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Interstitial Mononuclear Cell Infiltrates in Chronic Rejection of the Kidney and Correlation with Peripheral Blood*

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To investigate the characteristics of interstitial inflammatory cells and possible involvement of γδ T cells, 16 renal allograft biopsies showing chronic rejection were stained by immunohistochemical method and correlated with the data of peripheral blood evaluated by flow cytometry. For immunophenotyping, fresh frozen sections were stained with monoclonal antibodies against CD3, CD4, CD8, CD68, CD56, TCRδ1 and HLA DR. Paraffin embedded tissue was stained with CD45RO, CD20-Cy and CD68. Nine cases of nonspecific tubulointerstitial change and 4 cases of non-allograft tubulointerstitial nephritis were used as a control. Inflammatory infiltration was present in all cases studied. T cells predominated in the interstitium of chronic rejection and were followed by macrophages and B cells. The degree of interstitial infiltration of frozen section was not accordant with that of paraffin sections. Allografts with nonspecific tubulointerstitial changes or tubulointerstitial nephritis of native kidneys showed similar distribution pattern in terms of type and degree. However, the degree of infiltrate did not give any statistical significance among groups. The CD4/CD8 ratios in interstitial infiltrates were less than 1.0 in 6 cases and was not accordant with those of peripheral blood. Proportion of γδ T cells increased over 10% in 2 cases in tissue and in 3 cases in peripheral blood. In 3 cases of chronic rejection in which both tissue and blood results were available, there was no concordance of CD4/CD8 or γδT/CD3 between them. Tubular expression of HLA DR was, however, present only in 4 cases of chronic rejection. In conclusion, T lymphocytes were predominant regardless of diagnosis or disease activity. T lymphocyte subset did not give any suggestion as to the diagnosis or disease activity in chronic rejection. Furthermore γδ T cells had only limited value. Lymphocytic subsets in peripheral blood would not be predictors of tissue destruction in chronic rejection.

Key Words : *Renal transplantation, Interstitial mononuclear cells, γδ T cells, Flow cytometry*

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INTRODUCTION

As the renal allografts become destroyed in months and years after successful engraftment, the histological changes such as glomerulosclerosis, tubular atrophy, interstitial fibrosis and vascular wall thickening are par-

alleled. Mononuclear cell infiltration has been reported in the context of acute rejection mainly in the interstitium and with tubular invasion(tubulitis), which exerts a major role in acute graft dysfunction. The infiltrate consisits of monocytes/macrophages and T cells with contradictory results as to the predominant subsets either in tissue or in peripheral blood and as to the role of these cells (Platt et al.,1982 ; Hancock et al.,1983 ; Hall et al.,1984). The studies of inflammatory subset have been performed to determine whether it could be used to predict or confirm the onset of rejection (Carter et al., 1983). The infiltrate can be found in chronic rejection, either at perivascular or fibrotic interstitium and occasionally within atrophic tubules. The infiltrate can be seen in stable grafts in early posttransplant period, which cast doubts about the role and fate of these cells. Therefore the simple presence of inflammatory cells do not necessarily indicate active destruction, which may be influenced by cytokine release rather than inflammatory cells themselves or difference in inflammatory subtypes.

To elicit an immune response to alloantigen, host T cells receive information from antigen presenting cells bearing processed antigen via T cell antigen receptor (TCR). TCRs are mostly αβ, but in 5-10% are γδ subsets. γδ T cells are CD3+, but the great majority do not express CD4 or CD8. They are present in peripheral blood, lymph nodes and in the intestinal epithelium in human. γδ T cells are capable of nonspecific, MHC unrestricted cytotoxicity and function in concert with MHC restricted αβ T cells. It has been reported that infiltration of the γδ T cells was prominent or was correlated with progression of diseases in rheumatoid arthritis, IgA nephropathy and long standing cardiac allografts(Hohlfeld et al., 1991 ; Jacobs and Haynes, 1992). Therefore this might further suggest possible role of γδ T cells in rejection. However, in some reports they were reported to have no role in the progression of acute rejection and even downregulated immune response.

