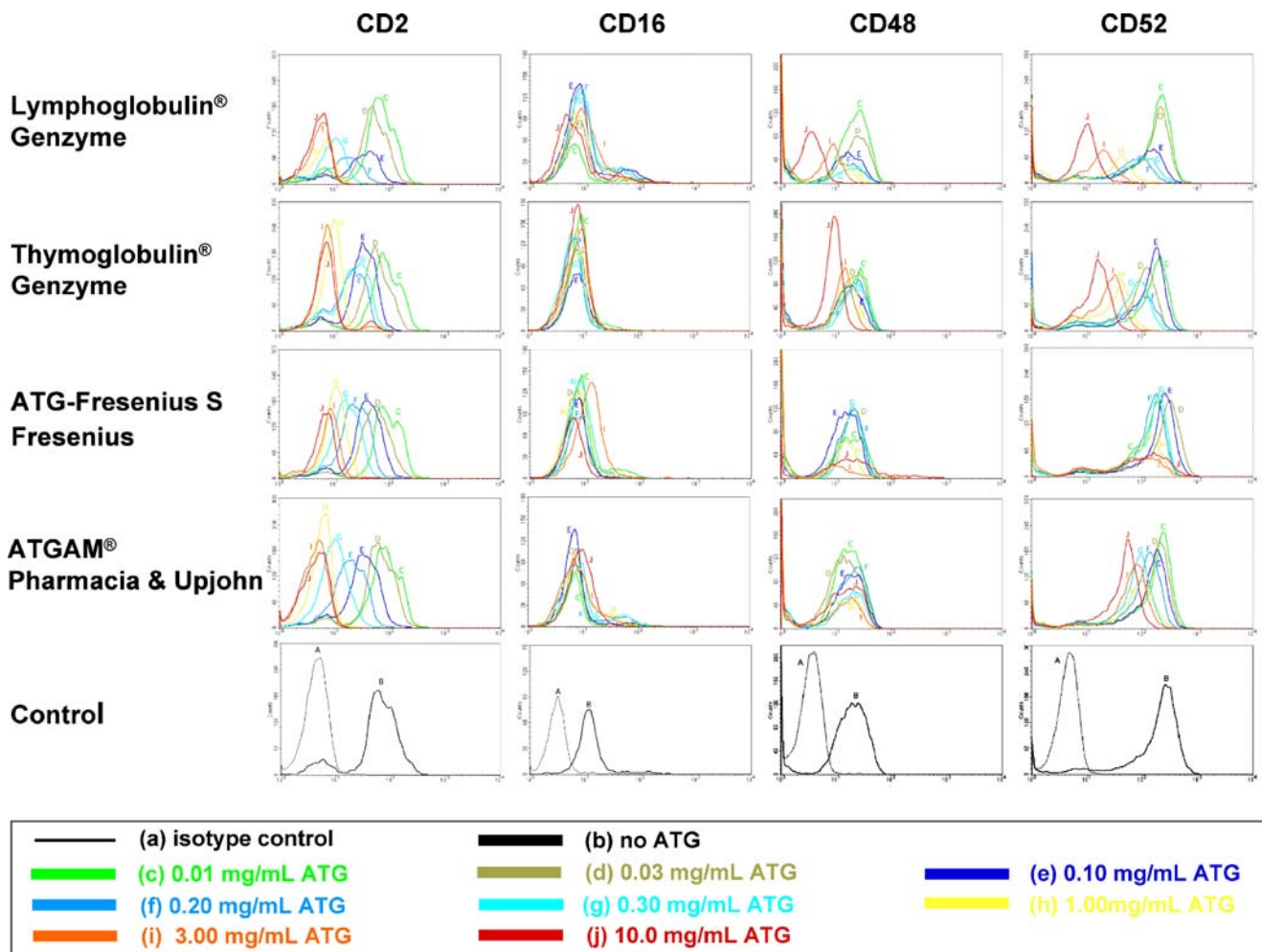


Fig. 1 Immunofluorescence (median channel) of CD2, CD16, CD48, and CD52 without ATG (b) and in presence of increasing concentrations of ATG. As target cells lymphocytes were used for CD2, CD48, and CD52, granulocytes for CD16

