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In vivo migration of lymphocytes in chronically rejecting rat kidney allografts

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Abstract Histological analyses have identified lymphocytes and macrophages as the predominant leukocyte populations that infiltrate organs undergoing chronic rejection. In order to define the time frame of this infiltration, we investigated the in vivo migration pattern of lymphocytes in a well-established rat model of chronic kidney allograft rejection. F344 kidneys were orthotopically transplanted into bilaterally nephrectomized Lewis rats. Recipients were treated with cyclosporin A (1.5 mg/kg/per day) for the first 10 days. After anti-CD18 or vehicle pretreatment, peripheral blood lymphocytes obtained from naive Lewis rats and labeled with 3H-uridine were injected into transplanted rats 12 and 16 weeks after transplantation. Organs were harvested 4, 8, and 12 h thereafter. After 12 weeks, proteinuria developed, accompanied by all signs of chronic rejection including glomerular sclerosis. Labeled lymphocytes rapidly infiltrated grafted kidneys 4 h after injection. Even more lymphocytes had accumulated in the grafts 12 h after injection. After 16 weeks, few lymphocytes had emigrated into the graft at 4 h, while infiltration was most pronounced by 12 h. Pretreatment with anti-CD18 inhibited the influx of lymphocytes. There was no difference between the patterns of lymphocytes derived from naive and transplanted rats. Our results emphasize the importance of endothelial cells in chronically rejecting kidneys for the control of leukocyte influx. β2-integrins may play a central role in determining the transendothelial migration during this process.

Key words Chronic rejection, lymphocytes · Lymphocytes, migration, chronic rejection

Introduction

Despite the increased short-term success of clinical transplantation during recent years, many renal allografts are lost due to chronic rejection. The declining number of long-functioning kidney grafts has remained unchanged despite improvements in tissue typing and immunosuppression [16, 25]. Chronic rejection is characterized by a gradual deterioration of graft function, as determined by proteinuria, hypertension, glomerulosclerosis, tubular atrophy, interstitial fibrosis, vascular obliterative changes, and an intense infiltration of mononuclear cells [7, 10, 19, 23]. However, the number of infiltrating cells is lower than during the early post-transplant period.

Leukocytes are known to cause glomerular damage; they can induce acute glomerular dysfunction and stimulate further inflammatory response [11]. Various investigators have studied migration patterns of leukocytes, taking both the speed of infiltration into vascularized organ grafts and the migration of immunocompetent cells throughout host tissues into consideration. However, these studies focused on acute rejection [13–15, 24]. The high number of lymphocytes detected immunohis-

tologically in grafts during chronic rejection raises the question of whether lymphocytes are attracted to the allografts due to specific alloantigen recognition, or whether they are retained indiscriminately due to independent events. It has also not been established whether increased influx, in situ proliferation, or a reduced efflux of these continually recirculating cells is responsible for the high number of lymphocytes and macrophages observed in the grafts.

The influx of leukocytes into sites of inflammation is regulated by a complex network of cytokines, chemokines, and adhesion molecules [3, 17, 23]. The β2-integrin family of leukocyte membrane glycoproteins (CD11/CD18) has been recognized as the principal surface molecules that regulate the adherence of lymphocytes and their subsequent transendothelial migration [1, 8, 9, 28]. These integrins seem to play a critical role in acute rejection since monoclonal antibodies against these molecules, such as antileukocyte function-associated antigen-1 (LFA-1), were effective in preventing acute rejection in rat cardiac allografts [20, 21].

This study was designed to examine the in vivo migration and distribution patterns of non-sensitized and sensitized peripheral blood lymphocytes during chronic rejection in rat kidney allografts. In addition, we investigated the kinetics of lymphocytes after anti-CD18 pretreatment.

Materials and methods

Animals

Naive male inbred rats weighing 200–250 g (Charles River, Germany) were used throughout the experiments. Lewis rats (LEW, RT^{l}) served as graft recipients, Fisher rats (F344, RT^{lv1}) as donors. Naive LEW rats served as controls. All animals were kept under standard conditions and fed rat chow and water ad libitum.