To investigate the subsets and pattern of the inflammatory cellular infiltrate and possible involvement of γδ T cells in chronic allograft dysfunction, renal allograft biopsies showing chronic rejection were stained by immunohistochemical method and correlated with the data of peripheral blood T lymphocyte subpopulation.

MATERIALS AND METHODS

Sixteen renal transplant biopsies showing chronic rejection and examined in the Department of Pathology, Yonsei University Medical Center from Oct. 1994 to Dec. 1995 were the subject of the study. Recipients were immunosuppressed basically with cyclosporine (CsA) and prednisone. Acute rejection was treated with methylprednisolone pulse, ALG or OKT3. In the cases showing high grade CsA toxicity defined by frequent occurrence of arteriolar hyalinosis, azathioprine was supplemented with dose reduction of CsA. The indications of biopsy were elevated serum creatinine over 30% of baseline level, significant proteinuria (over 1gm/24hrs) or persistent microscopic hematuria. Ultrasound-guided biopsies were obtained with a spring-loaded 18 gauge Biopty gun (Sweden) using aseptic technique under local anesthesia. The tissue was processed for light microscopy and immunofluorescent microscopy in all cases and electron microscopy if needed. The Banff working classification was used for the diagnosis and grading. For immunophenotyping both fresh frozen and paraffin embedded tissues were used. Frozen sections were cut on a cryostat, air dried and fixed for five minutes in cold acetone, then stained with monoclonal antibodies against CD3, CD4, CD8, CD68, CD56(Dako, Japan) and pan TCRγδ(TCRδ1, T cell diagnostics, Cambridge, MA, USA). Frozen sections were then washed and incubated with streptavidin biotin peroxidase conjugate and developed in a medium containing diaminobenzidine and freshly opened hydrogen peroxide. Paraffin embedded tissue was stained with CD45RO (UCHL-1, Dako, Japan), CD20-Cy (L26, Dako, Japan) and CD68 (Dako, Japan). For the tubular expression of HLA class II, the sections were stained with HLA DR (Dako Inc. Japan) as well. Biopsies taken from allografts showing nonspecific tubulointerstitial change (n=9) or from nonallograft kidneys with tubulointerstitial nephritis (n=4) were used as control. Cases showing nonspecific changes include focal interstital inflammation without tubulitis and/or focal tubular atrophy and interstitial fibrosis. One case of chronic rejection and 3 cases from the control group showed poor frozen section staining, which were ultimately discarded from analysis. Infiltrating cells in the cortical interstitium were individually counted using a point counting method, and counts were expressed as the number of positively stained cells per square mm. γδ T cells were calculated relative to CD3+ lymphocytes. For analysis of T lymphocyte subsets and γδ T cell distribution in peripheral blood, the blood was collected on the same day of biopsy. Among 27 cases tested, 14 of chronic rejection and 5 of nonspecific changes were included for the study. Only 3 cases of chronic rejection had comparable tissue results. The blood was diluted twice in RPMI

media. Mononuclear cells were separated by density gradient using Ficoll hypaque (Pharmacia, Sweden), and centrifuged for 30 minutes at 2,000rpm at room temperature. Ten microliter of fluorescein conjugated monoclonal antibodies against CD3, CD4, CD8 (Becton Dickinson, San Jose, CA) and TCRδ1(T cell diagnostics, Cambridge, MA, USA) was added to 10^6 cells, reacted at 4℃ for 30 minutes, washed twice with phosphate buffered saline containing bovine serum albumin, and fixed in 0.5% paraformaldehyde. Viable cells were analyzed using FACStar (Becton Dickinson, San Jose, CA).