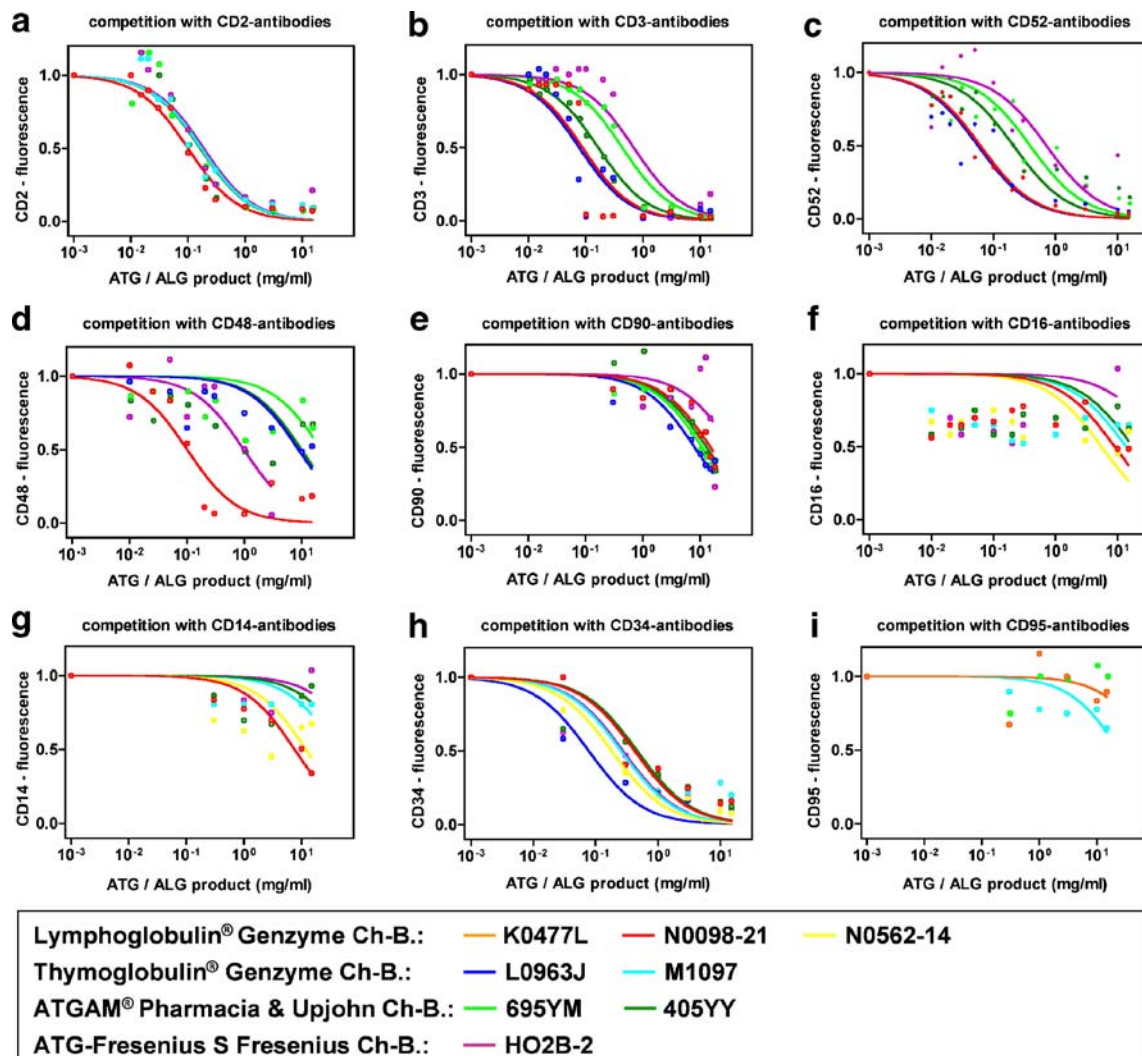


Fig. 2 Inhibition curves of binding fluorochrome-labeled monoclonal antibodies by increasing concentrations of commercial ATG/ALG products (Lymphoglobulin® Genzyme, ATGAM® Pharmacia & Upjohn, Thymoglobulin® Genzyme and ATG-Fresenius S) to the GPI-anchored proteins *c* CD52, *d* CD48, *e* CD90, *f* CD16, and *g* CD14 and to the non-GPI-anchored proteins *a* CD2, *b* CD3, *h* CD34, and *i* CD95

Table 1 Mean values of the 50% inhibition concentrations (mg/ml)

	CD2	CD3	CD52	CD48	CD90	CD16	CD14	CD34	CD95	CD157
Lymphoglobulin® (Genzyme)										
K0477L			0.027	0.446					>93.501	>87.986
N0098-21	0.060	0.053	0.062	0.200	11.556	7.762	8.52×10^{15}	1.240		
N0562-14					12.310	5.114	$>1.47 \times 10^{35}$	0.732		
ATGAM® (Pharmacia & Upjohn)										
695YM	0.111	0.228	0.216	33.670	6.260	2.111			Dnc	$>1.66 \times 10^{17}$
405YY	0.108	0.150	0.211	12.422	7.674	13.845	>66.943	0.513		
Thymoglobulin® (Genzyme)										
L0963J	0.109	0.087	0.057	4.981	5.692	1.388		0.195	>24.264	2.80×10^{13}
M1097					30.950	10.448	>43.599	0.202		
ATG-Fresenius S (Fresenius)										
HO2B-2	0.144	0.626	0.459	1.705	141.930	19.830	$>1.15 \times 10^{16}$	0.491		>28.580

Mean concentrations (mg/ml) of ATG required to give 50% inhibition of binding a specific fluorochrome-labeled monoclonal antibody. Dnc did not converge, > in one or more assays it was not possible to calculate an EC50 value

