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The frequency of B cells secreting antibodies against donor MHC antigens in rats rejecting renal allografts

S. Tiisala[1], D. Leszczynski[1], J. Halttunen[1], A. Nemlander[1], T. Paavonen[1], R. Renkonen[2], and P. Häyry[1]

[1] Transplantation Laboratory and [2] Department of Bacteriology and Immunology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland

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Abstract. We have estimated the frequency of B cells secreting antibodies against donor MHC antigens in rats rejecting histoincompatible renal allografts. In a major plus minor antigen-incompatible DA-to-WF combination on day 4 post-transplantation, reverse protein A plaque assay demonstrated that in the graft the frequency of lymphoid cells secreting Ig was 1:850. A major locus-incompatible and minor locus-compatible, congeneic LBN-to-Lewis strain combination was then applied to estimate the specificity of the secreted antibody. The lymphoid inflammatory cells were fused with mouse myeloma cells, cultured under limiting dilution conditions, and assayed by ELISA to donor and irrelevant strain spleen cells. Among cells infiltrating the graft, the fusion frequency was $1:172 \times 10^3$ and the frequency of Ig-producing hybrids $1:400 \times 10^3$ (i.e., this assay was approximately three log orders less sensitive than the reverse pA assay). The frequency of hybridomas secreting specific antibodies against donor MHC antigens was $1:720 \times 10^3$ (i.e., every second hybridoma deriving from inflammatory population produced specific Ig). In addition, there was at least one obviously polyspecific population of hybridomas, detectable only in the spleen and reactive with all rat strains tested with a frequency of $1:700 \times 10^3$. The inflammatory cells were also cultured directly under limiting dilution conditions, and the frequency of Ig-secreting cells was determined by ELISA. The frequency of inflammatory lymphocytes secreting detectable amounts of immunoglobulin in the supernatant was $1:14 \times 10^3$ in the graft (i.e., this assay was approximately one log order less sensitive than the reverse protein A plaque assay). Donor-strain reactive cells were not detected in the spleen or blood of nontransplanted Lewis (recipient) rats, nor were they detected in the blood of the transplant recipient. Our results show that B cells secreting antibodies against graft donor MHC antigens are present in the graft-infiltrating cells' inflammatory population. In the inflammatory population only approximately 1:850 of the inflammatory cells are engaged in Ig secretion, and at most 1:2 of these produce specific antibody to graft donor MHC antigens. This finding suggests that a nonspecific (polyclonal) Ig-producing population is also present in situ. These results are also concordant with the reported low frequency of specific T cells of both cytotoxic and helper type at the site of inflammation and emphasize the importance of nonspecific inflammatory ("delayed hypersensitivity") mechanisms in acute allograft rejection.

Key words: Beta lymphocytes, in the rat – MHC antigens, in the rat – Antibodies, Beta lymphocytes, in the rat – Renal allograft rejection, B cell antibodies, in the rat

Offprint requests to: P. Häyry

According to a classic concept, donor-directed T helper cells (THC), able to reconstitute the rejection response in thymectomized, irradiated, bone marrow reconstituted (AT × BM) "B" recipients, are of central importance in the initiation of allograft rejection [18, 24], while the cytotoxic T lymphocytes (CTL) recovered in high frequencies in the transplanted organs [2, 5, 21] provide most of the effector arm and are responsible for the damage of the allograft in situ [2, 22]. On the other hand, large quantities of immunologically noncommitted lymphoid cells and mononuclear phagocytes have always been recovered by, for example, enzymatic digestion from rejected allografts [10, 23], stressing the importance of inflammation effector mechanisms – often referred to as "delayed hypersensitivity" (DHS) type – suggested to be linked to rat renal allograft rejection [16].

Of central importance in the evaluation of the biological meaning of specific (i.e., donor-directed) vs. nonspecific effector mechanisms is the quantitation of the size of the specific response, that is, the frequency of donor-directed cells at the site of inflammation.

Three groups of investigators have previously used limiting dilution assays and estimated the frequencies of donor-directed THC and their precursors (pTHC), as well

as CTL and their precursors (pCTL), at the site of inflammation. These estimates suggest that at the peak of inflammation in a rat renal allograft, the frequency of THC/pTHC is only approximately 1:670–1320 in conditions favoring the proliferation of this cell type [17]. The frequency of CTL/pCTL in mouse sponge matrix allografts and dog renal allografts under conditons favoring the proliferation of this cell type is of the order of 1:500–1000, respectively [7, 20]. Although contrasting opinions have been presented [25], these results suggest that the frequency of donor-committed T cells in the transplant inflammatory infiltrate is of the order of 1% or less.

