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Immunoexpression of perforin and granzyme B on infiltrating lymphocytes in human renal acute allograft rejection

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SUMMARY

Graft destruction can be effected by direct cell-to-cell contact between activated effector T cell and a target graft resulting in delivery of cytotoxic molecules. Perforin and granzyme B can be used as activation markers for cytotoxic cells in allograft tissue. The aim of the study was to determine the immunoexpression of perforin and granzyme B by immune cells infiltrating renal tissue during acute allograft rejection and to evaluate any correlation between the phenotype of infiltrating lymphocytes and cells expressing cytotoxic granules as well as the severity of graft damage as defined by Banff 97 criteria. Immunoperoxidase staining was carried out using monoclonal antibodies anti- perforin, -granzyme B, -CD3 and -CD8 on renal allograft biopsy specimens from twenty one patients with acute renal transplant rejection: Banff 97 IA (n = 11) and Banff 97 IB (n = 10). As a control 11 biopsy specimens of renal transplant patient without any signs of rejection were used. All allograft biopsy specimens with acute renal transplant rejection contained a high number of CD3 + T cells (Banff IA: 437.4 ± 154.4 and Banff IB: 825 ± 339.9 vs *123.4 ± 52.5 in controls) and CD8 + T lymphocytes (Banff 97 IA: 177.6 ± 89.2 and Banff IB: 293.2 ± 112.4* vs *64.2 ± 37.1 in controls). Immunostaining for granzyme B and perforin was negative in controls. The immunopositivity for perforin was similar in Banff IA and Banff IB acute allograft rejection (1.5 ± 0.6* vs *1.8 ± 0.8, respectively). Granzyme B+ cell count was significantly higher in severe rejection group Banff IB (128.3 ± 74.3) than in Banff IA group (48.2 ± 18.3). Moreover, in acute allograft rejection Banff IB the number of granzyme B+ cells and perforin+ cells was correlated with the number of CD8 + T cells. In conclusion, our results suggest that in acute tubulointerstitial allograft rejection activated cytotoxic T lymphocytes play a major role. The strong immunopositivity for granzyme B on infiltrating cells in renal transplant tissue is suggested as a marker of severity of graft damage.*

Key words: ***Acute allograft rejection. Granzyme. Perforin. Cytotoxic T lymphocytes.***

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INMUNOEXPRESIÓN DE LA PERFORIZA Y LA GRANZIMA B EN LOS LINFOCITOS DEL INFILTRADO DEL RECHAZO AGUDO EN EL RIÑÓN HUMANO TRASPLANTADO

La destrucción del injerto renal se puede efectuar mediante el contacto directo célula-célula entre el linfocito T activado y una célula diana, que va a dar lugar a una liberación de moléculas citotóxicas. La perforina y la granzima B se pueden utilizar como marcadores de activación de las células citotóxicas en el tejido trasplantado. El objetivo de este estudio fue determinar la inmunoexpresión de la perforina y la granzima B por parte de los linfocitos que infiltran el tejido renal durante el rechazo agudo, y valorar la existencia de algún tipo de correlación entre el fenotipo de los linfocitos infiltrantes y las células que expresan gránulos citotóxicos, asi como con la severidad del daño del injerto definido por los criterios Banff 97. La tinción de la immunoperoxidasa se hizo con anticuerpos monoclonales anti- perforina, -granzima B, -CD3 and -CD8 en biopsias de 21 enfermos con rechazo agudo: Banff 97 IA (n = 11) y Banff 97 IB (n = 10). Como control se usaron 11 biopsias de enfermos trasplantados sin signos de rechazo. Todas las biopsias con rechazo agudo contenían un gran número de células CD3 + T (Banff IA: 437,4 ± 154,4 y Banff IB: 825 ± 339,9 vs *123,4 ± 52,5 en controles) y linfocitos CD8+T (Banff 97 IA: 177,6 ± 89,2 y Banff IB: 293,2 ± 112,4* vs *64,2 ± 37,1 en controles). La tinción para granzima B y perforina fue negativa en los controles. La positividad para la perforina fue similar en los rechazos agudos Banff IA y Banff IB (1,5 ± 0,6* vs *1,8 ± 0,8, respectivamente). El recuento de células granzima B+ fue significativamente superior en el grupo con rechazo agudo Banff IB (128,3 ± 74,3) que en los Banff IA (48,2 ± 18,3). Además, en los rechazos agudos Banff IB, El número de células granzima B+ y perforina + se correlacionó con el número de células CD8 + T. En conclusión, nuestros resultados sugieren que en el rechazo tubulointersticial agudo, los linfocitos T citotóxicos juegan un papel fundamental. Se sugiere que la fuerte positividad para la ganzima B en las células que infiltran el tejido renal es un marcador de severidad del daño causado al injerto.*

