Sequential cytokine dynamics in chronic rejection of rat renal allografts: Roles for cytokines RANTES and MCP-1

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ABSTRACT Chronic rejection, the most important cause of long-term graft failure, is thought to result from both alloantigen-dependent and -independent factors. To examine these influences, cytokine dynamics were assessed by semiquantitative competitive reverse transcriptase–PCR and by immunohistology in an established rat model of chronic rejection of renal allografts. Isograft controls develop morphologic and immunohistologic changes that are similar to renal allograft changes, although quantitatively less intense and at a delayed speed; these are thought to occur secondary to antigen-independent events. Sequential cytokine expression was determined throughout the process. During an early reversible allograft rejection episode, both T-cell associated [interleukin (IL) 2, IL-2 receptor, IL-4, and interferon γ] and macrophage (IL-1α, tumor necrosis factor α, and IL-6) products were up-regulated despite transient immunosuppression. RANTES (regulated upon activation, normal T-cell expressed and secreted) peaked at 2 weeks; intercellular adhesion molecule (ICAM-1) was maximally expressed at 6 weeks. Macrophage products such as monocyte chemoattractant protein (MCP-1) increased dramatically (to 10 times), presaging intense peak macrophage infiltration at 16 weeks. In contrast, in isografts, ICAM-1 peaked at 24 weeks. MCP-1 was maximally expressed at 52 weeks, commensurate with a progressive increase in infiltrating macrophages. Cytokine expression in the spleen of allograft and isograft recipients was insignificant. We conclude that chronic rejection of kidney allografts in rats is predominantly a local macrophage-dependent event with intense up-regulation of macrophage products such as MCP-1, IL-6, and inducible nitric oxide synthase. The cytokine expression in isografts emphasizes the contribution of antigen-independent events. The dynamics of RANTES expression between early and late phases of chronic rejection suggest a key role in mediating the events of the chronic process.

Despite progressive improvements in the initial success rate of clinical transplantation and increasingly detailed knowledge of immunobiology, the rate of long-term graft attrition has remained constant (1). Chronic rejection, a poorly understood process responsible for the majority of late graft failures, may be defined as progressive functional and structural deterioration of transplanted tissue occurring months or years after engraftment. In the kidney, its manifestations include progressive vascular obliteration, glomerulosclerosis, tubular atrophy, and fibrosis ultimately leading to organ failure (2–4). Neither the etiology nor pathophysiology of the phenomenon is fully characterized, although both antigen-dependent and antigen-independent factors have been implicated. Antigen-dependent factors include histocompatibility differences between donor and host, early acute rejection, and ongoing host alloresponsiveness; antigen-independent factors include the early injury of ischemia/reperfusion, cytomegalovirus infection, cyclospo-

rin A (CyA) toxicity, and the long-term effects of reduced functioning renal mass (4, 5).

In the rat kidney allograft model of chronic rejection used throughout these studies, morphological changes consistently evolve after 12 weeks to generalized fibrosis, with function declining progressively and the recipients dying of renal failure between 24 and 52 weeks (6). Although the numbers of host cells infiltrating long-term grafts are generally relatively sparse, the increased presence of macrophages seems critical to the development of later changes in the kidney grafts in this study and in vascular, hepatic, intestinal, and cardiac allografts examined by others (7–12).

The presence of macrophages in chronic rejection has also been associated with expression of macrophage-derived cytokines, particularly interleukin (IL) 1α, IL-6, tumor necrosis factor (TNF) α, monocyte chemoattractant protein (MCP-1) (8, 9, 13), and growth factors, including transforming growth factor (TGF) β and platelet-derived growth factor (11). Adhesion molecules are important in the events associated with the process; organs that maintain high levels of intercellular adhesion molecule (ICAM-1), for instance, inevitably progress to chronic dysfunction (14).

Few analyses of the time course of cytokine expression in chronic allograft rejection have been reported. Immunohistological studies of rejecting allografts have been performed with monoclonal antibodies to lineage-specific antigens of infiltrating cell populations, subpopulations, and their products (6). It has been difficult to define actual host events as they occur in the rejecting tissues. In this report, we analyze the kinetics of graft rejection and the severity of intragraft inflammatory responses by semiquantitative competitive reverse transcriptase (RT)–PCR of cytokines, chemokines, growth factors, and adhesion molecules (Table 1). We postulated that by serially analyzing tissues from kidney grafts for up-regulation of mRNA for cell products thought to play a role in rejection, we would be able to evaluate and dissect components of the antigen-dependent and -independent pathways in the pathophysiology of the chronic rejection process.

