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Blood lymphocyte subsets in ATG-treated and allografted rats

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Abstract. A single dose of rabbit antithymocyte globulin (ATG) was given as the sole immunosuppressive therapy in a model of strong MHC barrier rat heart allotransplantation. PVG/c hearts transplanted to Wistar/Kyoto (WKy) rats resulted in long-term surviving (LTS) grafts and cell-mediated lympholysis (CML) unresponsiveness in 50% of the animals. The effects of ATG treatment on the peripheral blood lymphocyte subsets were studied by flow cytometry. The absolute T-lymphocyte levels decreased to less than 5% and were normalized after 2 weeks. CD8-positive cells were normalized within 1 week, whereas CD4- and CD5-positive cells remained low. Rats with LTS grafts had low levels of all T-lymphocyte markers, especially the CD4- and CD5-positive cells. Rats rejecting their grafts showed an eightfold increase in levels of CD8- and CD5-positive lymphocytes and a twofold increase in levels of CD4-expressing lymphocytes. It is concluded that ATG treatment causes the immediate elimination of large lymphoid populations as well as long-lasting immunomodulation detectable in peripheral blood.

Key words: Antithymocyte globulin, rabbit, in rat heart transplantation – Heart transplantation in the rat, lymphocyte subsets, ATG – Lymphocyte subsets, ATG, in rat heart transplantation

The clinical efficacy of rabbit antithymocyte globulin (ATG) in immunosuppression and antirejection therapy is well documented. The function of ATG in experimental transplantation is also well described [5–7, 9, 13, 17]. We have used our own rabbit anti-rat thymocyte preparations in studies of rat heart transplantation across a strong MHC barrier (PVG/c to WKy). A single dose of ATG given within 10 days prior to transplantation resulted in long-term surviving (LTS) grafts with an optimal LTS rate of 50% if ATG was given 2 days before transplantation [9]. The carriers of LTS grafts demonstrated profound systemic tolerance detectable by cell-mediated lympholysis (CML) unresponsiveness [16] and acceptance of second syngeneic grafts. Uniform immunosuppressive qualities were obtained for the various ATG batches, which were standardized by in vitro cytotoxicity.

Recent studies have demonstrated that transplantation tolerance can be achieved in adult animals by the combination of debulking, i.e., reduction of cell numbers through mechanisms like antibody-dependent cytotoxicity (ADCC) and opsonization, as well as by modulation of specific cell surface receptors [15] in the presence of antigen.

To further understand the mechanisms behind the potent immunosuppressive capacity of ATG in our model, we followed the changes in rat peripheral blood lymphocyte subpopulations after ATG treatment alone and in grafted rats with and without rejection. The cell populations were quantified in absolute numbers since the postulated mechanisms behind successful treatment require reduction in circulating cell numbers and not only relative shifts in population sizes.

Materials and methods

Animals and test protocol

Wistar/Kyoto (WKy) rats ($RT1^{u}$; Möllegaard Avelslaboratorium AS, Skensved, Denmark) were transplanted with PVG/c ($RT1^{c}$) hearts (Bantin and Kingman, Hull, UK) to the cervical vessels using a nonsuture technique [10]. A single injection of 1 ml ATG was given in a tail vein 2 days prior to transplantation. Blood samples were analyzed from three groups of rats. Those in group 1 were ungrafted WKy rats, examined prior to and 1, 7, and 14 days after ATG treatment. Those in group 2 were ATG-treated WKy rats that had rejected their grafts or showed signs of ongoing rejection when tested on day 38 after transplantation. All rats had rejected their grafts by day 40. Group 3 included ATG-treated, grafted rats with well-functioning grafts, examined on day 38 after transplantation.

ATG

ATG was prepared by immunization of rabbits with rat thymocytes [9]. The ATG was absorbed with rat erythrocytes and rat liver powder. Finally, the titer of cytotoxicity against thymocytes was adjusted to 1:1024 with phosphate-buffered saline (PBS).

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Blood samples

Blood (0.6 ml) was drawn from the jugular vein of rats anesthetized with chloral hydrate intraperitoneally into heparinized syringes.

Leukocyte counts

The erythrocytes in 10 µl of whole blood were lysed with 250 µl 160 mmol/l NH_4Cl and the total leukocyte count was determined. Lymphocyte, monocyte, and granulocyte counts were made using May-Grünewald-Giemsa stained smears of peripheral blood.

