

Comparison of the Cytotoxicity of 4 Preparations of Anti-T-cell Globulins in Various Hematological Malignancies

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Abstract. Objective: The cytotoxic effects of 4 ATG preparations (Thymoglobulin®, ATG-Fresenius®, Lymphoglobulin® and ATGAM®) in hematological malignancies were compared. Materials and Methods: Myeloma, myeloid leukaemia and lymphoma cell lines as well as primary CLL and T-cell samples were used. Cells were incubated at $1 \times 10^6/\text{mL}$ with 50-500 µg/mL of various ATG preparations with or without complement. Cell viability was analyzed by flow cytometry. Results: All ATG preparations had potent and similar cytotoxic activity against T-cells, primary CLL cells, NHL cell lines and myeloma cell lines. Resistance of U266 myeloma cells to ATG-induced apoptosis is shared by all 4 ATGs and can be overcome by addition of complement. Conclusion: All 4 ATGs have similar cytotoxic activity against hematological malignancies *in vitro*. If ATGs are used to target hematological malignancies, the choice of which ATG to use would depend on whether the required ATG concentration can be achieved *in vivo*.

Inclusion of antithymocyte globulins (ATG) in conditioning regimens is known to reduce the risk of acute and chronic graft-versus-host disease (1-4). The potent immunosuppression induced may lead to a delay in immune recovery and increase the risk of infections (5-8), however so far they have not been accompanied by the theoretically expected increase in relapse rates.

By virtue of their mode of production, ATGs contain antibodies targeting a wide range of antigens expressed on various normal and malignant hematopoietic cells including T-, B-, NK, dendritic and plasma cells. Over the last few years there has been growing evidence that ATGs have potent cytotoxic effects particularly on lymphatic, and to a lesser extent, on

myeloid malignancies (9-12). This may in part explain the relatively low relapse rates observed in patients receiving ATG as part of conditioning regimens for hematological malignancies.

The mechanism involved in the cytotoxic effects of ATG include complement-dependent cytolysis, cell-mediated antibody-dependent cytotoxicity, opsonization and subsequent phagocytosis by macrophages, activation-induced cell death as well as apoptosis (9-12).

ATGs contain purified IgG sera produced by immunization of animals with human cells. Currently there are four commercially available preparations of ATG (Table I). ATG-Fresenius® (Fresenius-Biotech GmbH, Gräfelfing, Germany) is produced by immunization of rabbits with the Jurkat human T-lymphoblastic cell line. Thymoglobulin® (Genzyme, Cambridge, USA) is produced by immunizing rabbits with human thymocytes, while ATGAM® (Pharmacia, Upjohn, USA) and Lymphoglobulin® (Genzyme) are produced by immunizing horses with human thymocytes. Of the four ATG preparations, Thymoglobulin is best investigated. The human thymus is known to contain T-cells, dendritic cells and stroma cells as well B- and plasma cells (9). Due to differences in manufacturing procedures, the different types of ATG contain variable specificities and amounts of antibodies (13-15), explaining the wide variability in doses used in the clinical setting. For Thymoglobulin, a total dose of 4.5 to 8 mg/kg BW has been recommended for matched unrelated donor allogeneic hematopoietic stem cell transplantation (5), while the recommended dose for ATG-Fresenius is about 10 times as high (16, 17). Consequently the concentration of ATG detected in patients' sera varies broadly and may be as high as 1,000 µg/mL in patients treated with ATG-Fresenius (17). It is very important to consider clinically achievable concentrations when interpreting *in vitro* data. Here, for the first time, is a comparison of the antitumor effects of 4 commercially available ATG preparations at clinically relevant concentrations.

Materials and Methods

Cell lines. RPMI-8226, EJM, OPM2, KMS-12-BM, Jurkat and Raji were purchased from DSMZ GmbH (Braunschweig, Germany), HL60, K562, BV173, JVM-2 and U266 (ATCC TIB 196) were obtained from ATCC (Manassas, USA). EJM cells were cultured in

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Table I. Types of ATG.

Trade name	Animal immunized	Cells used
Thymoglobulin®	Rabbit	Human thymocytes
ATG-Fresenius®	Rabbit	Jurkat cell line
Lymphoglobulin®	Horse	Human thymocytes
ATGAM®	Horse	Human thymocytes

IMDM (Gibco, Karlsruhe, Germany) supplemented with 20% fetal calf serum (FCS). U266 and KMS12-BM were kept in RPMI-1640 supplemented with 20% FCS. All other cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum, FCS (Gibco). All cells were cultured at 37°C and 5% CO₂ under humidified conditions.