One way analysis of variance (ANOVA) was used to test difference among groups. Spearman correlation test was applied to assess the differences between parameters. Results were considered statistically significant at the $p<0.05$ level.

RESULTS

1. Phenotypic characterization of infiltrating lymphocytes

Inflammatory infiltration was present in all cases. T cells predominated in the interstitium of chronic rejection. The density of T cells were variable from case to case and from area to area (412.1±387.0; range 51-1621/mm^2) and were present scattered or in small clusters mainly in perivascular and periglomerular areas. Macrophages showed similar distribution to T cells. They outnumbered B cells by more than double in 7 cases, whereas B cells outnumbered in 2 cases. As the total number of B cells increased, they tended to aggregate. The findings from frozen tissue were similar, that is, T cells predominated over macrophages. However the degree of infiltration was not accordant with that of paraffin sections. Only 6 out of 12 cases showing significant cellular infiltrate in paraffin section had significant infiltrate in frozen tissue as well. Again 6 out of 10 cases with significant cellular infiltrate in frozen tissue had significant infiltrate in paraffin sections. CD3 positive cellular infiltrate showed similar tendency to CD45RO positive cells in terms of degree. Allografts with nonspecific tubulointerstitial changes or tubulointerstitial nephritis of native kidneys showed similar distribution pattern both in type and degree. Six out of 9 cases of allografts with nonspecific tubulointerstitial changes and 2 out of 4 cases of tubulointerstitial nephritis showed predominance of macrophages over B cells. The degree of infiltrate did not give any statistical significance among groups.

As to the subsets of lymphocytes, CD4 was predominant in 9 cases and CD8 was predominant in 6 cases and doubled its counterpart in 3 and 2 cases, respectively. CD8+ cells tended to be more widely scattered than CD4+ cells. The CD4/CD8 ratios in interstitial infiltrates were less than 1.0 in 6 cases and varied from those of peripheral blood.

γδ T cells were minimally infiltrated in an individually scattered pattern. Proportion of γδ T cells increased over 10% in 2 cases in tissue. CD56 positive cells were rarely found but, when present, were scattered(Table 1).

HLA DR was stained in endothelium of glomerular capillaries, interstitial peritubular capillaries and larger blood vessels in rejected and nonrejected kidneys. Approximately half of infiltrating cells were stained pos-

Table 1. Interstitial infiltrate in cases with chronic rejection, nonspecific infiltrate and tubulointerstitial nephritis.

	Chronic rejection	Nonspecific infiltrate	Tubulointerstitial nephritis
Paraffin sectioned tissue			
No. of cases	16	9	4
CD45RO	412.1±387.0[1,2]	222.4±128.5	494.3±625.6
CD20	114.3±147.8[2]	55.2± 53.5	111.5±162.5
CD68	107.6±108.2[2]	129.4± 68.8	198.3±194.6
Frozen tissue			
No. of cases	15	7	3
CD3	158.6±170.1	127.3±118.9	222.0±203.1
CD8	61.5± 79.2	41.3± 72.0	97.7±121.9
CD4	55.9± 59.9	55.4± 59.1	147.3±218.5
CD68	22.8± 26.8	21.9± 27.0	38.7± 65.2
γδTCR	15.7± 13.8	13.0± 23.3	11.7± 9.1
CD56	0.4± 0.8	0.3± 0.8	4.7± 6.4

1: Means±S.D.
No. of positive cells/mm^2 cortical interstitium.
2. $P<0.05$ between CD45RO and CD20, CD20 and CD68 and CD45RO and CD68

itively for HLA DR. Tubular expression of HLA DR was, however, present only in 4 cases of chronic rejection.