Monoclonal antibodies

Monoclonal antibodies (MAB) against five different rat lymphocyte surface antigens – OX6 (anti-MHC class II), OX8 (anti-CD 8, suppressor/cytotoxic T cells), W3/25 (anti-CD4, helper/inducer T cells), OX19 (anti-CD5, T cells and B-cell subpopulation) and W3/13 (anti-panT) – from Seralab, Sussex, UK, were used.

Staining procedure

Eighty µl of whole blood was mixed with 2 ml 160 mmol/l NH_4Cl in order to lyse the erythrocytes. The remaining cells were washed three times with PBS, incubated for 30 min at 4°C with 50 µl of the different MABs, and then washed three times with PBS. This was followed by incubation for 30 min at 4°C with 25 µl fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse antibodies (Becton Dickinson, Mountain View, Calif). Finally, they were washed twice with PBS.

Flow cytometry

Immediately after staining, the samples were analyzed by flow cytometry (Becton Dickinson FACStar 488 nm line, 250 MW, Filter set SL488). Data from 10 000 cells were acquired. Lymphocytes were gated according to forward and 90° light scatter, and the percentage of fluorescent cells was determined.

Statistical methods

The results are presented as mean ± standard deviation of the mean. The Mann-Whitney U-test was used to determine the significance of the difference between the groups.

Results

Group 1

The pretreatment leukocyte subset distribution in untreated rats is shown in Figs. 1 and 2. Prior to ATG treatment, the mean ratio between CD4- and CD8-positive cells was 1.96 ± 0.59, and 17% of the lymphocyte-like cells were not stained by the antibodies.

On day 1 after ATG treatment, there was a decrease of more than 90% in circulating lymphocytes and a decrease in total leukocytes, in spite of an increased granulocyte count. The decrease was most pronounced for CD4 cells (98.6%) and CD8 cells (97%), as compared to class II-expressing lymphocytes, which were reduced by 75% (Figs. 1, 2).

On days 7 and 14, the total leukocyte count was higher than before treatment, due to an increase in granulocytes and monocytes and a normalized lymphocyte level. The class II-expressing and the CD8 cell numbers were normalized or increased, whereas the CD4 cells remained low (Figs. 1, 2). The number of lymphocytes stained by the W3/13 antibody was normalized after 2 weeks, while lymphocytes expressing the CD5 antigen remained low.

Group 2

In ATG-treated rats that had rejected their grafts within 40 days, we observed on day 38 higher levels of CD8 cells and lower levels of CD4 cells than in the untreated controls (Fig. 3). The CD4: CD8 ratio was low. Class II-expressing lymphocytes and total leukocytes were higher than in the untreated controls. In two rats that rejected (i.e., no palpable heart beats) their grafts on day 40, these findings were seen on day 38.

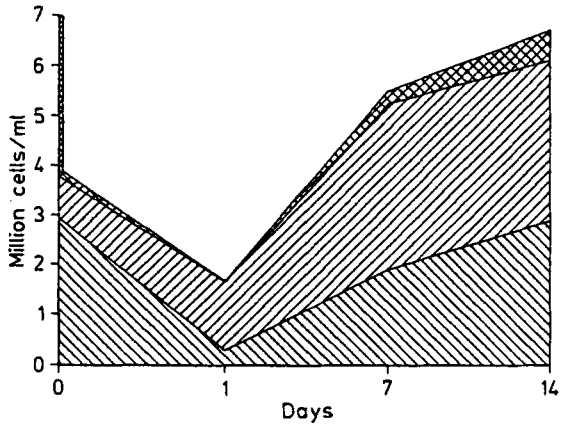

Fig. 1. Mean leukocyte subset levels in peripheral blood of six WKy rats before and at different time intervals after intravenous treatment with 1 ml ATG. ▨, Monocytes; ▨, granulocytes; ▨, lymphocytes