CLL samples, primary T-cells and human serum. CLL cells were obtained from patients' (n=5) peripheral blood after informed consent. Mononuclear cells were separated by ficoll gradient centrifugation (StemCell Technologies inc., Vancouver, Canada) and then cryopreserved. None of the patients was under treatment at the time of sample collection. Cells were cultured in RPMI-1640 medium (Gibco, Karlsruhe, Germany) supplemented with 10% heat-inactivated FCS (Gibco). Primary T-cells were obtained from peripheral blood from healthy volunteers (n=5) by incubation of whole blood with RosetteSep for T-cell separation (Cellsystems, St. Katharinen, Germany) and ficoll gradient centrifugation. Human serum pooled from peripheral blood of 5 healthy donors and stored at -80°C served as source of complement. This is referred to as active human serum or complement.

Informed consent was obtained from all participants. Research data were coded such that subjects could not be identified directly or through linked identifiers. Data have been anonymized prior to publication.

Antibodies and reagents. Rabbit anti-human thymocyte globulin, ATG-Fresenius was obtained from Fresenius Biotech GmbH (Gräfelfing, Germany), Thymoglobulin and Lymphoglobulin were purchased from Genzyme (Cambridge, UK), Germany), annexin-V-PE and 7-amino-actinomycin D (7-AAD), were purchased from BD Pharmingen (San Diego, USA).

Viability assays. For complement-mediated cell lysis, cells were cultured at 1×10⁶/mL in 96 well plates in RPMI-1640 medium supplemented with 50% active human serum and various concentrations of the polyclonal antibodies as indicated in the results section. After incubation for 45 minutes at 37°C and 5% CO₂, 2×10⁵ cells were resuspended in 400 μL of medium. Then 4 μL 7-AAD were added and cells were incubated for 5 minutes at room temperature prior to detection of viable cells by flow cytometry.

For complement-independent cell death, cells were cultured at 1×10⁶/mL in 96-well plates in RPMI-1640 medium supplemented with 10% heat inactivated FCS. Antibodies were added as indicated in Results section. Cells were then incubated for 20 h at 37°C and 5% CO₂ and stained as above.

In each experiment, a negative control with medium was performed. Further controls included rabbit and horse IgG. The percentage cytotoxicity was calculated by the following formula: [(Test-Med)/(100-Med)]×100, whereby Test=% dead cells in test

(e.g. Thymoglobulin 50 μg/mL), Med=% dead cells in medium with or without complement (active serum).

Flow cytometry. After appropriate staining cell viability was detected by flow cytometry (BD FACScan, Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed using Cellquest software. A minimum of 10,000 events were analyzed per sample.

Results

All ATG preparations possess potent cytotoxicity against primary T-cells. Since T-cells have been the main target of ATGs, the cytotoxicity of various ATG preparations on primary T-cells was initially examined. As seen in Figure 1, all 4 ATG preparations had potent and similar complement-dependent cytotoxicity against T-cells.

All ATG preparations possess potent cytotoxicity against myeloma cell lines. ATG-F and Thymoglobulin have been reported to possess cytotoxic effects against myeloma cells. As seen in Table II, all 4 ATG preparations showed cytotoxic activity against various myeloma cell lines.

Resistance to ATG-induced apoptosis is shared by all 4 ATGs and can be overcome by addition of complement. One of the advantages of ATGs is that they can kill malignant cells by multiple mechanisms. Cell lines such as U266 are resistant to apoptosis induced by all ATGs but become sensitive to all ATG preparations upon addition of complement (Figure 2).

All ATG preparations possess potent cytotoxicity against lymphoma cell lines and primary CLL samples. All ATG preparations showed potent cytotoxicity against lymphoma cell lines (Table III) and patients' CLL samples (Figure 3). Addition of complement strongly enhanced the effects of all ATGs (Table III).

ATG preparations have differential cytotoxicities against myeloid cell lines. As seen in Table IV, the cytotoxic effects of various ATG preparations on myeloid cells varied depending on the cell lines tested. Only very limited apoptosis was observed, while addition of complement led to an increase in cytotoxicity in most cases.