2. Flow cytometry and correlation with tissue infiltrate

The ratio of CD4/CD8 were less than 1.0 in 6 cases of 14 chronic rejection and 2 of 5 cases showing nonspecific changes. γδT/CD3 were above 10% in 3 of 11 chronic rejection, however, γδ proportion was not increased in all 4 cases with nonspecific changes(Table 2). In 3 cases of chronic rejection in which both tissue and blood results were available, there was no concordance of CD4/CD8 or γδT/CD3 between them(Table 3). In case 1, γδT/CD3 in the renal tissue was 60%, however, both the number of CD3 and γδ T cells were less than 10, therefore, the data was not appropriate for evaluation.

Table 2. Flow cytometric data

Diagnosis	Case No.	CD4 (%)	CD8 (%)	CD4 (%)	γδT/CD3 (%)
Chronic rejection	1	47.8	17.2	2.8	4.3
	2	53.8	33.7	1.6	4.2
	3	47.8	35.0	1.4	9.5
	4	62.6	25.0	2.5	4.6
	5	48.6	26.8	1.8	10.6
	6	31.9	36.2	0.9	8.4
	7	31.2	22.9	1.4	
	8	42.5	41.5	1.0	
	9	28.3	44.6	0.6	10.2
	10	31.5	41.6	0.8	4.9
	11	42.5	28.0	1.5	8.0
	12	25.1	33.4	0.8	10.3
	13	29.4	37.7	0.8	3.2
	14	30.3	33.5	0.9	8.4
Nonspecific	1	43.9	13.1	3.4	4.9
	2	53.8	14.8	3.6	6.4
	3	26.3	29.7	0.9	
	4	18.3	21.4	0.9	7.4
	5	36.2	32.7	1.1	9.6

Table 3. Comparison of tissue and flow cytometric data in 3 cases with chronic rejection.

Case	CD4/CD8 ratio		γδT/CD3(%)	
	Kidney	P.B*	Kidney	P.B.
1	1.2	0.6	60.0	10.2
2	0.7	1.5	12.0	8.0
3	0.3	0.8	8.3	10.3

* P.B. ; peripheral blood

DISCUSSION

Renal allografts with chronic dysfunction may have residual interstitial infiltrates in the vicinity of vessels and glomeruli, or lymph follicles at the corticomedullary junction. Among contributing factors of chronic rejection consistently known are histocompatibility and frequency or intensity of acute rejection (Tesi et al., 1993 ; Yilmaz and Häyry, 1993), that might suggest smoldering inflammatory infiltrate with focal activity causing irreversible renal damage. The presence of inflammatory cell infiltrate in a long-term renal allograft suggests a pathological process of potential clinical significance (Bohman et al., 1991). However, not all the inflammatory infiltrate may be involved in cytotoxicity, as revealed by inflammatory infiltrate in stable graft function (van Es et al.,1984 ; McWhinnie et al.,1986 ; Isoniemi et al.,1992 ; Rush et al., 1995). Our data confirmed this finding. Another evidence is the presence of functionally paralyzed inflammatory cells in acute rejection after antirejection treatment. These findings suggest that something other than simple presence of inflammatory cells may be important and that lymphocyte subset or cytokine may play a significant role in chronic allograft dysfunction.

To predict or diagnose the severity of rejection, immunohistologic studies have been performed, mostly in acute rejection. Raftery et al. (1989) reported that the presence of macrophages correlated best with severe rejection. Seron et al.(1991) reported that the percentage of naive T cells, neither macrophages nor memory T cells, increased with the severity of interstitial infiltrate. Others showed important role of T cells in acute rejection with either CD4 or CD8 predominance (Platt et al.,1982 ; Hancock et al.,1983 ; McWhinnie et al.,1986).

As McWhinnie et al. (1986) demonstrated that the magnitude of infiltrate would vary with time, graft status and immunosuppression, our data showed variability of cellular infiltrate from case to case. Even in the same case, degree of inflammation was variable. Therefore the focal nature of the lesion and its severity influence not only diagnosis but also inflammatory intensity. However, the phenotypic composition remains relatively constant with T cell predominance.

