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Intragraft events after heart transplantation: an experimental study comparing cytology in coronary sinus blood, peripheral blood, and daily histology

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Abstract Acute rejection is a frequent consequence after heart transplantation. To expand our knowledge of the rejection process and to investigate some intragraft events during acute rejection, the following experimental transplantation model was designed. Right cervical heart transplantation was performed in 12 mongrel dogs. Two experimental groups of six animals each received different immunosuppressive regimens. All animals were treated with daily triple drug therapy. In contrast to group 1, the animals in group 2 received high-dose steroids during rejection. The condition of the hearts was examined by daily transmural biopsies, graded according to the Billingham classification. To detect and quantify alterations in the mononuclear cell subsets of the myocardial venous return, blood samples from the coronary sinus blood (CS) and from peripheral blood (PB) were taken simultaneously with the biopsy. The total number of lymphoblasts and activated lymphocytes was determined and an activation index (AI) was calculated. The data referred to was established from 337 transmural biopsies. The AI of PB ($n = 287$) correlated well with the different stages of acute rejection (grade B0: AI = 2.2 ± 2.1; grade B1 + 2: AI = 6.3 ± 1.7; grade B3: AI = 10.0 ± 4.7; $P < 0.001$). The rejection kinetics of both groups, including the rejection-free interval following high-dose steroid administration in group 2, could be expressed accurately by the AI. The time course of the total number of lymphoblasts in CS versus PB demonstrated that the lymphoproliferative response started 4 days prior to the first intramyocardial signs of rejection ($\bar{x} = 3.8 \pm 0.7$; $n = 12$). The maximum number of lymphoblasts was seen on the day of rejection in group 1 and 1 day after the onset of histologically proven rejection in group 2 (group 1: $n = 6$: CS $\bar{x} = 40.1 \pm 7.5$; PB $\bar{x} = 12.2 \pm 4.1$; $P < 0.001$; group 2: $n = 6$: CS $\bar{x} = 39.4 \pm 8.8$; PB $\bar{x} = 12.9 \pm 3.7$; $P < 0.001$). Under rejection therapy in group 2 these cells decreased immediately, followed by a short rejection-free interval. In group 1 the total number of lymphoblasts diminished continuously, almost reaching the number in PB at the time of final rejection. In contrast, activated lymphocytes did not render adequate results. Comparison of daily histology and the data of PB proved there is a good correlation between the AI and the different histologic stages of acute rejection. The total number of lymphoblasts in CS during rejection is significantly higher than in PB. Acute rejection seems to be detectable almost 4 days before histology and PB cytology by cytologic evaluation of the CS. Therefore, we speculate that the differentation and proliferation of lymphoblasts during the initial phase of acute rejection takes place within the graft itself.

Key words Heart transplantation, dogs · Rejection, heart

Introduction

Heart transplantation represents the only means of treatment in cases of terminal heart failure that have proven to be refractory to medical or conventional surgical management. Despite recent developments in its detection and treatment, rejection remains an almost unavoidable complication encountered by graft recipients.

To investigate the immunologic situation during graft rejection, we quantified the alterations within the lymphocyte populations of coronary sinus blood in comparison to peripheral blood and daily histology. The quantitative analyses of different cell populations of the mononuclear concentrate out of the coronary sinus blood should enable us to receive accurate information about cellular flow at the beginning of graft rejection and to clarify still unknown aspects of its immunologic mechanisms and interrelations.

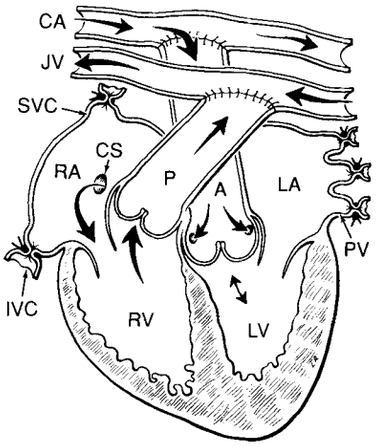

Fig. 1 Schematic diagram of the heterotopic transplant to indicate the specific blood flow. *A* Aorta; *P* pulmonary artery; *CA* common carotid artery; *JV* jugular vein; *SVC* superior vena cava; *IVC* inferior vena cava; *CS* coronary sinus; *LA* left atrium; *RA* right atrium; *LV* left ventricle; *RV* right ventricle; *PV* pulmonary veins