Discussion

There is increasing evidence that ATGs may have potent effects against hematological malignancies. Especially in the case of myeloma, where the phenotype of the malignant cells may strongly vary even in the same patient depending on their maturation stage, monoclonal serotherapy has proven to be of only very limited success (18-20). There have been recent reports on the complement-dependent

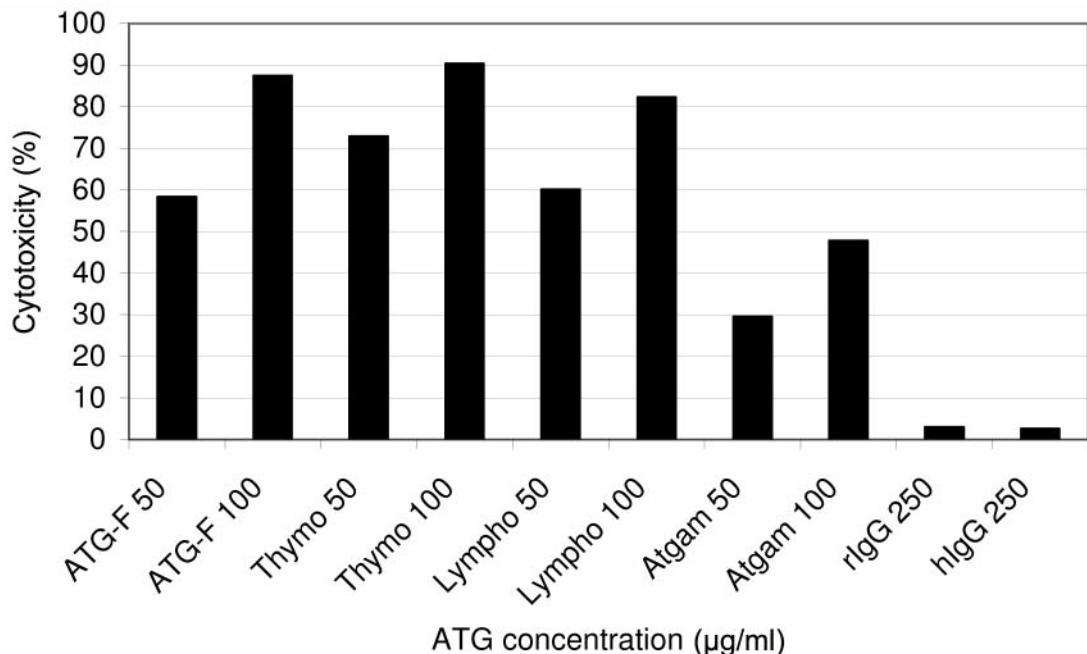


Figure 1. Complement-dependent cytotoxicity of ATGs in primary T-cells. Primary T-cells from peripheral blood of healthy donors ($n=5$) were incubated with various ATG preparations and 50% active serum. Results are presented as means from 5 independent experiments (1 per sample) each performed in duplicates.

Table II. Percentage cytotoxicity of various ATG preparations in myeloma cell lines with and without complement.

Concentration of ATG ($\mu\text{g/mL}$)	ATG-F 250	ATG-F 500	Thymo 250	Thymo 500	Lympho 250	Lympho 500	Atgam 250	Atgam 500	rIgG 250	rIgG 500	hIgG 250	hIgG 500
No complement												
OPM2	18.5	46	47	75.3	63.3	79	52.8	75.2	24	25.7	19.4	18.2
KMS12-BM	63.8	74.2	58.4	71.5	85.2	88.1	66.4	77.8	20.3	27.2	9.9	13
U266	5	7	2.8	9.8	5.5	8.5	3.1	4.1	1	2.9	3.7	4
EJM	2.8	10	13	23.2	13.8	19.5	4	4.9	5.5	6.2	3.3	3.3
RPMI-8226	37	61.1	41.3	52.6	50.6	57.1	26.9	36.9	31	44.1	25.7	36.5
With 50% complement												
OPM2	44.8	63.1	61.8	78.6	28.2	66.5	56.5	65.3	0.7	0.3	0	0.2
KMS12-BM	35.3	84.8	26	74.3	50.2	96	38.1	59.1	0.1	0.7	0	0.1
U266	64.5	83.6	64	79.9	42.6	76.2	30.4	62.2	1.3	1	1.3	0.3
EJM	35.3	54.2	42.8	63.6	27.7	38.3	12.3	38	0	0.6	1.1	0.5
RPMI-8226	52.7	82.6	77.2	89.4	37.5	75.1	29.3	60.1	2.1	0.3	0.2	0.1

cytotoxicity of ATG-Fresenius in myeloma cell lines and patient myeloma samples (10). Similar findings have also been reported for Thymoglobulin *in vitro* and in a xenograft model (11, 12).