curves are shown in Fig. 2. The mean EC50 values are shown in Table 1.

CD52 antibodies were detected in high concentrations in preparations from Genzyme Lymphoglobulin® (Ch-B.: K0477L, N0098-21) and Thymoglobulin® (Ch-B.: L0963J) with EC50 values range from 0.027 mg/ml to 0.062 mg/ml. Also ATGAM® (Ch-B.: 695YM and 405YY) (mean EC50, 0.213 mg/ml) and ATG-Fresenius S (Ch-B.: H02B-2) (mean EC50 of 0.459 mg/ml) contain CD52 antibodies.

Antibodies against the GPI-anchored antigen CD48 could be detected in high concentrations in Lymphoglobulin® Genzyme (Ch-B.: K0477L, N0098-21). For the rabbit ATG products ATG-Fresenius S and Thymoglobulin® Genzyme (Ch-B.: L0963J) higher concentrations were necessary to achieve 50% inhibition (1.705 or rather 4.981 mg/ml). In contrast, in both lots from ATGAM® antibodies against CD48 were detected in very low concentrations (EC50 > 10 mg/ml).

Antibodies against the GPI-anchored protein CD16 were present in ATGAM® Ch-B.: 695YM (EC50: 2.111 mg/ml) and in Thymoglobulin® Genzyme Ch-B.: L0963J (EC50: 1.388 mg/ml). In Lymphoglobulin® Genzyme (Ch-B.: N0098-21, N0562-14) 7.762 mg/ml and 5.114 mg/ml respectively ATG were necessary to reach the EC50. In other preparations the antibody was only detectable in negligible concentrations (EC50 > 10 mg/ml).

