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Negligible role for NK cells and macrophages in delayed xenograft rejection

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Abstract Hyperacute rejection (HAR) of a discordant xenograft can be avoided by complement manipulation, but delayed xenograft rejection (DXR) still leads to graft loss. It is generally assumed that macrophages and NK cells play key roles in DXR. In the present study the survival times and cellular infiltrate following guinea pig to rat heart transplantation was analyzed in the course of DXR, following aspecific and specific manipulation of macrophages and NK cells. HAR was overcome by a single injection of cobra venom factor 1 day before heart transplantation. To aspecifically reduce the inflammatory response dominating DXR, dexamethasone (DEXA) was given. Treatment with DEXA markedly reduced infiltration by NK cells, macrophages, and granulocytes. It also led to prolonged graft survival times (median survival of 0.4 days, $n = 10$, $P < 0.05$). In the second series of experiments the specific roles of NK cells and macrophages in DXR were further assessed. Monoclonal antibody 3.2.3 was used to selectively deplete NK cells. Liposome-encapsulated dichloromethylene biphosphonate was given to achieve macrophage depletion. Neither of these specific treatments, alone or combined, led to prolonged graft survival. Immunohistology revealed that at day 2 after transplantation no NK cells or macrophages were present in grafts from the combined treatment group. Only a mild infiltration of granulocytes was observed. Collectively, these results strongly suggest that NK cells and macrophages are not likely to be pivotal cell types in DXR.

Key words Xenotransplantation · Macrophages · NK cells · Guinea pig

Introduction

If a recipient of a discordant xenograft is treated appropriately to avoid hyperacute rejection (HAR), delayed xenograft rejection (DXR) occurs after a few days through as yet undefined mechanisms. In the guinea pig (GP) to rat combination, administration of cobra venom factor (CVF) will overcome HAR of GP hearts, which then will survive for up to 3 days [1]. DXR in this complement-depleted model is characterized by a prominent cellular infiltration and endothelial deposition of xenoreactive natural antibodies [2]. It has been shown that DXR involves a T cell-independent infiltration of macrophages and NK cells, in association with a marked expression of proinflammatory cytokines [3].

The aim of the present study was to investigate whether two different approaches to inhibit typical DXR-related infiltration, might prolong the survival of hearts in the GP to rat model. The aspecific approach consisted of treatment of recipients with dexamethasone (DEXA), a drug known for its capacity to inhibit inflammation in its broadest sense. In the more specific approach, NK cells and macrophages were selectively depleted by the following methods. Monoclonal antibody (mAb) 3.2.3 was used to deplete NK cells. This mAb recognizes a unique triggering structure present

on fresh and IL-2-activated NK cells [4]. Previous studies revealed that intraperitoneal treatment of rats with mAb 3.2.3 for 3 days can completely and selectively eliminate NK activity in the spleen and peripheral blood for at least 10 days [5]. To assess the role of macrophages, we eliminated macrophage function using liposome-encapsulated dichloromethylene biphosphonate (Cl_2MBP). On injection, these liposomes are rapidly phagocytosed by macrophages, leading to intracellular release of Cl_2MBP which eventually kills the cell. Cl_2MBP has been demonstrated to eliminate macrophages for about 7 days after intravenous injection [6, 7].

Materials and methods

Animals

Animals were obtained from Harlan-CPB (Austerlitz, The Netherlands). Male inbred Lewis rats were used as recipients and female Dunkin Hartley GPs as donors, GPs and rats weighed approximately 250 g when used. The experimental protocol was approved by the Committee on Animal Research of the Erasmus University.

Heart transplantation

GP hearts were transplanted heterotopically into rats according to the technique described by Ono and Lindsey [8]. Graft function was assessed by daily palpation. End of graft survival was defined as the day on which palpation indicated total loss of contractile activity, this was confirmed by inspection at laparotomy.

Complement depletion

To deplete recipients of complement CVF was used (*Naja naja kaouthia*, lot number 57362; ICN Pharmaceuticals, Zoetermeer, The Netherlands). CVF purification was performed according to Beukelman et al. [9]. A single dose of 0.1 ml CVF (with a complement-depleting activity of 30000 U/ml) dissolved in 0.9 ml PBS was administered i.v., 1 day prior to heart transplantation.

Dexamethasone

DEXA (Sigma, Zwijndrecht, The Netherlands) was reconstituted in PBS and injected i.m., daily at a dose rate of 0.5 mg/kg, starting 1 day before transplantation.

Graft-infiltrating cells

Immunohistological analysis of grafts undergoing DXR was performed at different intervals after transplantation, as described previously [10]. Immunoperoxidase staining on frozen sections was done using the following monoclonal antibodies: ED-1, W3/25, OX8, (Serotec, Oxford, UK), 3.2.3 (courtesy of Dr. Eggermont DDHK, Rotterdam, The Netherlands), and HIS48 (Pharmingen, San Diego, Calif., USA) to demonstrate monocytes/macrophages, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and granulocytes, respectively). The amount of infiltrating cells was scored semiquantitatively (from – to ++++).

Depletion of NK cells

Selective NK cell depletion was obtained by intraperitoneal administration of 0.1 ml purified mAb 3.2.3 (0.5 mg/ml) dissolved in 0.9 ml PBS on days –1, 0, and 1.

Depletion of macrophages

Liposome-encapsulated Cl_2MBP was used for selective depletion of macrophages. Liposomes were prepared as described previously [6]. Rats received 0.5 ml Cl_2MBP-liposomes suspension (5 mg Cl_2MBP/ml) i.v. on days –4 and –1.