A third lymphoid cell type with putative immunological specificity infiltrating an allograft in acute cellular rejection is the B cells. Although the frequency of B cells in, for example, rejected renal allografts is relatively small (of the order of magnitude of 10%–15% of all inflammatory leukocytes, as characterized by the surface and/or intracellular Ig expression), some 10%–20% of these cells undergo blastogenesis during the inflammatory episode [21] and about the same frequency of them secrete immunoglobulin in situ [19]. The presence of immunoglobulin (and complement) deposits have previously been reported in rejecting allografts [1]. Moreover, immunoglobulin-secreting cells [8] and antitubular basement membrane antibody-producing cells [11] have been recovered from human renal allografts. We therefore found it interesting to estimate the frequency of in situ donor-committed B cells during the inflammatory episode of rejection.

Materials and methods

Animals

The nuclei for the inbred rat strains Lewis ($RT1^l$), WF ($RT1^v$), and DA ($RT1^a$) were obtained from Prof. O. Sjögren, Department of Tumor Biology, University of Lund, Sweden and from Prof. J. L. Gowans, Dunn School of Pathology, Oxford, UK. The LBN ($RT1^n$) strain, congenic to Lewis and differing in the MHC only, was obtained from Dr. H. Binz, Department of Microbiology and Immunology, University of Zurich, Switzerland. The animals were bred and maintained in our own colony. Rats of both sexes, weighing 200–250 g and 2–4 months of age, were used for the experiments.

Transplantations

Renal transplantations were performed from LBN to Lewis strains. The donor kidney was grafted to the recipient aorta and inferior vena cava by end-to-side anastomosis [14]. The homolateral kidney was removed in order to perform an end-to-end ureteral anastomosis.

Media

RPMI 1640 (Gibco, Glasgow, Scotland) was used in all of the experiments. The incomplete medium was supplemented with 10 m*M* L-glutamine (Gibco), 50 μg/ml gentamycin (Gibco), and 10% fetal calf serum (FCS, Sera-Lab, Crawley Down, Sussex, UK). For selection of hybridoma cells, HAT-media (10% FCS/RPMI) supplemented with HAT media supplement (Sigma, St. Louis, Mo) was used. The final concentrations of hypoxanthine, aminopterin, and thymidine were 100 μ*M*, 0.4 μ*M*, and 16 μm, respectively. The cultures were incubated in an atmosphere of 5% CO_2 at 37°C.

Isolation of white cells

White cells were isolated from the renal graft, spleen, and peripheral blood. Transplanted kidneys were harvested on days 4 and 14 post-transplantation and immediately placed in ice-cold, serum-free RPMI 1640. The kidney capsule was carefully removed and the kidney was minced with a scalpel and forced through a stainless steel mesh (pore size 250 μm). The suspension was digested with 0.2 mg/ml collagenase (Millipore, Freehold, NJ) and 0.2 mg/ml DNase (Sigma) in serum-free RMPI 1640 at 37°C for 30 min with magnetic stirring. The suspension was filtered through a stainless steel mesh (pore size 50 μm), layered onto standard Ficoll-Isopaque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden), and spun at 400 *g* for 30 min to remove red cells, granulocytes, and cell debris. The cells at the interface were collected and washed with phosphate-buffered saline (PBS, 0.01 *M* phosphate buffer/0.9% NaCl, pH 7.4). Splenic lymphocytes were isolated by forcing the spleen through a stainless steel mesh (pore size 250 μm). The cells were spun to pellet and erythrocytes were lysed with lysing buffer [8.26 mg/ml NH_4Cl, 1.0 mg/ml $KHCO_3$, 0.037 mg/ml tetrasodium EDTA (tritriplex III)]. The lymphocytes were washed with PBS. Blood leukocytes were isolated from 5–10 ml heparinized blood by density centrifugation over Ficoll-Isopaque, leaving lymphocytes and monocytes at the interphase. The total recovery of nucleated cells were determined with a hemocytometer, and cytocentrifuged cell smears were made for differential counts. The smears were stained with May-Grünwald-Giemsa (MGG).