Kidney grafting

The left renal vessels of the recipient were isolated and clamped, and the native kidney was removed. The left donor kidney was removed, cooled, and positioned orthotopically in the host. Donor and recipient renal artery, vein, and ureter were then anastomized with 10–0 prolene sutures. Allograft recipients were treated with low-dose cyclosporin (1.5 mg/kg/per day) for the first 10 days after engraftment to suppress an initial acute rejection episode [5, 7]. The remaining native kidney was excised on day 10.

Lymphocyte isolation and labeling

Peripheral blood lymphocytes were chosen for the experiment as they circulate under physiological conditions. Lymphocytes obtained from naive male Lewis rats were used. Previous experiments demonstrated that the in vitro binding of peripheral or lymph node lymphocytes from grafted, naive, and third part animals to naive kidneys does not differ from the binding to chronically rejecting kidneys at any time during rejection [10]. To confirm this observation in vivo, additional studies were conducted with lymphocytes obtained from LEW recipients of a F344 graft 16 weeks after transplantation.

Lymphocytes were isolated by layering 1:10 diluted heparinized blood over a 61.8% Percoll (Seromed; density 1.124) / PBS gradient, centrifuging it at 1005 g for 20 min, and washing twice with PBS.

Cells were counted and resuspended (5×10^7/ml) in RMPI 1640 medium supplemented with 10% FCS. 5–^{3}H uridine (Amersham, specific activity 106 mCi/mg) was added to achieve a concentration of 5 μCi/ml, as described by Ford and Hunt [6]. After 1 h of incubation at 37 °C, cells were centrifuged, washed, and resuspended in PBS.

Migration studies

$2–3 \times 10^7$ 5–^{3}H Uridine-labeled lymphocytes, dissolved resuspended in 1 ml PBS, were injected slowly (1 min) into the left carotid artery in anesthetized, allografted, and naive animals after pretreatment with vehicle or anti-CD18 antibody (1 mg/kg). While planning the experiment, we reasoned that the antibody should be given shortly (5 min) before the transfer of labeled cells to assess the appropriate biodistribution of the antibody after intra-arterial injection.

Animals were sacrificed 4, 8, and 12 h later. The organs were removed, weighed, and cut into pieces (2–3 mm^3). Preliminary digestion was achieved by adding 1 ml of 2 M sodium hydroxide / 100 mg of tissue and shaking the mixture in a water bath (80 °C) for 35 min. After cooling, aliquots (0.5 ml) were transferred into new counting vials, bleached by adding H_2O_2 (0.3 ml), incubated overnight, and acidified by adding 0.3 ml of concentrated HCl. Fifteen milliliters of cold scintillant (Quickszint 1, Zinsser Analytic) was added to each sample, and triplicate samples were counted for 10 min in a 1217 Rackbeta liquid scintillation counter. A specimen of nonradioactive tissue was used as a background check. The radioactivity of each organ was expressed as follows:
% dose recovered/g = CPM/g of organ : total CPM/g of all organs.

Immunohistological analysis

Monoclonal Antibodies against T cells (CD5-OX19) and monocyte/macrophages (ED-1) were obtained from Serotec Camon Labor-Service (Germany); secondary (rabbit anti-mouse) and tertiary antibodies (mouse APAAP) came from DAKO (Denmark).

Kidney pieces were snap-frozen in liquid nitrogen and stored at −80 °C. Cryostat sections (4–6 μm) were stained individually with monoclonal antibodies from the above panel using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Stained cells were then counted with an ocular grid (400 ×, > 30 fields counted/section).

Experimental design

The present study was performed in three phases. The first phase involved ungrafted (naive) Lewis rats infused with labeled, nonsensitized, syngenic peripheral blood lymphocytes. Each rat received $2–3 \times 10^7$ 5-^{3}H uridine-labeled cells in 1 ml volume. Rats were sacrificed 4, 8, and 12 h after cell administration ($n = 6$/group/per time point) and the radioactivity of spleen, blood, kidney, liver, lungs, and heart was determined.

In the second phase, Lewis recipients of F344 renal allografts were infused with labeled cells as described above. Migration studies were performed 12 and 16 weeks after transplantation, during the most active phase of chronic rejection in this model. In each group, rats (n = 6/group/per time point) were sacrificed 4, 8, and 12 h after the injection of cells in order to determine radioactivity in the various organs.