The GPI-linked antibodies CD14 and CD157 could not be detected in significantly concentrations in all analyzed ATG preparations.

CD90 antibodies were found in minimal concentrations in ATGAM® and Thymoglobulin® Genzyme (Ch-B.: L0963J) with EC50 values ranging from 5.692 mg/ml to 7.674 mg/ml. In all other preparations we could not detect significant antibodies to the CD90 antigen (EC50 > 10 mg/ml) (Table 1).

In addition, we investigated whether ATG preparations also contain antibodies, against other non-GPI-anchored epitopes. Antibodies against the CD34 antigen were present in all ATG products (EC50, 0.195 mg/ml to 1.240 mg/ml).

The Fas antibody CD95 could not be detected in any ATG preparation.

There were no general differences between horse and rabbit ATG products. However, ATG-Fresenius S that is produced by immunization with the cell line Jurkat has the lowest concentrations of all tested antibody specificities, except the GPI-linked antibody CD48. The lowest CD48 concentration was found in ATGAM®. In some experiments with ATG-Fresenius S it was not possible to calculate the nonlinear regression curves with the median fluorescence values from high ATG concentration (10 mg/ml and 15 mg/ml) because in these concentrations the median fluorescence was much higher than the maximum binding in the absence of a competing antibody. The increased fluorescence intensity in assays containing high ATG

concentrations of 10 mg/ml or 15 mg/ml ATG may be caused by increased unspecific binding of the fluorochrome-labeled monoclonal antibodies or increased autofluorescence.

Discussion

Our data show that all analyzed commercial ATG products (Lymphoglobulin® Genzyme, Thymoglobulin® Genzyme, ATGAM® Pharmacia & Upjohn Company and ATG-Fresenius S Fresenius AG) contain high concentrations of CD52 and CD48 antibodies against GPI-anchored proteins except in the product ATGAM® where we could not detect competitive binding to the monoclonal anti-CD48 antibody J4-57. In our competitive binding assays, we included a single monoclonal antibody for each cluster of differentiation analyzed. Higher binding affinities to these clusters of differentiation may occur for epitopes different from those covered by the monoclonal antibodies used in this study. We also cannot exclude binding of the commercial ATG products to any other epitope of CD48 not covered by J4-57. In general, differences in binding affinities of the different ATG products might also be due to different grades of steric hindrance of monoclonal antibodies and the different ATG preparations.

Much higher concentrations of ATG were necessary to inhibit binding of CD16 or CD90 fluorochrome-labeled antibodies. Thus, content of antibodies was low in ATG preparations. Antibodies against the GPI-anchored proteins CD14 and CD157 could not be detected in significant concentrations by competitive binding assays.

Furthermore, we could confirm the presence of antibodies to the T-cell antigens CD2 and CD3. ATG preparations also contained antibodies against CD34. We could not demonstrate antibodies to CD95.

Several mechanisms of action of ATG have been postulated: an immunosuppressive component by T-cell receptor blockade and T cell depletion is generally accepted. Furthermore, an immunostimulatory effect of ATG has been demonstrated. ATG induce the production of hematopoietic growth factors and release cytokines IL-1 and IL-2 [17, 18]. In addition, it could be shown that ATG might regulate T-cells by expansion of CD4 + CD25 + T-cells [19]. Also ATG binds to all peripheral blood cells, as well as to bone marrow cells [20]. The effectiveness of ATG in treatment of AA could be also due to an increase of hematopoietic progenitor cells [21]. Beside this ATG can induce Fas-mediated apoptosis in human B- and myeloid cell lines and peripheral lymphocytes [22–25].