Palabras clave: ***Rechazo agudo. Granzima. Perforina. Linfocitos T citotóxicos.***

INTRODUCTION

Despite improved immunosuppressive regimens, renal transplantation is still complicated by acute rejection episodes, and what is more acute rejection has been shown to be the strongest predictive factor of subsequent chronic rejection[1]. Cytotoxic T lymphocytes are major mediators of allograft rejection. Graft destruction can be effected by direct cell-to-cell contact between activated effector T cell and a target graft resulting in delivery of cytotoxic molecule. Two principal mechanisms of T-cell mediated damage have been discovered: perforin - granzyme dependent killing and Fas - Fas-ligand dependent killing[2]. Perforins and granzymes are soluble mediators contained in the lysosome-like granules of cytotoxic T lymphocytes (CTLs). The role of perforin in cytotoxicity may be direct by forming holes in the target cell membrane and indirect by increasing the porosity of the target cell membrane and thereby enhancing the entry of granzymes[3]. «Granule enzymes» or granzymes, a family of serine proteases are stored as active enzymes, ready for exocytic release upon contact with target cell[4]. Several cell population may mediate cellular cytotoxicity: CD3+ CD8+ cytotoxic T lymphocytes, CD3+ CD4 + T helper cells and natural killer (NK) cells, thus the identification of effector cells such cytotoxic lymphocytes may enhance diagnosis or predict later dysfunction[5,6]. Tubulitis and vasculitis are the cardinal features of the rejection. Type I acute allograft rejection according to Banff classification is thought to be manifestations of cell-mediated rejection. In view of the above, the immunohistochemical analysis of cytotoxic granule protein may be useful in the evaluation of renal tissue in acute allograft rejection. The aim of the present study was to determine the immunoexpression of perforin and granzyme B by lymphocytes infiltrating renal tissue during acute allograft rejection. Moreover, relationship between the phenotype of infil-

trating T lymphocytes, and cells expressing cytotoxic granules as well as the severity of graft damage as defined by Banff 97 criteria was performed. Computerized image analysis of immunopositive cells was used to diminish subjectivity in evaluating the results.

MATERIAL AND METHODS

Patients

Twenty one renal biopsy specimens from patients with acute allograft rejection (AAR) were examined by percutaneous renal biopsy. As a control group 11 tissue specimens of renal transplant patients without any signs of rejection were used. All biopsies had been performed solely for diagnostic purposes. All of our patients were adults. In patients with acute allograft rejection the mean age was 40.2 ± 3.8, and male to female ratio was 15:6. In control group the mean age was 45.4 ± 8.5 and the male to female ratio was 8:3. The specimens were taken from 12 days to 5 months after engraftment (mean = 58.5 days). Acute rejection was considered clinically when patients showed unexplained increase in serum creatinine concentration. Morphological diagnosis of AAR was established independently by two experienced nephropathologists according to Banff 97 criteria[7] and based on light microscopy and immunofluorescence using standard protocols. Eleven specimens had Banff IA score: cases with interstitial infiltration (> 25% of parenchyma affected) and foci of moderate tubulitis (> 4 mononuclear cells/tubular cross section or group of 10 tubular cells). Ten specimens had IB score: cases with interstitial infiltration (> 25% of parenchyma affected) and foci of severe tubulitis (> 10 mononuclear cells/tubular cross section or group of 10 tubular cells). In all cases the standard immunosupression protocol was used.