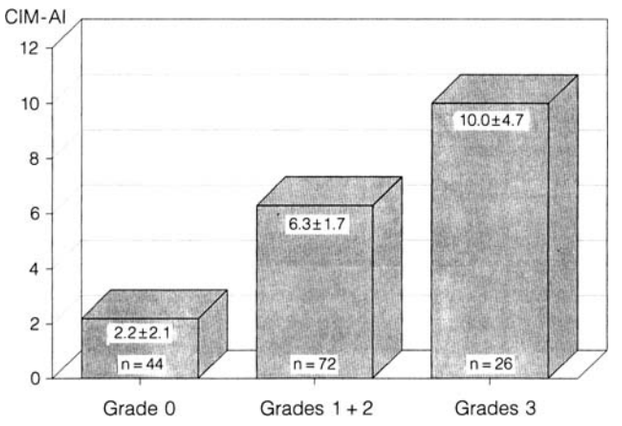

Fig. 2 Comparison of histological rejection grades and cytoimmunologic monitoring-activation index. Note that a distinction between grade 1 and 2 was not possible. $P < 0.001$

Materials and methods

All animals received humane care in compliance with the "Principles of Laboratory Animals" formulated by the National Society for Care and Use of Laboratory Animals, prepared by the National Academy of Science and published by the Institutes of Health (NIH Publication No. 80-23, revised 1978).

Right cervical heterotopic heart transplantation was performed in 12 mongrel dogs according to the procedure described by Mann et al. [11]. The caval veins and the pulmonary veins were ligated. No artificial shunts were created. Coronary circulation of the excised hearts was reestablished by anastomosing aorta and pulmonary artery of the donor hearts and-to-side to the recipients' common carotid artery and jugular vein, respectively. For this reason, it is evident that the only venous supply to the right atria is the blood from the coronary sinus (Fig. 1), provided both the tricuspid valve and the pulmonic valve are working sufficiently. Two experimental groups, each containing six animals, received identical basic immunosuppressive therapy consisting of triple drug therapy with methylprednisolone (0.2 mg/kg per day), azathioprine (1 mg/kg per day) and cyclosporin A. Cyclosporin A was administered approaching medium trough levels of 500–700 ng/ml. In contrast to the animals in group 1, the grafts in group 2 were additionally treated with pulsed steroids during acute rejection (125 mg methylprednisolone for 3 days). Hemodynamic function of the grafts was demonstrated by retrograde angiography and ventriculography.

Using two small skin incisions as access to the graft, two transmural punch biopsies were taken daily from the ventricles with the help of a prostate biopsy device (Biopty-cut TM Needle, Bard, Covington, Ga., USA). The myocardial tissue was fixed in formaldehyde, embedded in paraffin, stained with hematoxilin-eosin, and examined by light microscopy the same day, applying Billingham's criteria (state 1989) [3].

Cytoimmunologic monitoring (CIM) from peripheral blood (PB) and coronary sinus blood (CS) was performed daily, starting on the 2nd postoperative day and continuing until final rejection. CS could be obtained quickly and easily by aspiration of the right atria. The laboratory method has been described in detail previously [5, 7]. Heparinized blood (500 µl) was layered onto a ficoll-hypaque gradient (1.077 g/ml) and centrifuged. The mononuclear concentrate (MNC) thus obtained was washed twice and a smear was made by cytocentrifugation. After staining with May-Grünwald mixture, the lymphocytes were classified as either normal or activated. All slides were read by an observer with no prior knowledge of the grafts' condition. Some 300–500 lymphoid cells were counted. Various types of lymphoid cells were differentiated, depending upon the cell's size and cytoplasmic morphology. We focused our attention in particular on the number of activated lymphocytes and the number of lymphoblasts. Activated lymphocytes are larger than normal, resting lymphocytes, showing a wider basophilic cytoplasm border and relatively loose nuclear chromatin. Lymphoblast cells are markedly larger than activated lymphocytes, their cytoplasm is clearly basophilic, several nucleoli are detectable, and the chromatin structure is homogeneous. The ratio between the lymphoblasts and the total number of lymphoid cells in the MNC resulted in the so-called activation index (AI).

Continuous data are expressed as the mean ± standard deviation. All significance levels were determined using the Wilcoxon test. A P value under 0.05 was considered to be statistically significant.