Quantitation of immunoglobulin-secreting cells with pA plaque assay

The frequency of immunoglobulin (Ig)-secreting cells was quantitated by the reverse protein A plaque assay [9]. Sheep erythrocytes (ShRBC, stored in Alsever's solution) were washed three times with physiological saline (NaCl) before being coupled to protein A (Pharmacia). One part packed ShRBC and one part protein A (0.5 mg/ml) were added to ten parts of 2.5×10^{-4} *M* chromic chloride in NaCl. The mixture was incubated at 20°C for 1 h and subsequently washed three times in Hank's balanced salt solution (HBSS). For the plaque assay, the cell mixtures were prepared in microtiter plates (Nunc, Roskilde, Denmark). Twenty-five microliters of 30% protein A-coupled ShRBC in HBSS and 25 μl diluted monospecific (for IgG and IgM) rabbit anti-rat Ig (Miles Laboratories, Elkhart, Ind) were incubated at 37°C for 30 min. After incubation, 50 μl ShRBC preadsorbed guinea pig complement, diluted 1:6, and 100 μl lymphoid cell suspension ($1.0–1.2 \times 10^6$/ml) were added and mixed. The Cunningham chambers [6] were filled with the cell suspension, sealed with paraffin, and incubated at 37°C for 2 h. After incubation, the number of plaque-forming cells was counted with a transillumination microscope.

The total recovery of Ig-secreting cells from each organ was determined on the basis of the in vitro plaque assays, the number of cells used for the assay, and the total recovery of inflammatory (white) cells.

Preparation of feeder layer

Normal rats were anesthesized and killed by cervical dislocation. The peritoneal cavity was flushed with 20 ml PBS. The liquid was drained after gentle massage of the abdomen, and the peritoneal cells were spun down and resuspended in HAT medium. If erythro-

cytes were macroscopically detectable, they were lysed as above. The cells were dispensed in flat-bottomed microtiter plates (Nunclon; 20×10^3 cells/well in 0.1 ml medium) and incubated overnight.

Hybridization to X63-Ag-8.653 myeloma line under limiting dilution conditions

The X63-Ag-8.653 cells were at an exponential phase of growth. The myeloma cells and the lymphocytes were mixed together in a 1:1 ratio and spun together into a pellet. The supernatant was removed carefully. For 90 s, 2 ml of 41% polyethylene glycol (PEG) 1500 in RPMI was added while simultaneously agitating the tube; after this the tube was left still for 30 s. Four milliliters RPMI was added for another 90 s and the suspension was diluted with 40 ml RPMI. The cells were spun at 200 *g* for 5 min without brake, were suspended in HAT media, and were dispensed on top of the feeder layer at concentrations of 3, 10, 30, 50, 70, and 100×10^3/well in 32 replicates/dilution.

Direct culture of lymphocytes

The lymphocytes were suspended in 10% FCS/RPMI and plated on round-bottomed microculture plates (Nunclon, Copenhagen, Denmark) at concentrations of 1×10^6–1×10^2/well in 250 µl with 8–32 replicates. The cells were cultured for 48 h before testing supernatants for Ig secretion by ELISA.

Demonstration of immunoglobulin secretion and specificity of the cultures in ELISA

The frequencies of Ig-secreting cells was determined by ELISA. Rabbit anti-rat immunoglobulin (ICN Immuno Biologicals, Lisle, Ill) 25 µg/ml PBS was absorbed on 96-well ELISA plates (Nunc) at +4 °C overnight. The plates were washed three times with PBS containing 0.05% Tween and incubated at room temperature with 50 µl supernatant for 2 h. After being washed with PBS-Tween, 50 µl of peroxidase-conjugated rabbit anti-rat IgG (DAKO), diluted 1:1000, was added and the plates were left at room temperature for 1 h. After being washed, the substrate solution (0.01% H_2O_2/1 mg/ml 1,2-diphenylamine/0.1 *M* citrate buffer, pH 4.5) was added and the reaction was allowed to continue for 5 min at room temperature. The reaction was stopped with 0.1 *M* NaF and the plate was measured with Multiscan MC (LabSystems, Helsinki, Finland).

The frequency of the hybridized clones producing polyspecific or specific Ig was determined by ELISA using plates coated with the relevant spleen cells. Flat-bottomed 96-well microculture plates (Nunc) were coated by incubation with 50 µl poly-d-lysine (1 mg/100 ml PBS) for 30 min. Spleen cells were isolated as described above, then washed and plated on coated plates in 50 µl/well. The plates were centrifuged for 5 min at 2000 rpm and fixed by adding 50 µl of ice-cold 0.5% glutaraldehyde/PBS. Then they were incubated for 15 min at room temperature. The plates were washed with PBS, filled with 100 m*M* glycine/0.1% BSA/PBS, and incubated for 30 min at room temperature. After being washed twice with PBS, the wells were filled with 0.1% BSA/PBS and the plates were stored at −20 °C. The ELISA was performed as described above.