Immunohistochemistry

Paraffin sections were mounted onto superfrost slides, deparaffinized and antigen retrieval Streptavidin-biotin complex technique was employed. After rehydratation sections were reacted for 5 minutes with 3% hydrogen peroxide in distilled water and rinsed in Tris-buffered saline (TBS). Then, slides were incubated with monoclonal mouse anti-human granzyme B antibody (Clone GrB-7, DAKO, Glostrup, Denmark, dilution 1:50), monoclonal mouse anti-human perforin antibody (NCL-Perforin, Clone 5B10, Novocastra Lab. Ltd., UK, dilution 1:20), monoclonal mouse anti-human CD3 T cell antibody (DAKO, Glostrup, Denmark, Clone PC3/188A, dilution 1:50), and monoclonal mouse anti-human CD8 T cell antibody (DAKO, Glostrup, Denmark, Clone C8/144B, dilution 1: 25) in a moist chamber for 1 h at room temperature. Afterwards, sections were rinsed in TBS, and DAKO LSAB+/HRP Universal kit (Glostrup, Denmark) was used according to instruction of manufacturer. The positive immunoreactivity was visualised with DAB as chromogen. After washing in distilled water, sections were counter-stained with hematoxylin and coverslipped. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

Quantification of immunopositive cells were determined by counting in the renal tissue (glomeruli, tubuli and interstitium) CD8 + cells, CD3 + cells, perforin+ cells and granzyme B+ cells (semiautomatic function) in a sequence of ten consecutive computer images of 400x high power fields-0.0047 mm^2 each. Only immunoreactive cells with the clear identifiable nuclei were counted. Cells were scored positive when displayed a distinctly brown membrane or showed brown granular cytoplasmic pattern. The results were expressed as a mean number of immunopositive cells per mm^2. In each specimen staining intensity was recorded by two independent observers. The mean grade was calculated by averaging grades assigned by the two authors and approximating the arithmetical mean to the nearest unity.

Statistical methods

All data are expressed as mean ± SD (standard deviation). Differences between groups were tested using unpaired Student's test preceded by evaluation of normality and Levene's test. The Mann-Whitney U test was used where appropriate. Results were considered statistically significant if $P < 0.05$.

RESULTS

The number of CD3 + T lymphocytes, CD8 + T lymphocytes, cells containing cytotoxic granules: perforin and granzyme B in renal tissue in acute allograft rejection and in controls is summarized in table 1. In control group no positive immunostaining for perforin and granzyme B was seen. The intensity of perforin and granzyme B immunostaining was variable in renal tissue in patients with acute allograft rejection. Lymphocytes expressing cytotoxic molecules showed granular cytoplasmic staining pattern.

Table I. Number of perforin+ cells, granzyme B+ cells, CD3+T lymphocytes and CD8+T lymphocytes in controls and in renal biopsies with acute allograft rejection (AAR) classified as Banff 97 IA and IB

	Perforin+ cells/mm²	Granzyme B+ cells/mm²	CD3+T cells/mm²	CD8+T cells/mm²
Controls (n = 11)	0.0	0.0	123.4 ± 52.5	64.2 ± 37.1
AAR Banff IA (n = 11)	1.5 ± 0.6	48.2 ± 18.3	437 ± 154.4	177.6 ± 89.2
AAR Banff IB (n = 10)	1.8 ± 0.8	128.3 ± 74.3	825 ± 339.9	293.2 ± 112.4
P values:	$P < 0.0005^{(1)}$	$P < 0.0001^{(1)}$	$P < 0.0001^{(1)}$	$P < 0.001^{(1)}$
	$P < 0.0003^{(2)}$	$P < 0.0001^{(2)}$	$P < 0.0001^{(2)}$	$P < 0.0001^{(2)}$
	$P = 0.3$ (NS)$^{(3)}$	$P < 0.003^{(3)}$	$P < 0.005^{(3)}$	$P < 0.02^{(3)}$

[1]controls *vs.* AAR Banff IA, [2]controls *vs.* AAR Banff IB, [3]AAR Banff IA *vs.* AAR Banff IB
Data are expressed as mean ± SD.

