Antithymocyte and antilymphocyte globulins (ALG) are currently used as immunosuppressive agents in organ transplantation and for the treatment of acute graft-versus-host disease and aplastic anemia. Since any type of immunosuppressive treatment is known to carry the risk of developing B-cell lymphoproliferative disorders, we investigated the in vitro effect of ALG on human B-cell activation and proliferation. The data demonstrate that whatever the source of lymphocytes used for ALG preparation (thymocytes, thoracic duct lymphocytes, B- or T-cell lines), (1) ALG react with both B- and T-cell lines, and (2) ALG contain antibodies specific for B cells (eg, CD21) or common to T and B cells (eg anti-β2-microglobulin, anti-HLA-DR, CD18, CD11a) in addition to T-cell-specific antibodies. Unlike all other T-cell mitogens tested (Concanavalin A [Con A], Pokeweed mitogen [PWM]), ALG do not trigger B-cell differentiation into immunoglobulin-secreting cells at concentrations which induce maximum T-cell proliferation. This effect could be attributed to a direct interaction of ALG with B lymphocytes as shown by the capacity of ALG to block the response of purified B cells to a variety of activators. Furthermore, all the ALG tested were shown to inhibit the proliferation of six of the seven Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines and six of the seven Burkitt’s lymphoma cell lines studied. This selective B-cell antiproliferative property of ALG was not reproduced with CD11a, CD18, CD21, CD24, or anti-HLA-DR monoclonal antibodies (MoAbs). These results suggest that, although suppressing T-cell responses, ALG treatment may directly control B cell proliferation to some extent, in keeping with the relatively low risk of posttransplant lymphoproliferative disorders reported with ALG.

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MATERIAL AND METHODS

Antithymocyte globulins. Five batches of ALG that were obtained after immunization of horses with human thymocytes (ALG#7), thoracic duct lymphocytes (ALG#7), T lymphoblasts (ALG#5), and B-lymphoblastoid cell lines (ALG#11 and #12) were used in this study. Antituberculous toxoid horse γ-globulins (TTG) were used as control. Detailed characteristics of each batch have been reported in a previous publication.12

MoAbs. Five mouse MoAbs were purified from ascitis fluids by gel chromatography and stored at −20°C in phosphate-buffered saline after passage on 0.22-μm filters. BL2 (IgG2a, specific for a monoclonal determinant of HLA-DR), BL5 (IgG1, CD18),13 and BL13 (IgG1, CD21)14 were prepared by J. Brochier, and BE 104 (IgG, anti-human β2-microglobulin)15 was prepared by C. Vincent in our laboratory. IOT16a (IgG, CD11a) and Alb9 (IgG1, CD24)16 were obtained from Immunotech (Marseille, France). Purified MoAbs were biotinylated following the procedure of the manufacturer (BIF Biotechnics, Villeneuve la Garenne, France), and used for competitive immunofluorescence assays.

Cell preparation and culture. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by centrifugation of heparinized blood on a layer of Ficoll Hypaque (Lymphoprep; Nycomed, Oslo, Norway). Cells were washed three times in Hanks’ balanced salt solution before culture. Tonsil B-cell suspensions

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were prepared by two cycles of rosetting with aminoethyl isothiouronium bromide as previously described. The isolated population comprised greater than 98% CD19* or CD20* (B cells) and less than 1% CD2* (T cells) or CD14* (monocytes). Cells were resuspended in RPMI medium (Vietec, Saint Laurent de Mure, France) supplemented with 10% fetal calf serum (FCS; Seromed, ATGC, Noisy le Grand, France), 2 mmol/L L-glutamine, and antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL). PBMC were cultured at 1 × 10^6 cells/mL and B cells at 0.5 × 10^6 cells/mL, and were pulsed with 1 μCi/well of [methyl-^3]H]thymidine (H-TdR; CEA, Saclay, France) during the last 12 hours of a 3-day culture period. H-TdR uptake was measured using a Packard scintillation counter (Packard, Meriden, CT) by standard liquid scintillation counting after harvesting.