To test whether lymphocyte function is altered during chronic rejection, the migration pattern of lymphocytes obtained from rats with chronically rejecting kidney allografts was also determined 8 and 12 h after lymphocyte transfer in transplanted rats at week 16, the peak of chronic rejection in this model.

In the third part of the study, we evaluated the effects of anti-CD18 antibodies on the migration of labeled, naive lymphocytes in transplanted and naive rats at 12 h.

Statistical analysis

All data are represented as mean ± SD. Statistical comparisons were made using a one-way analysis of variance, followed by the Newman-Keuls multiple comparison test. Student's t-test was used to determine the effect of CD18 pretreatment. Statistical significance is indicated when $P < 0.05$.

Results

Kinetics of labeled lymphocytes in naive rats

The distribution of labeled, syngeneic, peripheral blood lymphocytes injected into naive Lewis hosts is shown in Table 1. Most radioactivity was detected in the spleen; in comparison, radioactivity recovered from the other organs was small.

An early accumulation of labeled, nonsensitized lymphocytes in the spleen was noted at 4 h, followed by a continuous decline. Radioactivity in the blood remained constant. The accumulation of labeled cells in kidney, liver, and heart was small but increased over time.

Kinetics of nonsensitized, syngeneic lymphocytes during chronic allograft rejection

In this rat model, the signs of chronic rejection developed at about 12 weeks after transplantation, at which time proteinuria became apparent. Infiltration of leukocytes into the grafts peaked at 16 weeks, and kidney function declined in parallel to progressive renal scarring thereafter [7]. Almost all leukocytes within the grafts were CD-18 positive at both time points [7].

12 weeks after transplantation

As in naive rats, most radioactivity was detected in the spleen (Table 2). However, the accumulation of labeled lymphocytes in the spleen was lower than in naive controls after 4 h (16.1 % vs 20.6 %) and remained similar at 8 h (17.6 %). There was a noticeable drop (9 %) after 12 h.

In allografted kidneys, radioactivity was higher at 4 h than in controls ($P < 0.05$) and then in allografts at week 16 ($P < 0.05$; Fig. 1). It decreased slightly after 8 h, and was most pronounced after 12 h (Fig. 1).

Radioactivity detected in the other organs was low with only minor changes.

16 weeks after transplantation

Though starting at a similar level as in controls (19.7 % vs 20.6 %), the radioactivity in the spleen decreased progressively to 9 % at 12 h (Table 3). In contrast, the radioactivity in the allografted kidneys increased over time, reaching the highest level at 12 h (Fig. 1).

Radioactivity in the other organs was moderate, with minimal changes during the period examined. After 8 h, the migration pattern of lymphocytes obtained from transplanted animals did not differ from that of naive lymphocytes in any organ. After 12 h, splenic activity was even lower (5.2 %) when lymphocytes from transplanted animals were used. Moreover, the intragraft accumulation of lymphocytes derived from transplanted animals was slightly lower than that of naive lymphocytes (Fig. 2).

Kinetics of nonsensitized syngeneic lymphocytes in naive rats and during chronic allograft rejection following anti-CD18 pretreatment

In all kidneys, radioactivity peaked at 12 h. While anti-CD18 pretreatment had no effect in naive rats (Table 1), anti-CD18 pretreatment of allograft kidneys resulted in significantly decreased radioactivity in the spleen (Tables 2, 3; Fig. 1).

Immunohistology

Immunohistological analysis of kidneys confirmed the patterns of lymphocyte infiltration as determined by radioactivity. Throughout the experiment, a significantly larger number of lymphocytes was present in allografts than in naive controls. Anti-CD18 pretreatment resulted in decreased lymphocyte infiltration.

ED-1 + macrophages were detected in small numbers in naive controls (Fig. 3). Their number was significantly increased in allografted kidneys. Interestingly, anti-CD18 pretreatment did not only reduce the number of lymphocytes, but also the number of macrophages found in chronically rejecting kidney allografts.