MATERIALS AND METHODS

Techniques. Inbred male rats (Harlan–Sprague–Dawley) were used throughout the experiments. For allografts, Lewis (LEW, RT1^1) rats served as recipients of Fischer 344 (F344, RT11v1) kidneys; for isografts, Lewis rats were used as both donors and recipients (15). Renal grafts (either allograft or isograft) were transplanted orthotopically to the left renal vessels and ureter of the hosts. The left kidneys of all recipients were removed during engraftment; the right kidneys were removed 10 days later (2, 9).

Table 1.   Sequence of rat primers (5′–3′)

Primer	Sense sequence	Antisense sequence	Size, bp
IL-1α	CTAAGAACTACTTCACATCCGCA	CTGGAATAAAACCCACTGAGGTAGG	623
IL-6	CAAGAGACTTCCAGCCAGTTGC	TTGCCGAGTAGACCTCATAGTGACC	614
IL-10	GTGAAGACTTTCTTTCAAA	TGATCAAGATGTCAAACTC	206
IL-2	TCAACAGCGCACCCACTTCAAG	GTTGAGATGATGCTTTGACAGA	403
IL-4	TCTCACGTCACTGACTGTA	CTTTCAGTGTTGTGAGCGT	406
IL-2Rβ	GCCAGCTGCTTCACCAACCA	GAGAAGAGCAGCAGGTCATC	224
MCP-1	ATGCAGGTCTCTGTCACG	AGTATGACAGAGAACTAG	255
NOSi	GTGTTCCACCAGGAGATGTTG	CTCCTGCCCACTGAGTTCGTC	576
RANTES	CCATATGGCTCGGACACCA	GCTCATCTCCAAATAGTTG	221
TGF-β1	CTTCAGCTCCACAGAGAAGAACTGC	CACGATCATGTTGGACAACTGCTCC	298
TNF-α	TACTGAACTTCGGGGGTGATTGGTCC	CAGCCTTGTCCCTTGAAGAGAACC	295
IFN-γ	ATCTGGAGGAACTGGCAAAAGGACGC	CTTAGGCTAGATTCTGGTGACAGC	288
ICAM-1	GGGTTGGAGACTAACTGGA	CCTCTGGCGGTAATAGGTG	201
β-Actin	TTGTAACCAACTGGGACGATATGG	GATCTTGATCTTCATGGTGCTAGG	764

Experimental Design. All allografted hosts were treated briefly with low-dose CyA (1.5 mg per kg per day, s.c. for 10 days) to reverse an initial acute rejection episode (2, 10); isografted animals were treated in similar fashion. Grafted animals were sacrificed at 1, 3, 5, and 7 days and 2, 4, 6, 8, 12, 16, 24, 32, and 52 weeks ($n = 3$ allograft and isograft recipients per time point).

Immunohistology. Frozen sample of kidneys were stained with appropriate antibodies and were then analyzed by the alkaline phosphatase–anti-alkaline phosphatase or peroxidase methods. Sections were counterstained with hematoxylin. Positive cell counts were expressed as the number of cells per field of view (c/FV; mean ± SD).