B- and T-cell mitogens. Culture of tonsil B cells in the presence of anti-CD40, presented on irradiated transfected L cells stably expressing FeR/CD432 molecule, and recombinant human IL-4 was performed as previously described. Insolubilized rabbit anti-human IgM was purchased from BioRad Laboratories (Richmond, CA) and was used at 1:400 for B-cell stimulation in association with 100 IU/mL of recombinant human interleukin-4 (rIL-4) (Schering-Plough, Dardilly, France). pokeweed mitogen (PWM), Concanavalin A (Con A), formalinized Cowan I strain Staphylococcus aureus (SAC), and phorbol myristate acetate (PMA) were obtained from Sigma Chemicals (St Louis, MO) and used, respectively, at: 1:300, 10 μg/mL, 1:200, and 10 ng/mL in culture. Several batches of Nocardia-delipidated cell mitogen (NDCM) were obtained from R. Barot (Orsay, France) and J. Brocher (Montpellier, France) and used at 100 or 200 μg/mL for PBMC cultures. OKT3 MoAb was purchased from Ortho Pharmaceutical (Raritan, NJ) and used at 100 ng/mL for mitogenic activity. The pair of MoAbs D66 and X11 was a generous gift of A. Bernard (Nice, France). Each ascis fluid was used at 1:400 for optimal mitogenic activity on human T cells.

Cell lines. The CD4* T-cell line Sub-T1, and the B-lymphoblastoid cell line Ri-BM established after infection with EBV of normal bone marrow cells were obtained from C. Desgranges (Iyon, France). The Estev lymphoblastoid cell line was a gift from J.M. Seigneurin (Grenoble, France). The 1970 and SUDHL-1 B lymphoblastoid cell lines, obtained via in vitro EBV transformation of normal peripheral blood B cells, as well as the Burkitt's lymphoma cell lines BL2, BL31, BL41, EBV-negative, and DAUDI, BL60, RAMOS, and RAJI, EBV-positive, were obtained from the International Agency for Research on Cancer (Iyon, France). The phenotype of BL2, BL31, BL41, BL60, DAUDI, and RAJI cell lines was described in a previous report. The IM9 cell line was obtained from ATCC, (Rockville, MD; #CCL159). Cell lines were maintained in RPMI medium supplemented with 10% FCS, 2 mmol/L L-glutamine, and antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL; and amphotericin, 0.25 μg/mL). For proliferation assay, cells were resuspended at 0.15 × 10^6 cells/mL in the presence of ALG or MoAbs, and were pulsed with [H-TdR (1 μCi/well)] during the last 20 hours of a 2-day culture period.

Determination of immunoglobulin secretion. Immunoglobulin secretion by cultured B cells was assessed with a sandwich-type enzyme-linked immunosorbent assay (ELISA) technique in microplates. Plates were coated with the immunoglobulin fraction of goat sera anti-human IgM (Zymed, Burlingame, CA), anti-human IgA, or anti-human IgG (390FT and 5081S Unipath, Bedford, UK). Binding of IgA, IgM, and IgG was detected using goat anti-human IgA (Southern Biotechnology Associates, Birmingham, AL) or goat anti-human IgM and IgG (B12419 and B12418, Biosys, Compiegne, France) antibodies labeled with horseradish peroxidase. Normal human serum was used as reference for calibration of the standard curve.

Detection of anti-IgM antibodies. Plates were coated with purified human IgM (Cappel, Organon Technica, Durham, NC). Fixation of anti-IgM antibodies present in polyclonal ALG was shown by using a goat anti-horse immunoglobulin antibody, coupled to horse-radish peroxidase (Biosys).