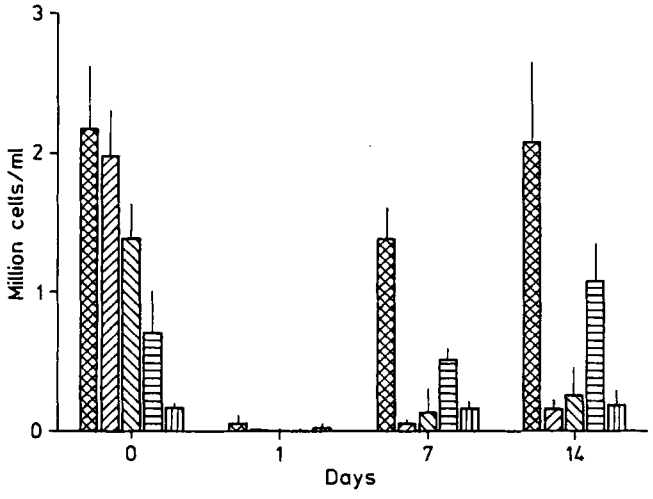

Fig. 2. Mean ± SD levels of four lymphocyte subsets and MHC class II expression on peripheral blood lymphocytes as measured with flow cytomerty with antigen-specific monoclonal antibodies. Analyses were made before and at different time intervals after IV treatment of six WKy rats with 1 ml ATG. ▨, W3/13; ▨, OX19; ▨, W3/25; ▨, OX8; ▨, OX6

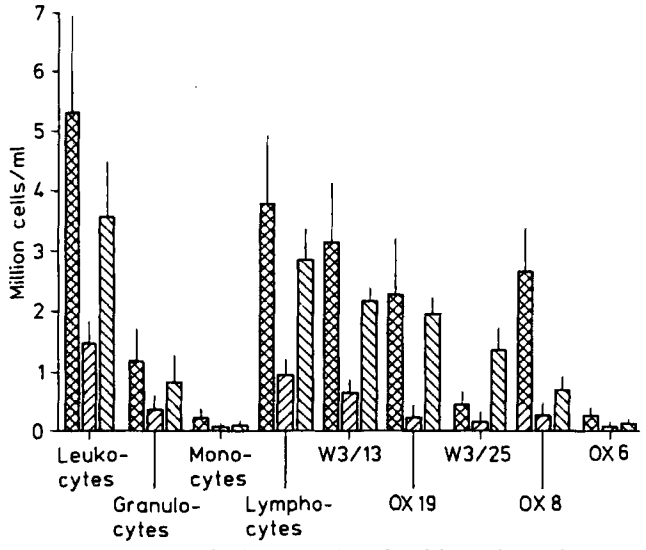

Fig. 3. Mean ± SD leukocyte subset level in peripheral blood of ATG-treated and PVG-grafted WKy rats at day 38 after transplantation and day 40 afterATG treatment. Group 1 (▨): rats with rejected grafts ($n = 7$); group 2 (▨): rats with long-term surviving (LTS) grafts ($n = 5$), and group 3 (▩): untreated, ungrafted control rats ($n = 6$)

Group 3

In rats with LTS grafts, tested on day 38, all leukocyte fractions were lower than in controls (Fig. 3). The CD4 cell number was only 16% of the controls, whereas the CD8 cells constituted 44%.

Pronounced differences were seen when groups 2 and 3 were compared. The CD8 and CD5 cell levels in group 2 were eight times higher than in group 3, whereas the CD4 cell level was two times higher.

Discussion

The above results show that our rabbit anti-rat ATG is efficient in vivo in reducing the number of circulating lymphocytes and monocytes, whereas no reduction was observed for granulocytes. Previous work has suggested that anti-T-cell antibodies may be particularly important in immunosuppression and the development of tolerance. Our data demonstrate that the ATG effect on T-cell subpopulations is not uniform. It is clear, for instance, that a more pronounced effect can be seen against CD4 cells than against CD8 cells.

After 2 weeks, the CD4-positive cells were still less than 20% of their pretreatment numbers. These results differ from those reported by Thompson et al. [14], who found that, following a single dose of antilymphocyte serum (ALS) given intraperitoneally, the total numbers of CD4 and CD8 cells decreased and recovered uniformly, and that the ratio between them was never altered. It seems that our ATG has a more pronounced effect on CD4 than on CD8-expressing cells compared to the ALS preparation. The depression of CD4 cells induced by ATG may be of great importance for the success of ATG as a pretreatment in allotransplantation.

Treatment with monoclonal anti-CD4 antibodies for 30 days after grafting PVG rats with PVG-RT1a hearts also induced indefinite graft survival [4]. Interestingly, Roser [12] recently showed that, of a series of four anti-CD4 antibodies, the one most potent in cell depletion was least effective in tolerance induction. The effects did not seem to relate to antibody isotype, affinity, or half-life. The IgG1 W3/25 antibody used to monitor CD4 in this study failed to induce tolerance or to clear CD4-expressing cells from the circulation, in spite of high affinity. It seems likely then that certain epitopes of the CD4 molecule are important for tolerance induction and that antibodies against these epitopes are present in our ATG.