RT–PCR. Frozen kidney and spleen RNA samples were prepared by using a guanidinium isothiocyanate/phenol/chloroform isolation method (ULTRA-spec.; Biotecx Laboratories, Houston) (16). Total RNA was used for first-strand cDNA synthesis with oligo(dT)$_{12-18}$ and RT under supplier-recommended conditions (GIBCO/BRL). Nonlooping non-overlapping oligonucleotide primer pairs from separate exons were prepared for each gene studied (Table 1) by Clontech or by Genosys (The Woodlands, TX). The competitive PCR for quantification of mRNA was performed as described (17). Amplification was begun with incubation at 94°C for 2 min and was optimized for each cytokine. PCR products (5 μl) were electrophoresed on an ethidium bromide-stained 1.5% agarose gel and gene-specific bands were visualized with UV (Fig. 1). We performed control experiments for each primer pair by substituting water for cDNA and omitting RT during the cDNA synthesis. The quantities of mimic or competitor fragments (Clontech) and target cDNA are compared by using a PC SCANJET with analysis by Adobe PHOTOSHOP software (Adobe Systems, Palo Alto, CA). Absolute amounts of cytokine PCR products as determined by competitive PCR were corrected by dividing by amounts of β-actin PCR product. Each sample was repeated twice and values are expressed as the mean ± SEM.
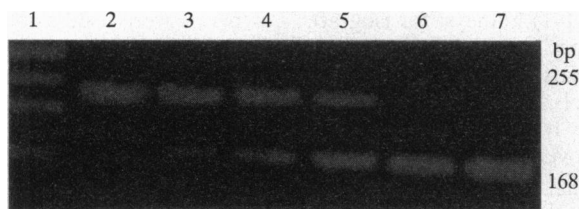

FIG. 1.   Example of serial dilution studies of MCP-1 in renal allografts at 8 weeks with the 255-bp MCP-1 product and 168-bp competitor MCP-1 DNA construct. Lanes: 1, low molecular weight markers; 2–7, MCP-1 competitor DNA construct at 0, 0.001, 0.004, 0.008, 0.02, and 0.1 pg, respectively.

RESULTS

Chronic Allograft Rejection. *Morphology and immunohistology.* Large numbers of CD5$^+$ lymphocytes infiltrate the grafts transiently in the first 2 weeks during a reversible acute rejection episode, diminishing to relatively low and stable levels thereafter ($\approx$30 c/FV) (Table 2). This population was almost never encountered in glomeruli, remaining distributed throughout the interstitium. CD4$^+$ cells predominated over CD8$^+$ cells throughout the course. ED-1$^+$ monocyte/macrophages in high numbers ($\approx$95 c/FV) localized preferentially in glomeruli and perivascular areas between 12 and 16 weeks. After 12 weeks, glomerular sclerosis and intimal proliferation of arteries and arterioles become increasingly evident. The presence of macrophages in glomeruli and around vessels was associated with upregulation of several cytokines, including IL-1α, IL-6, TNF-α, and TGF-β (6).

Cytokine expression in kidney allografts. Semiquantitative competitive RT–PCR was used to measure the induction patterns and relationships of cytokines (Table 1) during the process of chronic rejection of rat kidney allografts. Fig. 1 displays an example of the dilution series performed on a particular cytokine, MCP-1, by using cDNA from a renal allograft at one time period.

Despite immunosuppression with low-dose CyA during the first 10 days after engraftment, the typical components of an acute T-cell-mediated rejection episode were noted (Fig. 2), with increased production of IL-2, IL-2 receptor (IL-2R) β chain, and interferon (IFN) γ. During this early period, IL-2 (peaking at 2.5 times β-actin at 3 days) and IL-2R (peaking at 2 times β-actin at 1 week) are present. IFN-γ is induced in parallel to IL-2 during this initial period (Fig. 2); both are secreted by T$_h$1 T helper cells. In contrast, IL-4, a product associated with T$_h$2 T helper cells, is minimally detectable (0.5 times β-actin expression) (data not shown). T-cell products, which were in a relative period of quiescence, reappear during peak macrophage infiltration between 12 and 16 weeks (Fig. 2), the time during which there are functional changes (proteinuria) and beginning structural changes of chronic rejection (2, 6). At 16 weeks, IL-2 first increases, followed by IL-2R expression at 24 weeks. An initial burst of IL-1α and IL-6 occurs 5 days after transplantation during the acute rejection episode (Fig. 3). IL-2Rs peak 2 days after IL-1α induction (Figs. 2 and 3).