Immunofluorescence. The competitive immunofluorescence assay has been described in a previous report. Briefly, samples of 3 × 10^6 cells were washed two times with isotonic NaCl/Pi buffer containing 1% bovine serum albumin and 0.2% NaN3 (PBS/BSA/azide) between each incubation with the following reagents: 40 μL of ALG for 30 minutes at 4°C; 20 μL of biotinylated MoAb for 30 minutes at 4°C; then 10 μL of streptavidin phycoerythrin (Becton Dickinson, Mountain View, CA) for 30 minutes at 4°C to show binding of the MoAb. Finally, cells were fixed with 1% formaldehyde in PBS/BSA/azide. Analysis was performed on a FACSscan flow cytometer (Becton Dickinson).

For determination of the binding of ALG to B- and T-cell lines, cells (3 × 10^6) were incubated with increasing amounts of ALG (from 1 to 500 μg/mL), then with a fluorescein isothiocyanate-conjugated (FITC) goat antihorse immunoglobulin antibody (Biosys). Results were expressed as specific mean fluorescence intensities (MFI) after subtraction of fluorescence obtained in the absence of ALG.

RESULTS

Presence of B-cell antibodies in polyclonal ALG. Since ALG were prepared using different cell sources as antigen (eg, thymocytes, thoracic duct lymphocytes, B- or T-cell lines), we first determined the binding capacity of ALG to B- and T-cell lines, respectively. As shown in Fig 1, the fixation of antibodies, as evaluated by MFI, increased with the concentration of ALG from 1 to 500 μg/mL. Regardless of the source of cells used as immunogen, the five preparations of ALG were found to bind to the lymphoblastoid cell lines 1970, Ri-BM, and to Burkitt’s lymphoma cell lines RAJ1 and RAMOS. The binding was comparable to that observed with the T-cell line Sup-T1. Little or no binding was observed with control TTG. To assess the presence of antibodies directed against molecules expressed in high amounts on both B and T cells, such as CD11a, CD18, HLA-DR, and β2-microglobulin, or exclusively on B cells such as CD21, the receptor for EBV, or complement receptor type 2, we performed a competitive immunofluorescence assay. Cells were preincubated with increasing amounts of ALG and then the fixation of the labeled MoAb (IOT16a, BL5, BL13, BL2, or BE104) was assessed. A dose-dependent decrease of the MFI could be demonstrated with the five MoAbs (Fig 2). Specificity control with TTG showed weak or no inhibition. These results indicate that ALG contained antibodies directed against the same epitope recognized by each tested MoAb or a closely related epitope.

Finally, the presence of anti-human IgM antibodies assessed by an ELISA method could be demonstrated in only two ALG (#11 and #12) obtained with B-lymphoblastoid cell lines as immunogen, but not in the other ALG preparations.

Inhibition of B-cell differentiation into immunoglobulin-secreting cells by ALG. ALG preparations were tested for their ability to stimulate the proliferation of PBMC from normal donors, as measured by H-TdR incorporation after
3 days of culture. The five ALG batches under study, except ALG #5, induced a strong $^{3}H$-TdR incorporation in PBMC cultures (Fig 3). The proliferative response was dose-dependent, with a maximum at 250 or 500 µg/mL. Higher ALG concentrations (1,250 and 2,500 µg/mL) induced little or no $^{3}H$-TdR incorporation. Of note, ALG #5, which was not mitogenic, did not contain antibodies to CD2 or CD3 molecules, whereas antibodies of these specificities were demonstrated in the other ALG preparations.

T-cell mitogens such as the lectins PWM or Con A, and the CD3 or CD2 MoAbs, were reported to induce T-cell-dependent B-cell proliferation and terminal differentiation of B cells into immunoglobulin-secreting cells. We therefore examined whether ALG had the same property. B-cell differentiation was assessed by measurement of IgM, IgG, and IgA concentrations in 7-day culture supernatants. As shown in Table 1, PWM, Con A, OKT3, or the pair of CD2 MoAbs D66 and X11, all induced a marked increase of IgM, IgG, and IgA secretion above control levels. Conversely, none of the ALG preparations introduced at maximal mitogenic concentration induced any differentiation into immunoglobulin-secreting cells. This could be attributed either to a blockade of B-cell response, or to a lack of appropriate stimulation of T cells for induction of B-cell activation. To test these hypotheses, we added ALG to PBMC cultures stimulated by PWM mitogen and we observed an inhibition of IgM, IgG, and IgA synthesis (Table 2). We then performed the same experiment using NDCM, which can induce human B-cell proliferation and differentiation in the absence of T cells. ALG was found to completely inhibit immunoglobulin synthesis induced by NDCM. These results pointed to an inhibition of human B-cell differentiation by ALG, but they did not indicate whether ALG could block an early stage of activation and proliferation or only terminal differentiation.