We and others have previously described that immunotherapy with the Campath-1H antibody, an excellent tool for T-cell depletion, which is directed selectively against the

GPI-anchored antigen CD52, might lead to a transient emergence of GPI-deficient lymphocytes and monocytes with the phenotypic and molecular characteristics of PNH cells caused by an escape of immune-mediated attack [14–16]. These observations and the fact that many AA patients have minor GPI-AP deficient cells at diagnosis or emergence after immunosuppressive therapy [8–13, 26–28] was the reason for us to investigate the antibody composition of ATG to GPI-anchored proteins. Indeed we could demonstrate the presence of high concentrations of CD52 antibodies and, somewhat lower, CD48 antibodies in all ATG products.

CD52 antibodies might contribute to the immunosuppressive effect by T-cell depletion in ATG preparations. Furthermore, antibodies found against GPI-anchored proteins in ATG products might explain the expansion of pre-existing GPI-AP-deficient populations in some AA patients after ATG therapy.

In contrast to data from Rebellato et al. who demonstrated high activity of anti-CD16 antibodies in rabbit antithymocyte globulin, which are known to influence immunoregulation by antibody-dependent cellular cytotoxicity, we could detect anti-CD16 antibodies only in lower concentrations in all tested ATG preparations. In agreement to data from Rebellato et al. we could not identify antibodies against the GPI-anchored antigen CD14 [3].

In accordance with previously reported data [1–3, 29] all polyclonal ATG preparations contain antibodies against the pan-T-cell antibodies CD2 and CD3. Both might be involved in the immunomodulatory effect of ATG via T-cell activation and lymphokine release. In addition, it has been demonstrated that ATG-induced apoptosis depends on the availability of CD2 and CD3 antibodies [23].

In line with Bourdage and Hamlin it is difficult to compare concentrations of the single antibodies between different laboratories, because of the different technique of the competitive binding assays (for example different incubation time, washing, and monoclonal specific antibodies) [2].

It is known that ATG binds to peripheral blood cells as well as to bone marrow cells [20]. It has been supposed that an important mechanism of ATG for hematological recovery in AA might be a dose-dependent increase of hematopoietic progenitor cells [21]. We could confirm that ATG binds to hematopoietic progenitor cells. Anti-CD34 antibodies could be found in all ATG preparations.

We could not detect anti-CD95 antibodies by our assays. This death receptor was postulated to play an important role in pathophysiology of ATG since CD95 expression is increased in bone marrow of aplastic anemia patients [30, 31].

It has been speculated that the immunogen used for production is a critical factor [2]. No significant differences could be demonstrated between rabbit and horse ATG. There were slight variations of the EC50 value between lots. However, overall the antibody composition was very consistent between lots.

In conclusion we found GPI-anchored proteins in commercial ATG products. They might contribute to immunosuppressive effect in treatment of AA (e.g. CD52 and CD48 antibodies). In addition, they could be responsible for expansion of pre-existing GPI-deficient populations caused by an escape of an immune attack against GPI-anchored cells. However, this selection of antigen negative cells might not be the only mechanism by which ATG modulates hematopoiesis. ATG-mediated stimulation of hematopoietic progenitor cells either by direct action or via release of cytokines is an additional mechanism [32, 33].

This is the first description of relevant concentrations of various antibodies against GPI-anchored proteins in ATG preparations. It is not clear whether these antibodies are harmful (e.g. by favoring of outgrowth of PNH clones) or helpful (e.g. by immunosuppressive effect or CD34 cell stimulation). We were able to find competition of anti-CD34 antibody clone 8G12 and ATG-preparations, making it likely that early hematopoietic progenitor cells might be affected by ATG independent from CD52 expression, leading to clonal selection. However, further studies with ATG on patients without hematopoietic disorders, (e.g. renal transplant patients) are necessary to prove clonal selection by ATG.

Therefore, further clinical studies are necessary to clarify whether there is a correlation between the amount of antibodies against GPI-anchored proteins in ATG and the appearance of GPI-deficient populations or the evolution to secondary clinical PNH after use of ATG for treatment of AA or conditioning for stem cell transplantation or treatment/prevention of graft rejection after solid organ transplantation.

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