After the acute rejection event, RANTES (regulated upon activation, normal T-cell expressed and secreted), a chemokine for T cells, monocytes, and eosinophils, produced by T cells, mesangial cells, monocytes, fibroblasts, renal tubular epithelial cells (18, 19), and pulmonary endothelial cells (20), has been implicated as important in ongoing inflammatory processes of renal tubular cells (18). It is up-regulated most highly at 2 weeks (Fig. 2). ICAM-1 expression begins to

Table 2. Immunohistology of kidney allografts and isografts

Time, weeks	ED-1, c/FV	CD-5, c/FV	CD-4, c/FV	CD-8, c/FV	MHC-II, c/FV	ICAM-1, c/FV	IL-2R, c/FV
			Kidney allografts				
8	17 ± 2	11 ± 5	ND	ND	ND	ND	ND
12	47 ± 3	25 ± 3	48 ± 9	22 ± 3	54 ± 14	47 ± 5	6 ± 1
16	95 ± 11	37 ± 5	49 ± 7	29 ± 8	78 ± 10	100 ± 4	11 ± 1
24	66 ± 6	30 ± 4	73 ± 9	23 ± 10	60 ± 11	56 ± 5	4 ± 1
			Kidney isografts				
32	30 ± 3	21 ± 1	17 ± 4	10 ± 3	1.5 ± 0	3.0 ± 0	0
52	36 ± 7	26 ± 5	19 ± 4	14 ± 3	2.0 ± 0	3.3 ± 0	0

Time is given as the number of weeks after transplantation. Cells are infiltrating cells, tubular cells, or endothelial cells. Isografts at 8, 12, 16, and 24 weeks had normal morphology. ND, no data; $n = 5$ per time period.

increase at 2 weeks and then peaks at 8 weeks. The presence of this adhesion molecule precedes the induction of cytokines associated with macrophage activation (Figs. 2 and 3).

After the initial induction of RANTES and IFN-γ expression (Fig. 2), MCP-1 begins a burst of expression between 4 and 12 weeks (Fig. 3). MCP-1 is a potent chemotactic factor specific for monocytes and may contribute to the chronic inflammatory response of transplant arteriosclerosis (13). In the present model, its expression exceeds that of any other cytokines tested (10 times β-actin at 12 weeks) and remains high throughout the 52-week follow-up period (Fig. 3).

The peak expression of inducible nitric oxide synthase (NOSi), IL-6, and TNF-α at 16 weeks (Fig. 3) suggests enhanced transcription by cells that have been recruited to the donor kidney (i.e., macrophages). The maximum expression of NOSi follows macrophage activation and is synchronous with IL-6, IL-2, and TNF-α induction. NOSi expression decreases with an associated increase in TGF-β.

The expression of two cytokines, IL-10 and TGF-β, increases slightly in the latter phases of the chronic process, after 32 weeks (Fig. 3). The increased expression of IL-10 in these studies is concomitant with a decrease of NOSi, MCP-1, RANTES, IL-1α, and TNF-α (Figs. 2 and 3). TGF-β slowly increases throughout the course of chronic rejection, reaching its maximum at 52 weeks (the last point sampled).

Renal Isografts. *Morphology and immunohistology.* The role of antigen-independent events in chronic graft dysfunction has been emphasized by the changes developing in long-term isografts that mirror those occurring earlier in chronically rejecting allografts (ref. 21 and Table 2). For 24 weeks after engraftment, isograft morphology remained relatively normal except for a persistent minor periglomerular and perivascular mononuclear cell infiltrate. By 52 weeks, virtually all arteries showed luminal narrowing or obliteration. Over 30% of glo-

meruli had developed segmental or global sclerosis by 72 weeks, and tubular atrophy and interstitial fibrosis were obvious. Immunohistologically, the early cell infiltrate consisted primarily of macrophages (>80%) and small numbers of CD4$^+$ T lymphocytes (<20 c/FV); after 32 weeks, ED1$^+$ macrophages entered vessel walls and adjacent perivascular areas. By 52 weeks, large numbers of macrophages ($\approx$40 c/FV), primarily intraglomerular and around vessels, were noted; IL-1α, IL-6, IL-8, IFN-γ (data not shown), and ICAM-1 expression was also strikingly upregulated during this period.

Cytokine expression in kidney isografts. As the typical T-cell products (IL-2, IL-2R, IL-4, RANTES, and IFN-γ) are minimally expressed in isografts by RT–PCR, any increased expression was not significant (data not shown).