*Inhibition of B-cell proliferation to anti-IgM plus rIL-4 by polyclonal ALG.* It is well established that highly purified human B cells can be stimulated with a combination of anti-IgM antibodies and a variety of T-cell-derived growth factors, including IL-4. We tested the effect of ALG on the proliferation of highly purified tonsil B-cell suspensions. Even at low concentration (10 µg/mL), ALG profoundly inhibited DNA synthesis stimulated with anti-IgM plus...
ANTI-B CELL PROLIFERATIVE PROPERTY OF ALG

Fig 3. Mitogenic effect of different batches of ALG on PBMC of a normal donor. 3H-TdR uptake was measured during the last 12 hours of a 3-day culture period. Results are triplicates from a single experiment representative of three others. Same symbols as Fig 1.

Table 2. Inhibition of E-Cell Differentiation by ALG

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Immunoglobulin Levels (ng/mL)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>None</td>
<td>127</td>
</tr>
<tr>
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<tr>
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<td>279</td>
</tr>
<tr>
<td>PWM + TTG</td>
<td>4,500</td>
</tr>
<tr>
<td>NDCM</td>
<td>23,000</td>
</tr>
<tr>
<td>NDCM + ALG 11</td>
<td>&lt;24</td>
</tr>
<tr>
<td>NDCM + ALG 12</td>
<td>152</td>
</tr>
<tr>
<td>NDCM + TTG</td>
<td>20,000</td>
</tr>
</tbody>
</table>

Immunoglobulin levels (ng/mL) were determined by an ELISA method, in 7-day culture supernatants. PWM and NDCM were used at 1:300 and 200 μg/mL, respectively. ALG and TTG were used at 500 μg/mL.

No inhibition was observed with control TTG at 10 or 100 μg/mL (Fig 4), but a nonspecific inhibition was obtained in the presence of TTG at 500 μg/mL (not shown). Finally, the proliferative response of highly purified B-cell suspensions to SAC plus rIL-2 was also inhibited by ALG at 10 μg/mL (data not shown).

Inhibition of B-cell proliferation to anti-CD40 plus rIL-4 by polyclonal ALG. It was recently described that IL-4 potentiates the proliferation of human resting B cells stimulated by anti-CD40 presented on irradiated transfected L cells, stably expressing FcγRII/CDw32 molecule. Addition of ALG #11 to this system resulted in a dose-dependent inhibition of B-cell proliferation (Fig 5A). Inhibition was also observed in the absence of IL-4 (Fig 5B). Inhibition was observed even at low ALG concentration (1 μg/mL). Control TTG at 1 to 100 μg/mL was not inhibitory.

Inhibition of the proliferation of B-cell lines by polyclonal ALG. To test whether the antiproliferative effect of ALG on activated human normal B cells could also be demonstrated on EBV-infected lymphoblastoid cell lines and on Burkitt's lymphoma cell lines, we measured 3H-TdR incorporation into seven B-lymphoblastoid cell lines and seven Burkitt's lymphoma cell lines. As depicted in Fig 6A and B, proliferation of B-cell lines was inhibited in a dose-dependent fashion by ALG #11. Note that the SUDHL-1 lymphoblastoid cell line and RAJI Burkitt's lymphoma cell line were found to be resistant. Results reported in Fig 7 demonstrated that proliferation of B-cell lines was inhibited not only by ALG #11 and #12 (antilymphoblastoid cell line), but also by other ALG (anti-T cells). However, ALG #11 and #12 were more potent inhibitors than other ALG. Control TTG had no effect on lymphoblastoid cell lines, but induced a moderate nonspecific inhibition of proliferation on RAMOS and BL31 cell lines at the highest concentration (500 μg/mL). The proliferation of the Sup-T1 T-cell line was either not modified or enhanced in presence of ALG #5, #7, or #9, and was marginally reduced in presence of ALG #11 and #12. Note that, at the end of the culture, the number of viable cells was lower in ALG-treated sensitive cell lines than in SUP-T1 and with TTG.
Fig 5. Effect of ALG #11 on B-cell proliferation induced by MoAb 89 (anti-CD40) alone (A) or in association with IL-4 (B). ALG #11 (1) or control lTG (0) were added at the beginning of the culture. \(^{3}H\)-TdR uptake was assessed during the last 20 hours of an 8-day culture period.