These cells were scattered in the renal tubular epithelium and interstitium (fig. 1). The cells expressing perforin and granzyme B were also shown within glomeruli. In renal acute allograft rejection determined as Banff IB clusters of granzyme B positive cells were seen in places of severe tubulointerstitial damage (fig. 2). The perforin positive cell count was similar in Banff IA and in Banff IA renal allograft biopsy (1.5 ± 0.6 *vs* 1.8 ± 0.8, respectively). The number of granzyme B positive cells was significantly higher in AAR Banff IB group than in AAR Banff IA group (128.3 ± 74.3 *vs* 48.2 ± 18.3, respectively. $P < 0.003$).

All renal allografts tissue showed extensive infiltration with CD3+ and CD8 + T lymphocytes (figs. 3 and 4) in comparison with controls. Statistical analysis revealed significant differences in the number of CD3 positive T cells and CD8 positive T cells between AAR Banff IA and AAR Banff IB groups. In

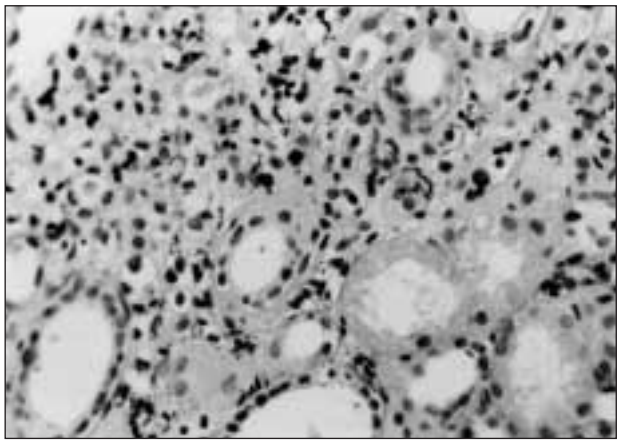

Fig. 2.—Immunostaining with anti-granzyme B monoclonal antibody. Numerous granzyme B immunopositive cells in places of severe tubulointerstitial damage in renal acute allograft rejection determined as Banff IB (x 400).

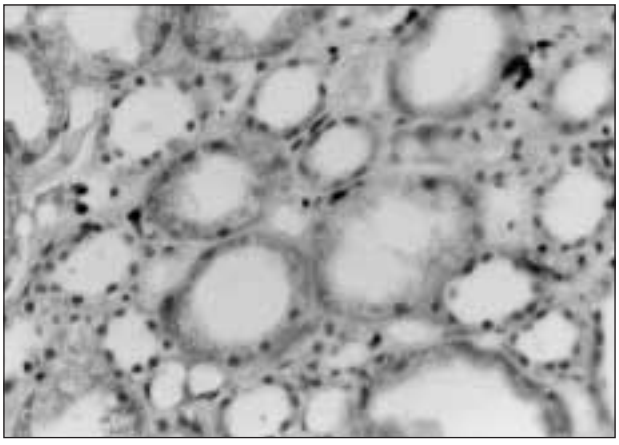

Fig. 1.—Immunostaining with anti-perforin monoclonal antibody in biopsy specimen with acute allograft rejection classified as Banff IA. Perforin immunopositive cells are scattered in the renal interstitium and tubular epithelium (x 400).