In contrast to allografts, TGF-β expression begins to increase after 8 weeks in isografts (Fig. 4) to $\approx$3 times that of β-actin at 52 weeks, a value higher than that in allografts (Fig. 3). The increased presence of this factor at this late period seems to correlate with the progressive tubular atrophy and fibrosis occurring at that time (21). MCP-1 also is increased at 52 weeks, although 4 times less than in allografts. IL-1α and β-actin reach similar levels at 52 weeks in both isografts and allografts. Figs. 3 and 4 indicate that IL-10 peak expression does not vary significantly between renal allograft and renal isograft samples.

Cytokine Expression in Host Spleen. We analyzed whether the events of chronic rejection were localized primarily in the engrafted donor organ or whether they were also manifest in host lymphoid tissues. Unlike the situation in acute rejection where there is much extraallograft activity (22), we found that every cytokine, regardless of donor organ source, was expressed below the internal control of β-actin in spleens except IL-2R, which was up-regulated to 1.5 times at 52 weeks in the spleens of the allograft recipients (data not shown).
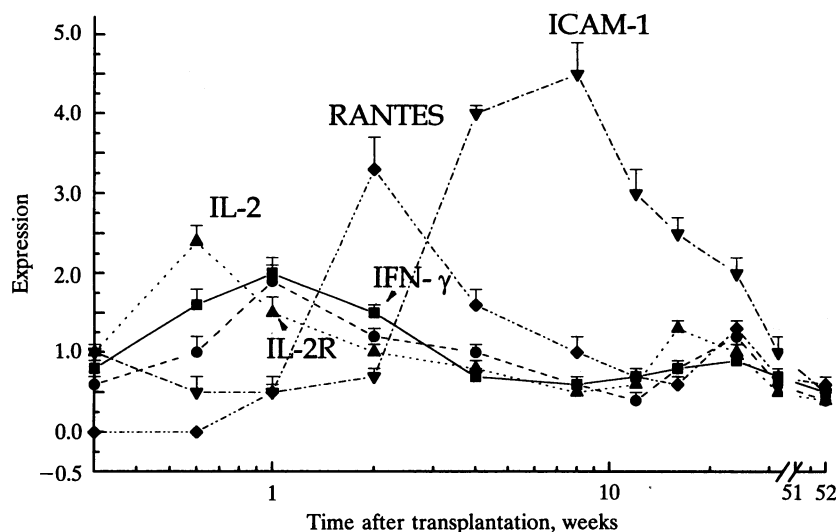

FIG. 2. Cytokine expression in chronically rejecting renal allografts during the early phase. Data are derived from at least three experiments in duplicate, with at least two animals in each group. Expression relative to that of β-actin is shown.
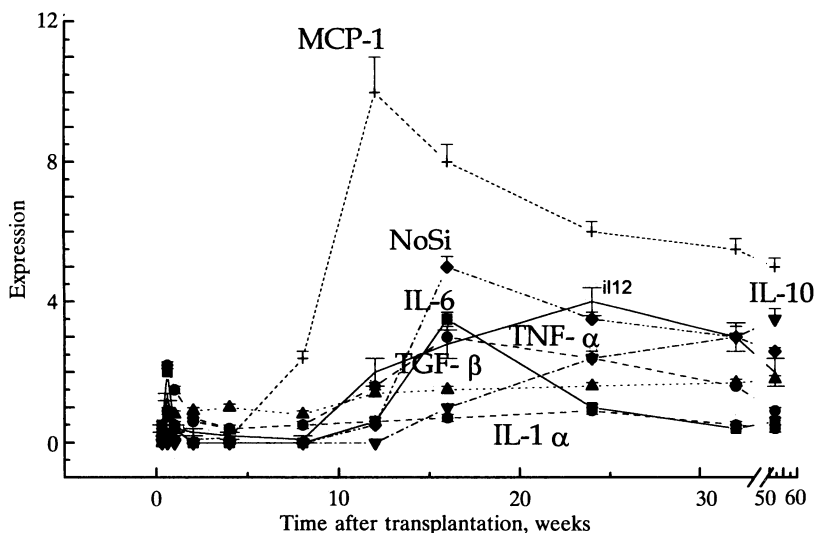
FIG. 3. Cytokine expression in chronically rejecting renal allografts during the late phase. Expression relative to that of β-actin is shown.