Whether this reflects a toxic or only antiproliferative effects is presently under investigation.

Effect of MoAbs on the proliferation of B-cell lines. MoAbs directed against HLA-DR (BL2), CD11a (IOT16a), CD18 (BL5), CD21 (BL13), and CD24 (Alb9) were added at concentrations ranging from 1 to 50 \(\mu\)g/mL. None had any effect on the proliferation of the B-lymphoblastoid cell lines Ri-BM and 1970, the T-cell line Sup-T1, and the Burkitt's lymphoma cell line RAJI. The proliferation of the two Burkitt's lymphoma cell lines RAMOS and BL31 was also unaffected by addition of these MoAbs, except in the presence of a high concentration of the CD11a MoAb IOT16a, which reduced the proliferation of RAMOS and BL31 cell lines by 17.6% and 33.6%, respectively. Proliferation of the six cell lines tested was also not affected by addition of CD21 and CD24 MoAbs in association (data not shown).

Fig 7. Effect of ALG on the proliferation of B- and T-cell lines. The B-lymphoblastoid cell lines Ri-BM and 1970, Burkitt's lymphoma cell lines RAMOS, BL31, and RAJI, and the CD4+ T-cell line Sup-T1 were cultured in the presence of ALG #5 (O), #7 (O), #9 (1), #11 (A), #12 (A), or control lTG (O). At 1, 10, 100, and 500 \(\mu\)g/mL, \(^{3}H\)-TdR uptake was assessed during the last 20 hours of a 48-hour culture period.

DISCUSSION

ALG have been prepared from different types of lymphocyte suspensions, such as human thymocytes, thoracic duct lymphocytes, and B- or T-lymphoblastoid or lymphoma cell lines. Furthermore, different procedures of absorption have been used to remove undesirable antibodies. One is, therefore, expecting a marked heterogeneity in the content of specific antibodies among various preparations of ALG. Such heterogeneity represents an important drawback in efforts toward standardization of ALG. Nevertheless, the five ALG preparations used in the present study were found to react strongly with B-cell lines, as well as T-cell lines. Of note, the two ALG that were prepared using human B-lymphoblastoid cell lines as immunogen did not react more strongly with B cells than with T cells. However, these two preparations were the only ones that contained anti-IgM antibodies. Staining of T and B cells can be attributed to the presence in ALG of antibodies directed against surface molecules expressed on both T and B cells such as HLA class I, CD11a/CD18, or CD45. However,
the ALG preparations were shown to also contain B-cell-specific antibodies (eg, CD21), as well as T-cell-specific antibodies (eg, CD2, CD3, CD4, and CD8). In confirmation of early reports, we found that ALG were potent in vitro T-cell activators. Among the five preparations tested, the only exception was ALG #5, which was reported in a previous study to lack demonstrable CD2 and CD3 specificities. Antibodies to these molecules are likely to account for the mitogenic activity of ALG. Most T-cell mitogens induce T-cell–dependent polyclonal B-cell activation with subsequent differentiation into immunoglobulin-producing cells. In the present report, we showed that ALG does not induce B-cell maturation in vitro, which was surprising since either the CD3 MoAb OKT3 or the pair of CD2 MoAbs D66 and X11 triggered strong immunoglobulin secretion in vitro in agreement with previous reports. The absence of B-cell response can be attributed to a direct effect of ALG on B cells, because fixation of ALG prevented the maturation of B cells into immunoglobulin-secreting cells in PBMC cultures stimulated either by T-dependent (PWM) or T-independent (NDCM) polyclonal B-cell activators. Furthermore, ALG completely inhibited the proliferation of highly purified B lymphocytes stimulated either by anti-IgM or anti-CD40 in association with recombinant IL-4, or SAC plus recombinant IL-2. This remarkable lack of B-cell activation and maturation was achieved at ALG concentrations that were strongly mitogenic for T cells.