There is disagreement as to the most prominent T cell subset found in biopsies from rejecting grafts. Platt et al. (1982) using an immunofluorescence technique and Hancock et al. (1983) using an immunoperoxidase technique found that CD8+ cells were more frequent than CD4+ cells in acute rejection. McWhinnie et al.

(1986) described that T lymphocytes accounted for approximately 35% of infiltrating cells and CD8+ cells were more common than CD4+. However, normal or elevated ratios of T4 to T8 cells in peripheral blood were associated with rejection(Cosimi et al., 1981). The T4/T8 ratio of infiltrating cells showed no significant change in acute tubular necrosis, acute rejection, chronic rejection or renal disease in the native kidneys of nontransplant patients (Burdick et al.,1984 ; Waltzer et al.,1987). Proportions of T cells, T cell subsets, B cells and macrophages were similar in sequential biopsies. These implicate the inadequacy of this immunological index in allograft monitoring. Diversity of findings are found in peripheral blood or tissue section. The monitoring of peripheral blood lymphocyte subsets have no benifits either in diagnosis or predicting disease activity.

A significant macrophage infiltration was found in chronic rejection as demonstrated by our results. In severe acute rejection, macrophages probably acting as effector cells were preponderant over T cells as compared to mild or moderate rejection (Hancock et al 1983). However in chronic rejection they may play roles in fibrosis rather than active destruction.

In acute rejection, T cells with NK-like activity rather than specific alloreactive T cells contributes to destruction of kidney grafts. NK/K cells were less than 9% of the infiltrate (McWhinnie et al., 1986) but they were increased in early posttransplantation (Hancock et al., 1985). We could not demonstrate any significant infiltrate of CD56 positive cells. The negative staining for CD56 showed two possibilities. In part because these appear exclusively in early phase of acute rejection, and possibly because of the lack of sensitivity of our antiserum to detect them.

The mean percentage of TCRδ1+ per CD3+ cells were 7.0 - 9.0% in peripheral blood (Bucy et al., 1989 ; Falini et al., 1989). Increased percentages of γδ T cells have been reported in a number of infectious or autoimmune inflammatory conditions including rheumatoid arthritis, Kikuchi's and tuberculous lymphadenitis. γδ T cells are found exclusively in the kidneys of patients with IgAN who progressed to renal failure (Falk et al., 1995). Raasveld et al. (1992) investigated distribution of γδ T cells in early posttransplantation period and demonstrated no significant increase in the percentage of γδ T cells in fine needle aspirate even during acute rejection. They concluded that γδ T cells seem to play no major role in transplant rejection. Antirejection treatment did not have any effect on the percentage of γδ T cells either in fine needle aspirate or blood. A significant decrease in γδ T cells in the peripheral blood may be caused by immunosuppressive drug treatment and may be responsible for the decreased natural killer cytotoxic activity. Volk et al.(1989) showed increased γδ T cells in long-term renal transplantation. γδ T cells may act as mediators of cellular immunity that recognize antigens directly, without the need for specialized antigen presenting cells, or they might be involved in the down-regulation of the cellular immune response(Vaessen et al., 1991). Unusual accumulation of γδ T cells may reflect a general breakdown in immune regulation, rather than simple expansion of T cells in response to direct stimulation in adults. γδ T cell population was elevated in 3 of 11 cases while no such increase was observed in 4 nonspecific cases. We do not know whether these subsets might have exerted some role in graft damage at least in these three cases. We were unable to demonstrate CD56 positive cells in our biopsy materials, and γδ T cells appear to have no or a limited role in chronic rejection in our experience.

In summary, T lymphocytes were predominant regardless of diagnosis or disease activity. T lymphocyte subset did not give any suggestion as to the diagnosis or disease activity in chronic rejection. Furthermore γδ T cells had only limited value and may not be involved in cytotoxicity. Lymphocytic subsets in peripheral blood are not predictors of tissue destruction in chronic rejection.

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