Results

The cells were analyzed at two different, representative points in time: on day 4 post-transplantation, when the inflammation had reached its peak values [10], and on day 14 post-transplantation, when rejection was completed and the kidney had been irreversibly rejected.

Table 1. Recovery and frequency of Ig-secreting cells after renal transplantation in DA-to-WF major plus minor locus-incompatible strain combination

Days post-tx	Source of cells	Recovery of white cells $\times 10^6$	Recovery of Ig-secreting cells[a] $\times 10^3$	Frequency of Ig-secreting cells
0	Graft[b]	7.4 ± 3.1	7 ± 7.9	1:1000
	Spleen	185 ± 35	73 ± 55	1:2500
	Blood	2.4 ± 1.3	–[c]	–
4	Graft	46 ± 30	54 ± 40	1:850
	Spleen	300 ± 80	348 ± 170	1:860
	Blood	3.7 ± 2.0	–	–
14	Graft	26 ± 11	13.6 ± 10.1	1:1900
	Spleen	225 ± 32	525 ± 527	1:430
	Blood	2.5 ± 1	–	–

[a] Recovery of inflammatory white cells forming plaques in reverse protein A plaque assay with sheep erythrocytes
[b] Donor kidney prior to implantation (representing passenger white blood cells)
[c] Not detectable

The frequency of Ig-releasing cells was analyzed using a major and minor incompatible strain combination, DA-to-WF ($RT1^a$ and $RT1^v$), and reverse pA plaque assay.

In order to examine the specificity of the B-cell response, we chose to determine the frequency of B cells specifically directed against MHC antigens, which are known to be of prime importance in the rejection process. We therefore used the congeneic LBN-to-Lewis combination, which differs in MHC only, and MHC antigen-expressing target cells. The frequency of Ig-secreting cells were determined by ELISA.

Normal Lewis or WF rats were used as controls. The fusion frequency and the frequencies of Ig-secreting cells and cells secreting specific antibodies were determined according to limiting dilution principles [15].

Evaluation of Ig-secreting cells

Reverse protein A assay demonstrated that prior to transplantation 1:2500 spleen cells, 1:1000 kidney passenger leukocytes, and nondetectable numbers of blood cells were secreting immunoglobulin. Upon rejection, the frequencies increased to 1:860 and 1:850, respectively. After rejection was completed, the frequency of immunoglobulin-secreting cells still increased in the spleen to 1:430 but declined in the necrotic graft to just 1:1900. In the blood they remained undetectable (Table 1).

By the direct culture method, the frequency of Ig-secreting cells in the spleen of a nontransplanted rat was $1:65 \times 10^3$ and this increased to $1:24 \times 10^3$ on day 4 post-transplantation. In the blood of a nontransplanted rat, the frequency was $1:80 \times 10^3$ and it declined at peak of inflammation to $1:150 \times 10^3$. In the allograft, a frequency of $1:14 \times 10^3$ was detected (Table 2).

Table 2. Frequency of antibody-secreting B cells in DA-to-WF (pA assay) and LBN-to-Lew (direct culture) rat renal allografts, spleen, and blood of transplant recipient[a]

Days post-tx	Source of cells	Frequency of Ig-secreting cells	
		pA Assay	Direct culture[b]
0	Graft[c]	1:1000	[c]
	Spleen	1:2500	1:65000
	Blood	–[c]	1:80000
4	Graft	1:850	1:14000
	Spleen	1:860	1:24000
	Blood	–	1:150000
14	Graft	1:1900	[c]
	Spleen	1:430	[c]
	Blood	–	[c]

[a] Calculated from the number of inflammatory (white) cells after removal of granulocytes by Ficoll centrifugation (FIP)
[b] One representative experiment out of four
[c] Not detectable

Evaluation of specific cells

The plot for determining the fusion frequency of inflammatory LBN-to-Lewis leukocytes at peak of inflammation to the mouse myeloma line X63-Ag-8.653 under limiting dilution conditions is presented in Fig. 1. Plots for frequency of Ig-secreting cells as well as donor-specific cells are also depicted.