Study design

Rats were divided into the following groups: group 1 ($n = 19$): no treatment, group 2 ($n = 19$): complement depletion by CVF, group 3 ($n = 10$): treatment with CVF and DEXA, group 4 ($n = 4$): complement depletion by CVF and NK cell depletion by 3.2.3, group 5 ($n = 5$): complement depletion by CVF and macrophage depletion by liposomes, and group 6 ($n = 9$): treatments as in groups 4 and 5 combined. Analysis of infiltrating cells was done on day 2 after transplantation on grafts from animals treated as in groups 2, 3, and 6, using 3 animals per group. Day 2 was chosen as the best representative for the presence of DXR (see Table 1).

Statistical analysis

Student's t-test was used to evaluate the differences (considered significant when $P < 0.05$) between group mean values. Survival times are given as median survival time (MdST) and range.

Results

Survival times

The survival times obtained in groups 1–6 are given in Table 1. Control GP grafts in group 1 had a MdST of 16 min (range 8–55 min). Treatment with CVF resulted in a MdST of 2 days (range 1–4 days). Addition of DEXA (group 3) not only led to a significantly prolonged MdST of 4 days (range 2–5 days) but also to an improved performance of the heart grafts. The hearts showed less edema and hemorrhage and kept on beating vigorously until 1 day before rejection. This compares favorably with hearts from group 2, which performed poorly from day 1 onwards, exhibiting extreme hemorrhagic edema. Depletion of NK cells (group 4) or macrophages (group 5) in recipients treated with CVF had no effect on graft survival time. The MdSTs were 2 days, similar to those following treatment with CVF alone. Also the combined treatment (group 6) did not lead to prolonged graft survival.

Table 1 Graft survival times in the guinea pig to Lewis rat combination. Group 3 vs group 2: $P < 0.05$. Monoclonal antibody 3.2.3 to deplete NK cells: 0.1 ml on days − 1, 0, and 1. Liposome-encapsulated dichloromethylene biphosphonate to deplete macrophages: 0.5 ml on days − 4 and − 1. (*MdST* Median survival time, *CVF* cobra venom factor, *DEXA* dexamethasone 0.5 mg/kg per day)

Group	Treatment	MdST	Range	*n*
1	None	16 min	8–55 min	19
2	CVF	2 days	1–4 days	19
3	CVF and DEXA	4 days	2–5 days	10
4	CVF and 3.2.3	2 days	2–3 days	4
5	CVF and liposomes	2 days	2–3 days	5
6	CVF and 3.2.3 plus liposomes	3 days	1–3 days	9

Table 2 Graft-infiltrating cells on day 2 following guinea pig to Lewis rat heart transplantation. For treatment, see Table 1. Infiltrating cells are given as mean scores, $n = 3$/group. (*T* $CD4^+$ and/or $CD8^+$ cells, *NK* 3.2.3-positive cells, *Mo/Ma* ED-1-positive cells, *Gran* HIS48-positive cells

Group	Treatment	T	NK	Mo/Ma	Gran
2	CVF	±	++++	+++	+++
3	CVF and DEXA	−	−	−	±
6	CVF and 3.2.3 and liposomes	−	−	−	+

Graft-infiltrating cells

The results are summarized in Table 2. In animals treated with CVF only, graft infiltrating cells mainly consisted of NK cells, macrophages, and granulocytes. Only a few T cells were demonstrable. Hearts from animals treated with CVF and DEXA contained no T cells, NK cells, and macrophages and only a few granulocytes. Hearts grafts from group 6, treated with 3.2.3 and macrophage-depleting liposomes, showed a similar picture, no T cells, NK cells, or macrophages but only a mild infiltration of granulocytes.

Discussion

It has been demonstrated repeatedly that when HAR of a discordant xenograft is blocked by preventing the activation of complement, grafts are still rejected after a few days [1, 2, 11]. The mechanism underlying this rejection process, called DXR, is still not clear. Immunohistological examination of grafts undergoing DXR has revealed that macrophages and NK cells are abundantly present, which suggests that these cells participate in the rejection process. Our present results confirm that grafts subjected to DXR are heavily infiltrated by macrophages and NK cells. From earlier studies we known that NK cells are already demonstrable from the day of transplantation onward, whereas macrophages start infiltrating from day 1 [12]. The assumption that the presence of macrophages and NK cells would imply their actual involvement in DXR sounds logical but is not supported by our current results. Firstly, when DEXA was given to CVF-treated recipients virtually no infiltrating cells could be demonstrated. Such grafts survived significantly longer than CVF-treated controls, but still were rejected within an additional 2 days. The major gain of DEXA treatment was that the hearts performed much better because edematous enlargement of the grafts was postponed. It is known that DEXA is capable of inhibiting various aspects of the inflammatory response, including production of IL-6 and TNFα [13, 14]. In earlier experiments we showed that DEXA had a profound inhibitory effect on the early TNFα response, which suggests that the early hemorrhagic deterioration of a graft during DXR may be mediated by this cytokine [11]. The second line of evidence that macrophages and NK cells are innocent bystanders rather than the actual executors of DXR comes from our results obtained with NK cell and macrophage depletion. Neither the removal of NK cells nor the depletion of macrophages had any effect. Also, when both treatments were combined, no effect on survival was observed. In addition, immunohistochemical analysis of grafts from the latter group demonstrated the efficacy of the treatment, since no NK cells and macrophages were found to be present. Several other investigators have tried to define in vivo the effector cells in DXR. Fryer et al. [15] used similar macrophage-depleting liposomes, as we did in the present experiments, and also failed to improve discordant xenograft survival in two different GP to rat models. From our studies we conclude that DXR in the rather harsh GP to rat model is not likely to be mediated by macrophages and NK cells.

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