Discussion

In chronic rejection, lymphocytes and macrophages are the predominant cell populations in clinical and experimental settings [2, 10, 25, 26]. The current study pro-

Table 1 Homing patterns of 5-^{3}H uridine-labeled, nonsensitized, syngeneic peripheral blood lymphocytes injected into naive Lewis rats (% dose recovered/g)

Tissue	4 Hours	8 Hours	12 Hours	12 Hours following anti-CD18 pretreatment
Spleen	20.577 ± 1.474	17.774 ± 4.708*	14.982 ± 1.635*	15.370 ± 3.984
Blood	0.408 ± 0.044	0.376 ± 0.084	0.456 ± 0.018	0.393 ± 0.048
Kidney	0.449 ± 0.087	0.717 ± 0.385	0.753 ± 0.077*	0.706 ± 0.354
Liver	0.675 ± 0.059	0.846 ± 0.155*	0.836 ± 0.037	0.879 ± 0.138
Lung	2.231 ± 0.459	2.010 ± 0.556	1.754 ± 0.097	2.002 ± 0.465
Heart	0.329 ± 0.085	0.389 ± 0.155	0.562 ± 0.04*	0.367 ± 0.032

* $P < 0.05$ vs 4 h

Table 2 Homing patterns of 5-^{3}H uridine-labeled, nonsensitized, syngeneic peripheral blood lymphocytes injected into allografted Lewis rats 12 weeks after transplantation (% dose recovered/g)

Tissue	4 Hours	8 Hours	12 Hours	12 Hours following anti-CD18 pretreatment
Spleen	16.114 ± 4.881	17.580 ± 4.052	8.792 ± 1.984*,**	16.074 ± 0.171***
Blood	0.547 ± 0.162	0.329 ± 0.047	0.591 ± 0.063	0.464 ± 0.024
Kidney	0.686 ± 0.137	0.550 ± 0.07	1.010 ± 0.124*,**	0.664 ± 0.072***
Liver	0.660 ± 0.07	0.895 ± 0.225	0.869 ± 0.033	0.757 ± 0.045
Lung	1.716 ± 0.36	1.500 ± 0.201	1.506 ± 0.117	1.772 ± 0.085
Heart	0.409 ± 0.129	0.398 ± 0.175	0.587 ± 0.091	0.407 ± 0.098

* $P < 0.05$ vs 4 h; ** $P < 0.05$ vs 8 h; *** $P < 0.05$ vs 12 h

Table 3 Homing patterns of 5-^{3}H uridine-labeled, nonsensitized, syngeneic peripheral blood lymphocytes injected into allografted Lewis rats 16 weeks after transplantation (% dose recovered/g)

Tissue	4 Hours	8 Hours	12 Hours	12 Hours following anti-CD18 pretreatment
Spleen	19.658 ± 3.934	12.185 ± 4.713	8.549 ± 0.194*	12.955 ± 0.571***
Blood	0.401 ± 0.043	0.433 ± 0.095	0.315 ± 0.019	0.659 ± 0.18
Kidney	0.438 ± 0.057	0.868 ± 0.357*	1.613 ± 0.176*,**	0.767 ± 0.118***
Liver	0.478 ± 0.06	0.917 ± 0.283*	1.172 ± 0.103*	0.746 ± 0.09***
Lung	1.348 ± 0.238	1.686 ± 0.2	1.256 ± 0.151	1.676 ± 0.556
Heart	0.279 ± 0.04	0.715 ± 0.382	0.708 ± 0.118	0.467 ± 0.652

* $P < 0.05$ vs 4 h; ** $P < 0.05$ vs 8 h; *** $P < 0.05$ vs 12 h

vides several important insights into the mechanisms underlying the in vivo migration of lymphocytes into transplanted kidneys. The time pattern of lymphocyte migration into the kidney differed between naive and grafted animals. In addition, the radioactivity in renal allografts differed between 12 and 16 weeks after transplantation. Twelve weeks after transplantation, the number of lymphocytes that had accumulated in the grafts 4 h after injection was higher than in any other group. By contrast, 16 weeks after transplantation, the increase in graft radioactivity was slow but had reached higher levels than in any other group after 12 h. Thus, at week 12, the influx of lymphocytes into the grafts was fast but accompanied by a strong efflux. At week 16, on the other hand, the influx was slow, but the efflux was markedly reduced. Corroborating these results, immunohistological analysis of chronically rejecting kidneys revealed the peak of lymphocyte infiltration at 16 weeks [10].