The fusion frequency of spleen and blood cells of a nontransplanted Lewis recipient was $1:30 \times 10^3$ and $1:97 \times 10^3$, respectively. At the peak of inflammation, the fusion frequency of inflammatory leukocytes was $1:172 \times 10^3$, whereas the fusion frequencies of recipient blood and spleen cells declined to $1:138 \times 10^3$ and $1:165 \times 10^3$, respectively. After rejection was completed, fusion frequencies comparable to those in the pre-transplantation period were again observed in the recipient spleen and blood (Table 3).

Most of the clones obtained secreted immunoglobulins (Table 3). The highest frequencies were obtained for a nontransplanted rat. In a nontransplanted rat spleen and blood, as well as in the spleen on day 14 post-transplantation, all clones were secreting Ig. Half of the clones were producing immunoglobulins in the allograft, spleen, and blood on day 4 and in the blood on day 14 after transplantation.

Specific clones were undetectable in the spleen and in the blood of the forthcoming (nontransplanted) Lewis recipient (Table 3). At the peak of the inflammatory response, the frequency of clones producing specific donor-directed antibody in the kidney allograft was $1:720 \times 10^3$. In the spleen, the frequency was $1:220 \times 10^3$ (Table 3). Thus, the frequency of clones producing specific antibodies among those clones that secrete antibodies was 1:2 and 1:1 for graft and spleen, respectively.

Evaluation of polyspecific cells by hybridization method

Prior to transplantation, a very low frequency ($1:1.5 \times 10^6$) of clones producing "polyspecific" antibody, reactive with at least three rat strains – LBN, Lewis, and DA – was observed in the nontransplanted Lewis recipient spleen, a frequency that increased during rejection (Table 3). Such polyspecific reactive clones were not observed in the inflammatory infiltrate of the renal allograft during rejection, nor were they detectable in the recipient blood.

Discussion

Studying the specificity of the B-cell response towards the graft is limited by two major factors. First, B cells are very difficult to grow and maintain in culture, even in the presence of T cell-derived B cell growth factor (BCGF) [13]. This leads to the second difficulty that the amounts of antibodies secreted by a single B cell are minute and, therefore, the possibilities to test their specificity is very limited.

We first tried to reproduce the method used by Cerottini and Brunner [4] and to demonstrate alloantibody-secreting cells to nucleated target cells in a plaque assay. When this proved unsuccessful, we selected the following alternative as second best. We first estimated the quantity of the Ig response by calculating the frequencies of Ig-secreting cells in a major and minor locus-incompatible strain combination that is presumed to give a maximal response. Thereafter, we analyzed the clonality of the response using another strain combination that differed in the major locus only.

Table 3. Frequency of cell fusion, Ig-secreting cells, and donor-specific B cells in LBN-to-Lew rat renal allografts, spleen, and blood of transplant recipient[a]

Days post-tx	Source of cells	Fusion with X63-Ag-8.653 $\times 10^3$	Frequency of Ig-producing clones $\times 10^{3b}$		
			Total	anti-LBN	anti-LBN + Lew + DA
0	Spleen	1:30	1:30	0	1:1550
	Blood	1:97	1:120	0	0
4	Graft	1:172	1:400	1:720	0
	Spleen	1:138	1:210	1:220	1:700
	Blood	1:165	1:210	0	0
14	Spleen	1:67	1:59	0	1:1000
	Blood	1:100	1:240	0	0

[a] One representative experiment out of three. Calculated from the total number of inflammatory (white) cells after removal of granulocytes by Ficoll centrifugation (FIP)
[b] Hybridomas producing Ig and reactive with LBN or LBN + Lew + DA spleen cells in ELISA

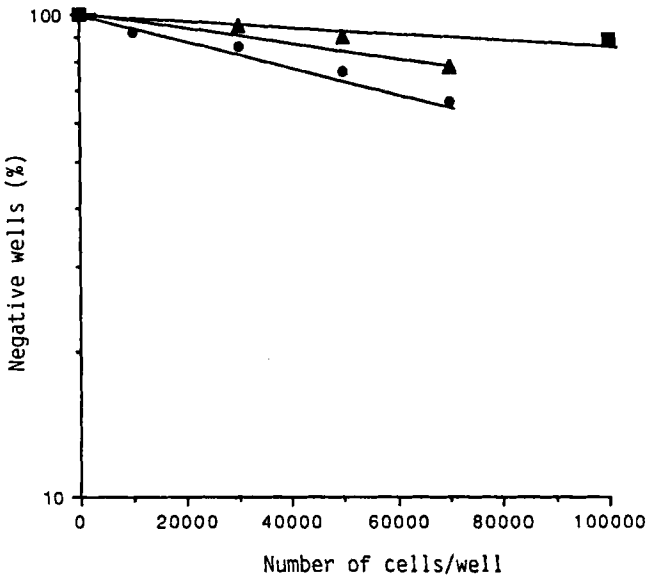

Fig. 1. Representative plot of limiting dilution assay. Fusion frequency (●), frequency of Ig-secreting cells (▲), and donor-specific B-cells (■) among cells isolated from a 4-day rejecting kidney allograft are shown. Frequency was determined with the hybridization method. One representative plot out of five is shown