DISCUSSION

There is evidence to suggest that two processes contribute to the etiology of chronic rejection in this kidney model. (*i*) The process is primarily an antigen-dependent effect influenced by early immunological injury such as acute rejection and later persistent host alloresponsiveness. (*ii*) Antigen-independent factors may contribute to the progressive changes (2–5). In this study, we confirm by RT–PCR that both influences are important in the pathoimmunology of the chronic process by comparing renal allografts and isografts.

That the advancing course of renal transplant dysfunction may be, at least in part, independent of immune mechanisms has also been suggested by similar changes noted in various forms of chronic kidney failure of disparate etiologies. Potentially influential factors include early injury secondary to prolonged ischemia and reperfusion, the long-term effects of diminished functioning kidney mass, disorders of lipoprotein metabolism, and the nephrotoxic effects of various immunosuppressive drugs and cytomegalovirus infection (2–4). In fact, the term "chronic rejection" may itself be a misnomer; "chronic graft dysfunction" may be a more accurate description, as it is less specific and suggests a multifactorial etiology. Lymphocytes and macrophages may persist in both organ allografts and isografts with and without clinical evidence of dysfunction. In this *in vivo* study, we used an established and reproducible chronic rejection model in rat kidneys that develops progres-

sive changes similar to those in human renal allografts (22, 23) to analyze sequential cytokine expression.

The use of RT–PCR allowed us to compare and contrast the cytokine profiles in allografts and isografts (Figs. 2–4). We found that the up-regulation of T-cell-associated products occurs in the early phase of the chronic rejection model in renal allografts, not isografts (Figs. 2 vs. 4). RANTES peaked at 2 weeks in the renal allograft, after general activation of T cells and expression of IL-2 and IL-2R (Fig. 2). This factor was not significantly detected in the renal isografts (Fig. 4). Another inducible gene product in the early phase of chronic rejection in allografts is ICAM-1. Between 6 and 8 weeks, ICAM-1 becomes maximally expressed (Fig. 2) and is present on glomerular capillary endothelium, renal mesangium, and other renal structures (Table 2). In the renal allografts (Fig. 2), ICAM-1 expression follows RANTES peak expression and immediately precedes MCP-1 expression, presumably directing the position of infiltrating host cell populations within the graft substance. In the isografts, ICAM-1 peaks at 24 weeks to 3 times less expression than in the allografts; this precedes the delayed infiltration pattern of macrophages and the increase in macrophage-associated products (i.e., MCP-1) at 52 weeks in the isograft (Table 2 and Fig. 4).

Several key features were noted in the late-phase analysis of differential cytokine up-regulation in the allograft and isograft models. Macrophages seem particularly important in the etiology of chronic rejection, regardless of immunogenicity of the
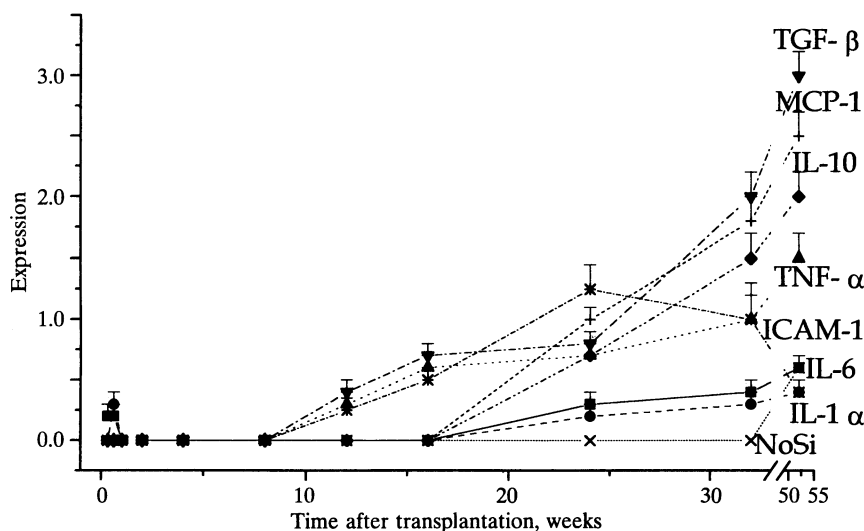

FIG. 4. Cytokine expression in chronically rejecting renal isografts. Expression relative to that of β-actin is shown.