In the present study, the cytotoxic effects of 4 commercially available ATG preparations on lymphoma cell lines, patients CLL samples, myeloid cell lines, myeloma cell lines and normal primary T-cells are compared. It must be taken into

consideration that the clinically achievable concentrations of ATG range from about 75 $\mu\text{g/mL}$ for Thymoglobulin (21) to up to 1,000 $\mu\text{g/mL}$ for ATG-Fresenius (17).

The cytotoxicities of all 4 ATGs on T-cells were comparable. This may appear to contradict the broad differences in doses of the different ATGs used in the clinical setting. However, as the mechanisms involved in ATG-mediated immunomodulation are better understood, it is becoming clear that T-cell depletion is

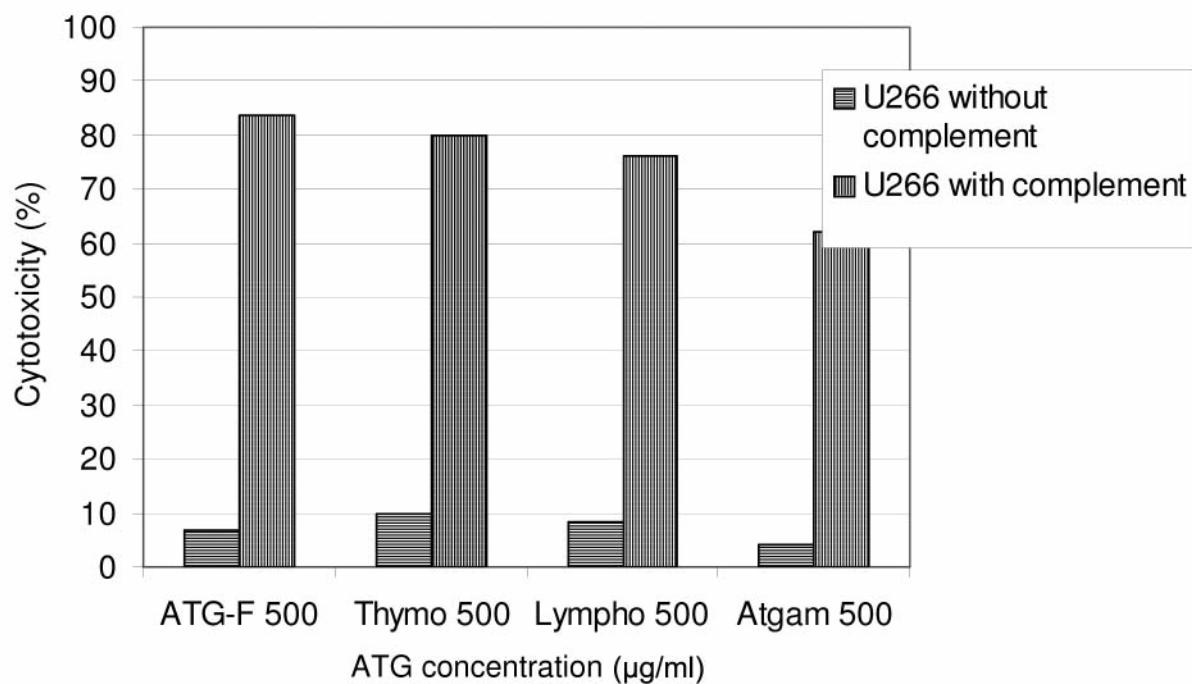


Figure 2. Complement-dependent cytotoxicity can overcome resistance to apoptosis. U266 cells were incubated with various ATG preparations with or without 50% active serum. Results are presented as means from 3 independent experiments (1 per sample) each performed in duplicates.

Table III. Percentage cytotoxicity of various ATG preparations in lymphoma cell lines with and without complement.

Concentration of ATG ($\mu\text{g/mL}$)	ATG-F 50	ATG-F 100	Thymo 50	Thymo 100	Lympho 50	Lympho 100	Atgam 50	Atgam 100	rIgG 250	hIgG 250
No complement										
Raji	15.5	30.5	24.1	34.1	25.1	31.1	16.1	49	2.8	6.6
JVM-2	0.7	2.1	1.3	5.6	8.7	16	4	14.9	10.6	5.5
Jurkat	12.3	39.8	30.9	47.9	26.2	61.1	24.7	42.1	4.1	2.5
With 50% complement										
Raji	78.3	85.9	85.1	90.6	86.1	86.3	48.2	64.6	4.3	4.9
JVM-2	5.2	19.7	10.4	25.5	13.4	20.2	28.6	41.1	0.5	8
Jurkat	56.7	90.0	20.8	55.6	57.8	89.9	36.5	68.3	3.5	3.7

Table IV. Percentage cytotoxicity of various ATG preparations in myeloid cell lines with and without complement.