Results

Retrograde ventriculography and coronary angiography ($n = 4$) showed sufficient hemodynamic function (Psyst.: $\bar{x} = 101.5 \pm 12.0$ mmHg. Penddiast.: $\bar{x} = 6.5 \pm 3.5$ mmHg) and a normal oxygen saturation ($\bar{x} = 88.5\% \pm 3.5\%$). Coronary perfusion was normal. The contrast medium ejected rapidly after 20 s ($\bar{x} = 19.7 \pm 3.5$), making a "pooling phenomenon" unlikely. Tricuspid and pulmonic valves did not show any signs of insufficiency.

The histologic data consisted of 337 transmural biopsies. A total of 287 blood samples of the PB and of the CS were obtained. CIM-AI correlated well with the corresponding pathohistological findings (grade B0: AI = 2.2 ± 2.1; grade B1 + 2: AI = 6.3 ± 1.7; grade B3: AI = 10.0 ± 4.7; $P < 0.001$; Fig. 2). Daily comparison of CIM-AI and histology demonstrated that the method reliably describes the rejection kinetics of both test groups, including the rejection-free interval following high-dose steroid application in group 2.

The time course of the total number of lymphoblasts in CS was compared to the total number in PB, referring to the 1st day of positive histology (Figs. 3, 4). Due to the higher number of lymphoblast cells in CS, both test groups suggest that the intragraft lymphoproliferative response starts 4 days before the first histologic signs of rejection ($\bar{x} = 3.8 \pm 0.7$; $n = 12$) and 2 days before peripheral activation becomes apparent.

The peak in the number of lymphoblasts in CS was seen on the day of acute rejection in group 2 and 1 day after the onset of histological rejection in group 1 (group 1: $n = 6$, CS $\bar{x} = 40.1 \pm 7.5$, PB $\bar{x} = 12.2 \pm 4.1$, $P < 0.001$; group 2: $n = 6$, CS $\bar{x} = 39.4 \pm 8.8$, PB $\bar{x} = 12.9 \pm 3.7$, $P < 0.001$). Simultaneously, the number of lymphoblasts in CS was 3.5 times that found in PB. The difference was mostly expressed 3 days before the diagnosis of histology rejection, when the number of lymphoblasts in CS was 15 times higher than in PB.

Rejection therapy (group 2) immediately reduced the number of lymphoblasts in CS (Fig. 4), reaching normal values after 5.7 days, on the average. During the following rejection-free interval of nearly 1 week, the lymphoblast population did not exceed the lowest level. In group 1 (Fig. 3), the number of lymphoblasts in CS diminished continuously after the mentioned peak, almost reaching the level of lymphoblasts in PB at the time of final graft rejection.

The other cell populations investigated did not seem to be involved in the rejection process in such large quantities. In any case, they did not render adequate results.

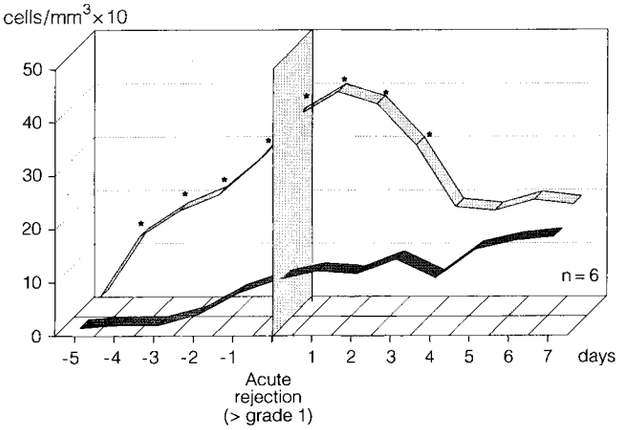

Fig. 3 Daily comparison of lymphoblasts from peripheral (■) and coronary sinus (□) blood in group 1 before and during acute rejection. Cell proliferation starts at least 4 days prior to onset of rejection and ends immediately after the histologic appearance of acute rejection, even though the immunoreaction persists, finally leading to graft failure. * $P < 0.001$ vs peripheral blood