organ. Subendothelial plaques consisting of macrophages in foamy transformation have been noted in chronically rejecting human renal allografts (12). The macrophage product MCP-1 is maximally expressed at 10 times that of β-actin in rat kidney allografts at 12 weeks, preceding macrophage infiltration (Table 2). MCP-1 has been suggested to be a chemotactic and activation factor in studies performed in a cardiac allograft model (13). In isografts, MCP-1 is present and peaks at 52 weeks (last point sampled) but is expressed at 4 times lower levels than in the allograft (Figs. 3 vs. 4). In both the allograft and isograft models, we believe MCP-1 is playing a crucial role in the chemoactivation and chemoattraction of macrophages.

Many cytokines and growth factors are up-regulated in the allografts but not the isografts (Figs. 2–4). NOSi peaks at 16 weeks in allografts but does not reach significant values in the isografts; its production by activated macrophages has been shown to play a key role in the development of rat immune complex glomerulonephritis (24). In our allograft studies, NOSi is expressed after macrophage activation and infiltration into the graft tissues and related temporally to increased tubular atrophy and glomerulosclerosis. Also maximally up-regulated at 16 weeks in the allografts, IL-6 is synthesized by monocyte/macrophages, activated T cells, and other cells in response to IL-1α and TNF-α (25). It is a potent mitogen of glomerular mesangial cells and may contribute to glomerulosclerosis not only in chronic rejection but also in other states of renal dysfunction (25).

One IL, IL-10, is expressed in similar intensities in both allografts and isografts. It is produced by T cells, macrophage, and B cells, has been shown to be a potent suppressor of macrophage functions (26), and has been associated with the induction of peripheral tolerance to alloantigens (9). IL-10 may also function as a potent modulator of monocyte/macrophage function and may suppress the production of proinflammatory cytokines such as TNF-α, IL-1α, IL-6, and IL-8 (8, 9). Our studies demonstrate increased IL-10 expression in both renal allografts and isografts.

We have shown by immunohistology that TGF-β is increased in allografts and isografts at 16 and 32 weeks, respectively, and is associated with fibrosis and tubular atrophy (6, 20). It is of interest that TGF-β peaks to 1 times that of β-actin in the allograft yet peaks to 3 times that of β-actin in isografts. This cytokine has been associated primarily with fibrogenesis and fibrosis, which are the end results of chronic graft dysfunction, regardless of type of organ (27). Recently, Khanna *et al.* (28) have reported that CyA enhances the production of functionally active TGF-β in T-cell cultures; this enhanced TGF-β expression could contribute to renal disease.

By knowing the cytokine expression patterns in the T-cell-dominated early phase and in the macrophage-mediated late phase of chronic rejection, one could construct a model for the process that incorporates both antigen-dependent and antigen-independent events. At first, T cells are activated by alloantigen and secrete RANTES along with other cytokines; this could be a specific local chemoattractant within the renal interstitium to cause cell adherence to the vascular endothelium. T cells and monocytes are attracted to the site of inflammation, bind to the endothelium, and then enter the graft substance. RANTES may facilitate T-cell and macrophage accumulation by direct chemotactic effects or by regulating the expression of macrophage products. Once activated macrophages have infiltrated the tissues and released IL-1α and TNF-α, the expression of RANTES by renal tubular epithelial cells and by mesangial cells in turn would increase. Macrophages and their products, particularly IL-6, NOSi, and MCP-1, may change the function of contractile mesangial cells in the glomerulus, increase their proliferation, and cause overproduction of the mesangial matrix. Gradual functional deterioration secondary to the development of glomerulosclerosis and vascular obliteration may also produce systemic

hypertension, which may itself cause the remaining functioning glomeruli to hyperfilter before they eventually fibrose, establishing a self-sustaining vicious cycle.

Our semiquantitative competitive RT–PCR experiments noting the sequential patterns of cytokine expression among allografts and isografts may offer several points of insight into the etiology of chronic dysfunction. We demonstrate that an early phase of chronic rejection where T-cell-associated cytokines predominate is followed by a late phase in which macrophage-associated cytokines dominate. RANTES could play an important intermediary role between the two phases and may be a link in inducing chronic changes.

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