Previous experiments involving challenges to tolerant rats using fresh grafts have suggested that tolerance is not due to graft adaptation but rather to an altered immune repertoire. Experiments with thymectomized animals have failed to induce tolerance after anti-CD4 treatment, and no return of new populations of depleted cells occurred [2, 12]. It has been suggested that depletion or inactivation of CD4 cells sparing the CD8 cells was essential for tolerogenic protocols [12]. This hypothesis fits well with our results, where ATG caused a preferential prolonged depression of CD4-expressing cells.

Whether or not it is necessary to reduce the number of available antidonor cytotoxic precursor cells at the time of transplantation may vary with, for example, the strain combination, possibly reflecting the degree of MHC disparity. It may be relevant to this discussion that cyclosporin A does not allow for LTS induction in our model, whereas it functions well with identical protocols in PVG to DA rats (M. Olausson, unpublished observation). The ATG is strongly cytotoxic in vitro, and the results of this study suggest a similarly potent effect in vivo. The mode of action of ATG is not known in detail, but it is likely that mechanisms like opsonization and removal by the reticuloendothelial system may be important as well.

The long-term modulatory effects on the T-cell subpopulations suggest that ATG may be lytic also for lymphoid precursors in the bone marrow. It seems likely that in our model there is a combined effect of initial debulking, dramatically reducing the number of preformed alloreactive cells, and immunomodulation, which may be responsible for the long-term effects. We have previously demonstrated the importance of the presence of antigen (graft) during this period [11].

Expression of the CD5 antigen, recognized by the MAB OX19, seems to be required for lymphoid proliferation and generation of cytotoxicity in response to alloantigens in vitro [8]. The OX19 antibody given as a single dose after grafting LEW rats with DA × LEW hearts prolonged graft survival [1]. We found that expression of the CD5 antigen was depressed after ATG treatment, apparently reflecting the CD4 rather than the CD8 levels. Before treatment, 91% of the T lymphocytes, recognized by the MAB W3/13, seemed to co-express the CD5 antigen. Two weeks after treatment, the T-lymphocyte level was

normalized, but less than 9% had detectable CD5 antigen. It has been proposed that the CD5 antigen is in much higher density on CD4- [8] than on CD8-expressing lymphocytes. Modulation of this antigen may play a role in the ATG-mediated immunosuppression allowing for tolerance induction.

The targets for the immunosuppressive activity of conventional rabbit anti-rat ALS are not known in detail [3]. In general, our results are consistent with data from different MAB protocol experiments. Since our polyclonal ATG has a potent immunosuppressive action and induces permanent tolerance, the present results can assist in defining antibodies, alone or in cocktails, for future therapeutic use.

Fifty percent of the rats treated with 1 ml ATG 2 days prior to transplantation rejected their grafts, and this occurred between day 25 and day 40. Among the rats that had rejected their grafts by day 40, and compared to untreated controls, there was an increase in all leukocyte subtypes, except granulocytes and CD4-positive lymphocytes, on day 38. On the other hand, compared to controls, rats accepting their grafts showed decreased levels of all leukocytes except monocytes on day 38. When comparing ATG-treated, grafted rats that became tolerant with those rejecting their grafts, the latter had an eightfold increase in level of CD8- and CD5-expressing cells, while the number of CD4 cells doubled. Since the CD8 cells are thought to be the main effector cells in graft rejection, the increased level is not unexpected. The CD5 expression is more surprising since cytotoxic cells have been said to lose CD5 expression during activation [8]. Further experiments with simultaneous two-color analysis will be required to study whether co-expression of CD8 and CD5 occurs during rejection after ATG treatment in our model.

LTS rats tested around 10 months after transplantation showed low lymphocyte levels similar to those of non-rejecting rats on day 38 (data not shown).

One may conclude that ATG treatment induces a strong turbulence in the leukocyte subpopulations. The most pronounced and long-lasting effects are reduced CD4- and CD5-expressing T-cell numbers. These changes in the repertoire may be of great importance for tolerance induction following ATG treatment prior to allotransplantation. Graft rejection causes a strong increase in peripheral blood CD8-expressing cells, possibly co-expressing the CD5 antigen. The distribution of peripheral blood leukocytes apparently reflects the functional status of the immune system, in terms of graft rejection or survival, immediately as well as late after transplantation.

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