The importance of week 12 for the induction and development of chronic rejection in this model has been highlighted by in vitro lymphocyte binding studies in kidney allografts; lymphocyte binding peaked during the course of chronic rejection at week 12, while lymphocyte infiltration peaked at week 16, a time of reduced binding [1, 10]. The binding of lymphocytes directly correlated to the expression of ICAM-1 (CD54) [26]. One possible reason for the accumulation of radioactivity in the grafts is the destruction of lymphocytes. Yet, this seems unlikely, as antibodies against CD18 effectively reduced their infiltration into the kidneys.

In all groups, labeled peripheral blood lymphocytes migrated rapidly from the bloodstream into the spleen, followed by their continuous emigration from

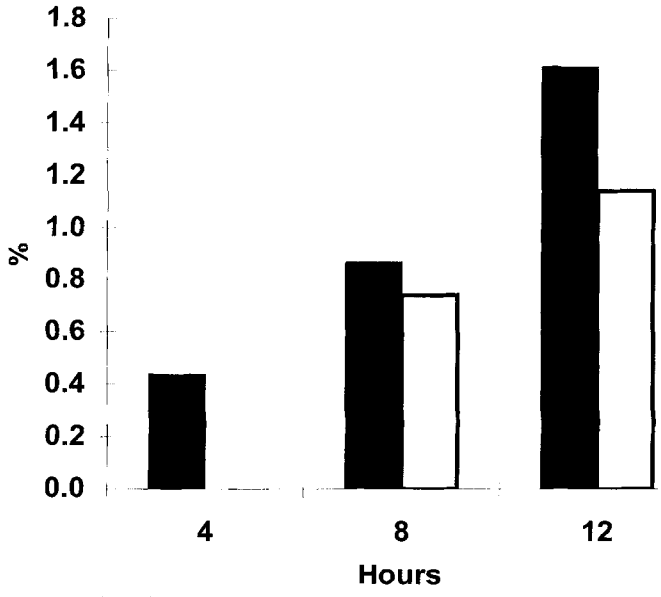

Fig. 1 Radioactivity in the grafts 16 weeks after transplantation 4, 8, and 12 h after injection of lymphocytes isolated from naive (■) and transplanted (□) animals (*CPM* counts per minute; % CPM/g of organ : total CPM/g of all organs)

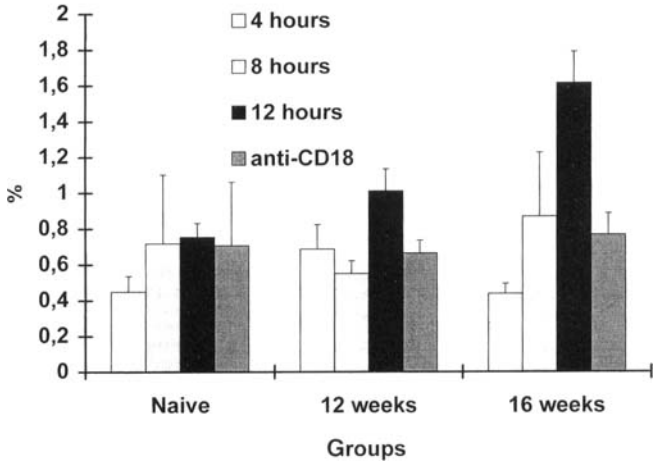

Fig. 2 Radioactivity in naive kidneys and grafts undergoing chronic rejection 4, 8, and 12 h after injection. The effect of anti-CD18 was determined 12 h following the administration of lymphocytes (*CPM* counts per minute; % CPM/g of organ : total CPM/g of all organs)

this organ. It can be assumed that lymphocytes primarily migrated from the spleen to other lymphoid organs. This hypothesis is supported by in vivo migration studies performed in naive animals. Oluwole et al. observed a decreased splenic radioactivity (from 50.6% 3 h after injection to 32.1% 18 h after injection) in parallel to an increased activity in mesenteric lymph nodes (from 12.5% to 35.2% at 3 and 18 h)