In view of the data obtained with normal human B cells, we investigated the antiproliferative effect of ALG on B- and T-cell lines. We report that ALG preparations used in this study could inhibit the proliferation of lymphoblastoid cell lines obtained from normal human peripheral or bone marrow B cells infected with EBV in vitro, as well as EBV-positive and EBV-negative Burkitt’s lymphoma cell lines. With regard to the 14 cell lines tested, only one lymphoblastoid (SUDHL-1) and one Burkitt’s lymphoma cell line (RAJ1) were found to be resistant to ALG. The proliferation of the T-cell line Sup-T1 was not inhibited.

It is not possible at present to attribute the potent antiproliferative effect of ALG on B cells to a known antibody specificity. Indeed, as shown in the present study, only the CD11a MoAb IOT16a had a small inhibitory effect on the growth of RAMOS and BL31 cell lines. The other MoAbs tested were ineffective, including CD21 and CD24 MoAbs, either alone or in association. This finding, which is in agreement with a previous report by Blanche et al, should be discussed in view of the suggested therapeutic effect of these MoAbs in posttransplant lymphoproliferative disorders. It is possible that this activity involves in vivo antibody-dependent cell cytotoxicity by effector cells that are not present in our in vitro system. Several MoAbs directed against B-cell surface functional molecules have been reported to interfere with activation and proliferation of normal B cells, but not B-cell lines. Hence, a CD19 MoAb was reported to block human tonsil B cells in the late G1 or early G2 phase of the cell cycle. A CD23 MoAb was shown to inhibit B-cell activation induced by IL-4 in association with SAC or anti-IgM antibodies, but not in the presence of IL-2. A CD21 MoAb could inhibit B-cell proliferation induced by anti-IgM plus BCGF. CD45 MoAbs were shown to prevent B-cell activation induced by anti-IgM and T-cell cytokines or activation by CD40 MoAb plus PMA. Divergent results were obtained with CD24 antibodies. It is noteworthy that, whenever tested, these MoAbs had no demonstrable effect on lymphoblastoid B-cell lines. Therefore, the antiproliferative effect of ALG on B cells and B-cell lines reported here appears to be a unique property of ALG. Further studies are required to define the antibody specificities that may be involved in this effect.

The antiproliferative effect of ALG on B cells demonstrated here by using different in vitro models may be relevant to some in vivo properties of ALG. Hence, ALG treatment that involves repeated injections of large doses of heterologous γ-globulins was reported to induce only a moderate and often transient antibody response in the majority of renal allograft recipients. Blockade of B-cell responses, along with additional immunosuppressive chemotherapy, could have contributed to this relatively low antibody production. Furthermore, the low incidence of lymphoproliferative disorders reported in heart allograft recipients who received ALG as compared with the group treated with OKT3 may be related to the antiproliferative activity of ALG on EBV-transformed B-cell lines. Finally, from the results of an open prospective multicenter trial, Fischer et al suggested that administration of the anti-B-cell MoAbs CD21 and CD24 may be effective in controlling diffuse severe oligoclonal B-cell proliferation occurring after bone marrow or organ transplantation. Similar trials with ALG in organ transplantation could be considered in view of the present results, inasmuch as ALG, unlike anti-B-cell MoAbs, could avoid the risk of rejection linked to complete withdrawal of immunosuppressive therapy.

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Antiproliferative effect of antilymphocyte globulins on B cells and B-cell lines

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