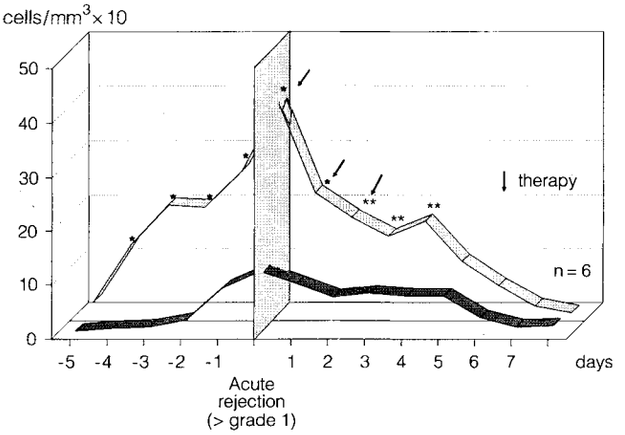

Fig. 4 Daily comparison of lymphoblasts from peripheral (■) and coronary sinus (□) blood in group 2 before and after treated rejection. Cell proliferation starts at least 4 days prior to onset of rejection. Under the influence of pulsed steroids, starting on the day of rejection, the blast cell proliferation seems to be blocked, leading to a rejection-free interval. * $P < 0.001$ / ** $P < 0.01$ vs peripheral blood

Discussion

As a consequence of the very large number of biopsies taken within a relatively short observation period, the usually need for four to six biopsy specimens in order to prevent sampling error was not feasible. The resulting iatrogenic lesions would have been considerable for graft function. The quantitative yield in cardiac tissue, however, is in accordance with that obtained from four to six endomyocardial specimens due to our transmural biopsy cylinders. Beyond that, we were able to obtain evidence

about the intramyocardial distribution pattern of rejection [14].

Cytologic evaluation of the lymphocyte populations in PB during graft rejection, described as CIM, has already been investigated by various transplantation centers [8, 10]. This test makes it possible to adequately detect acute rejection, particularly during the early postoperative course. Unfortunately, up until now, CIM has not been able to provide evidence about the actual grade of rejection. Treatment of acute rejection is, however, primarily instituted as soon as moderate or severe rejection is present. Thus, an ideal noninvasive test should be sensitive enough to differentiate various intensities of the rejection process.

The data obtained suggest that CIM-AI could be a suitable tool to discriminate different grades of acute rejection in the early post-transplant period. This would be particularly important since other noninvasive rejection tests lack this feature [1, 6]. Unfortunately, the distinction between Billingham grades 1 and 2 especially, which can be equated with the threshold of rejection therapy, was not feasible using the AI. Moreover, we have to consider that the meanwhile established, more dissimilar international grading for rejection is very demanding on the distinctness of CIM for rejection grading [4].

Up to now the time and place of the initial lymphocytic activation and proliferation, subject of the afferent limb of the rejection process after successful heart transplantation, is not clarified. A special feature of the heterotopic transplantation model is that there is no venous blood flow to the right atria, except from the blood obtained from the coronary sinus. This beneficial situation was used to measure the alterations in the mononuclear cell subsets of CS, in particular in correlation to PB and daily histology.

The results obtained from the CS indicate that the maturation and proliferation of the blast cells, as a correlate to the initial steps of cellular graft rejection, seem to take place within the graft itself, thus supporting the hypothesis that rejection of primarily vascularized organs is an intragraft event [13].

In a clinical study, the T-cell subsets in CS were tested in comparison to PB and endomyocardial biobsy (EMB) using monoclonal antibodies [9]. There was a four-fold increase in CD4 receptor positive cells on the days histologic rejection was diagnosed whereas the CD8 positive cells remained unchanged. Neither differentiation of the maturation stage of the cells involved nor an analysis of the CS cells in comparison to daily histology was possible.

Based on these observations, it seems that the afore mentioned significant increase in CS lymphoblasts compared to those in PB depends on the maturation and proliferation of CD4 positive cells. Their outstanding role in the initiation and precipitation of graft rejection has been described previously [2, 12].

In addition, it became apparent that the increase in the number of lymphoblasts in CS started almost 4 days before the first cellular infiltrates could be detected within the myocardial tissue. Therefore, we speculate that the cellular rejection process in fact starts some days before histologic examination becomes sensitive. Regarding the decrease in lymphoblasts after their peak in group 1, it seems conceivable that the marked proliferation is specific for the onset of rejection, whereas their involvement in the effector phase of graft rejection might be of secondary importance only.

In conclusion, there are some points in favor of the theory that the maturation and proliferation of CD4 positive cells during rejection is an intragraft event. The initiation of graft rejection seems to be earlier than the onset of histologic rejection has made us believe thus far.

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