Experiments with the DA-to-WF combination clearly demonstrated an increase in the number of Ig-secreting cells from 1:1000 to 1:850 during rejection and a decline to 1:1900 after rejection was completed. In the spleen, the frequency of Ig-secreting cells increased from 1:2500 to 1:860 and further to 1:430 after rejection was completed. The question remains as to whether all of them or only a fraction of them produce antibodies directed to graft donor antigens.

The same pattern was also observed for the LBN-to-Lewis strain combination measured by ELISA from supernatants of directly cultured lymphoid cells. This method remained, however, at least one log less sensitive than the pA assay. We attempted to determine the frequency of specific B cells by a complement-mediated cytotoxicity assay using an LBN MHC class I-expressing hybridoma as targets. Our attempts, however, failed. This was presumably due to the fact that in direct culture, the concentration of antibodies in the culture supernatants remains too low to be able to lyse the targets efficiently.

In order to estimate the frequency of B cells secreting specific antibodies against donor MHC antigens, B cells were immortalized by hybridization with mouse myeloma X63-Ag-8.653, cultured, and assayed for antibody production and specificity.

The major weakness of the fusion assay is that although the mouse myeloma strain X63-Ag-8.653 is known to have a very high fusion frequency [12], there is no way to determine which cells fuse and which are lost during the hybridization process. To make an exact estimate, all cells should have the same ability to fuse, something which would lead to their proportional distribution among the hybrid population. The high frequency of Ig secretors among the hybrids suggests, however, that this is not the case.

Blast cells, representing approximately 10% of the Ig-containing population [21, 26], are known to have a higher tendency to fuse than resting cells. They, therefore, most likely become enriched in the hybrid population. Our results show higher frequencies than those actually present in situ.

On the other hand, the fusion frequency was seen to decline at the peak of inflammation compared to that observed prior to transplantation. This may be due to rapid differentiation caused by transplantation, something which brings the cells beyond the blast stage into a nonfusing antibody-secreting plasma cell.

Our results suggest that nearly all B cells in the recipient spleen produce antibodies directed against donor MHC antigens. In the graft, however, at most half of the Ig-secreting cells secrete specific donor-directed antibodies. The function of the rest of the Ig-secreting cells is unknown. They may, in part, represent polyclonal activation products, generated as a result of the massive T-cell response, and in part graft-related antibodies other than MHC, such as endothelial cell antigens [3].

Interestingly, we did, in fact, detect a population of polyspecific B cells secreting antibodies that reacted with antigens present on cells from host (Lewis), donor (LBN), and third party (DA) rat strains. These cells were detected only in the spleen of both normal rats and graft recipients. This suggests the possibility that autodirected cells exist at low concentration in the spleen but that they most likely are not released into the circulation. During rejection, when the immune system is activated, their frequency increases due to a general increase in cell division among spleen cells. Because such cells are not detectable in the graft and in the circulation, their possible role in rejection remains unknown.

Assuming that the PFCA is the most accurate method for determining frequencies, we "corrected" the estimates obtained with the hybridization method by comparing them to frequencies of Ig secretors obtained by PFCA. Using PFCA, the frequency of immunoglobulin-secreting cells in the infiltrate and spleen was estimated as 1:850 and 1:860, respectively. Of those cells that fused and secreted immunoglobulins, 1:1–2 were specific in the hybridization assay, giving a frequency of cells secreting specific immunoglobulins of approximately 1:1500 and 1:860 for the infiltrate and spleen, respectively.

We therefore assume that within the inflammatory cell population of a rejecting renal allograft, the frequency of B cells producing immunoglobulins specific to the graft donor is low and thus comparable to the reported frequencies to THC/pTHC or CTL/pCTL in the inflammatory infiltrate of rat renal allografts, mouse sponge matrix allografts, or dog renal allografts [7, 17, 20]. Taken together, these results support both the concept that the frequency of specific lymphoid cells at the site of inflammation is quite low (at most 1%–2% of all inflammatory cells) and the role of DHS in transplantation.

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