Concentration of ATG ($\mu\text{g/mL}$)	ATG-F 250	ATG-F 500	Thymo 250	Thymo 500	Lympho 250	Lympho 500	Atgam 250	Atgam 500	rIgG 250	hIgG 250	hIgG 500
No complement											
K562	15.3	20.9	11.7	17.2	37.9	73.9	13.4	25.2	5.2	7.9	6.7
BV-173	10.5	12.3	17.7	20.7	21.3	20.1	14.1	18.2	8.7	8.6	8.1
HL60	16.6	34.2	14.3	37	19.8	60	8.5	20.3	6.7	14.6	4.1
With 50% complement											
K562	37.5	79.9	11.5	41.6	12.7	65.7	3.8	12.3	2.6	2.5	1.8
BV-173	11.8	9.6	52.7	57.2	33.3	46.7	16.9	25	3.7	3.6	12.3
HL60	96.6	97.0	97.0	97.6	96.7	96.5	64.3	88.7	0.4	1.3	7.5

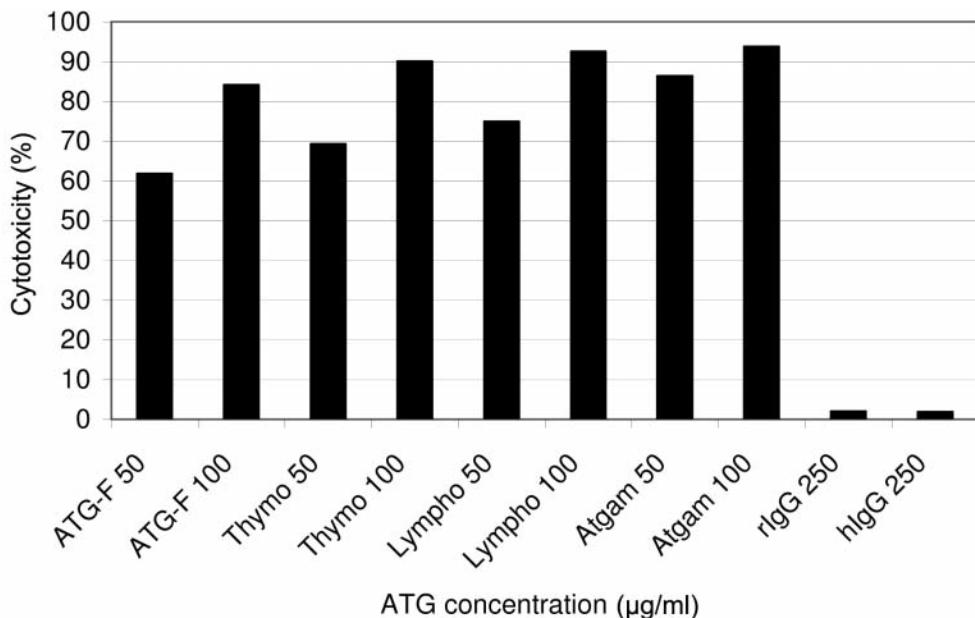


Figure 3. Complement-dependent cytotoxicity of ATGs in primary CLL cells. Primary CLL cells from patients' peripheral blood ($n=5$) were incubated with various ATG preparations and 50% active serum. Results are presented as means from 5 independent experiments (1 per sample) each performed in duplicates.

only part of the story. Other important immunoregulatory phenomena which are associated with ATG such as proliferation of regulatory T-cells and down-regulation of adhesion molecules needed for T-cell trafficking occur at low ATG concentrations of between 1 and 10 $\mu\text{g/mL}$ (22). Due to differences in their methods of preparation, the different ATGs may possess differential immunomodulatory potentials independent of their capacity to deplete T-cells.

In the case of malignant cells it may always be necessary to deplete them, thus requiring higher concentrations of ATG which are achievable with ATG-Fresenius at the doses used in allogeneic stem cell transplantation. The dose of Thymoglobulin used in allogeneic stem cell transplantation will be adequate to kill CLL cells by CDC and apoptosis but not myeloma or malignant myeloid cells. Whether other mechanisms such as ADCC will play a relevant role in ATG mediated killing of malignant cells is currently being investigated.

These findings indicate that all 4 commercially available preparations of ATG have similar cytotoxic activity against hematological malignancies *in vitro*. Based on this, the choice of which ATG to use would depend on whether the required ATG concentration can be achieved *in vivo*.

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