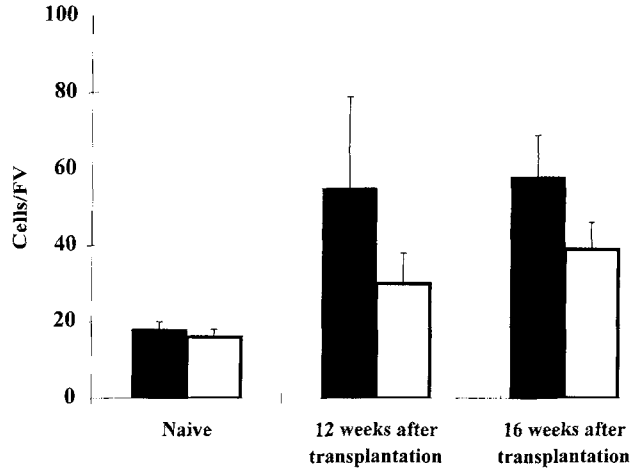

Fig. 3 Infiltration of macrophages/monocytes into naive and grafted kidneys, as determined immunohistologically, 12 h after the injection of vehicle or anti-CD18 (*cells/FV* cells per field of view at 400 × magnification) . ■ Vehicle, □ anti-CD18

[18]. While lymphocyte emigration from the spleen was slow in naive animals, it was rapid in animals undergoing chronic rejection.

Our results indicate that the migration pattern of lymphocytes from transplanted animals undergoing chronic rejection does not differ from that of naive animals. These in vivo results confirm the data from in vitro experiments in which the binding of lymphocytes from different sources to allografts undergoing chronic rejection did not differ [10]. It may indicate that during chronic rejection only a minor proportion of lymphocytes are activated. More likely, changes in the graft – probably on the endothelium – induce the increased lymphocyte extravasation into the grafted organ during this process.

ICAM-1 and its ligand, the β2-integrin LFA-1 (CD11a/CD18), are known to be involved in acute rejection. Monoclonal antibodies against ICAM-1 increased graft survival in monkeys [4] and in mice [29]. However, the absence of ICAM-1 expression in allografts or recipients was insufficient to prolong cardiac allograft survival in ICAM-1 knock-out mice [22]. On the other hand, anti-LFA-1 reduced the number of infiltrating lymphocytes and macrophages, and was effective in preventing acute rejection in rat cardiac allografts [20, 21]. Our results suggest that β2-integrins are critical for lymphocyte infiltration during chronic rejection. In our experiment, anti-CD18 pretreatment reduced lymphocyte infiltration in allografts to the level observed in naive controls and did not affect lymphocyte infiltration in naive animals. The failure of anti-CD18 pretreatment to reduce lymphocyte infiltration in naive animals suggests that, under normal conditions, lymphocyte influx into the kidney is primarily CD18-independent.

This is supported by Van Epps et al., who also failed to note an effect of anti-CD18 antibodies on the random migration of lymphocytes. However, anti-CD18 antibodies were effective in inhibiting transendothelial migration of T cells induced by chemotactic factors (IL-2, LCF) [27]. In contrast, Kavanaugh at al. observed that LFA-1-deficient T-cell clones showed less transendothelial migration, regardless of the activation status of the endothelium [12]. Thus, we have to assume that lymphocytes become activated when interacting with endothelial cells in chronically rejecting kidneys. This results in an increased avidity of β2-integrins on leukocytes, and may alter their transendothelial migration.

Neutrophils, monocytes, and NK cells also express β2-integrins. Thus, it was not surprising that anti-CD18 pretreatment resulted in decreased infiltration of these monocytes macrophages in chronically rejecting kidney allografts. However, the degree of this reduction suggests a dynamic influx of monocytes into chronically rejecting allografts.

Whether or not long-term treatment with CD18 can inhibit the progression of chronic rejection remains unclear. We could only use mouse antibodies, thus we can safely assume that, as the production of anti-mouse antibodies follows prolonged treatment, a modified antibody would be necessary to answer this question.

In conclusion, our results emphasize the importance of endothelial cells in chronically rejecting kidneys for the control of leukocyte influx. β2-integrins may play a central role in determining the transendothelial migration during this process.

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