AAR Banff IB renal tissues mean number of CD3 + cells was higher than in AAR Banff IA group (825 ± 339.9 *vs* 437 ± 154.4, respectively, $P < 0.005$). Moreover, CD8+ cells count was significantly higher in severe rejection group (in Banff IB: 293.2 ± 112.4, and in Banff IA: 177.6 ± 89.2, $P < 0.02$). The relationship between perforin immunopositive cells, granzyme B immunopositive cells and CD3+ as well as CD8+ T lymphocytes appear from table II. Statistical analysis revealed significant positive correlation between perforin+ cells and CD8+ T cells ($P < 0.03$), and between granzyme B+ cells and CD8+ T lymphocytes ($P < 0.002$) in renal tissue in Banff IB acute allograft rejection. In acute renal allograft rejection Banff IA these correlations were also positive but they not reached statistical significance. As is shown in table II no correlation between the number of granzyme B+ cells and CD3+ T cells was found, as well as between perforin+ cells and CD3+ T cells.

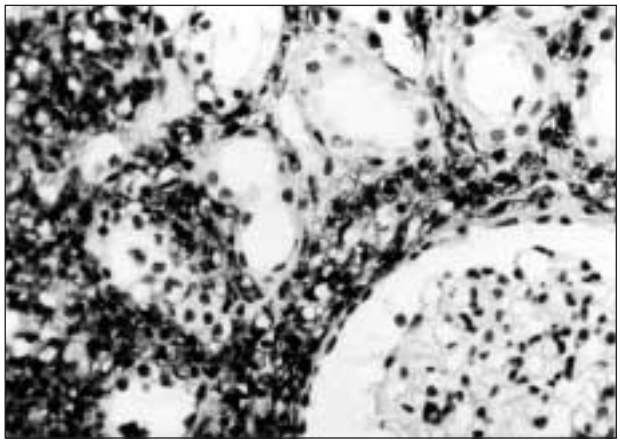

Fig. 3.—Immunoperoxidase staining with monoclonal antibody anti-CD3. Clusters of CD3+T lymphocytes in acute renal allograft rejection type Banff IB (x 400).

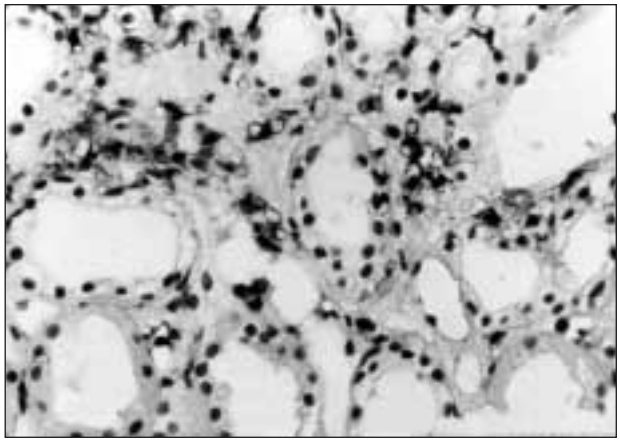

Fig. 4.—Immunoperoxidase staining for CD8+T lymphocytes in the renal interstitium and tubular epithelium in acute allograft rejection Banff IA (x 400).

DISCUSSION

The presence of activated lymphocytes are considered an important indicator of acute rejection. Approximately 50% of infiltrating cells in acute allograft rejection are CD3 + T cells, and both CD8+ and CD4 + T-cell subsets are present in varying proportions. CD8 + T cells often associated with tubulitis may be one of the major effector cells involved in target cell killing effectuated by perforin or release of granzymes[8]. It is commonly accepted that the secretion of the granzymes is induced by target cells during the cytotoxic cell response[9]. Biopsies obtained from well-functioning renal allografts at 3 and 6 months have frequently shown mononuclear leukocytic infiltrates[10,11] without heightened expression for cytokines, perforin or granzyme B[12]. The present study revealed immunopositivity for perforin and granzyme B in cells infiltrating renal tissue in patients with acute tubulointerstitial allograft rejection. In control group of renal transplant patients without any signs of rejection no immunopositivity for cytotoxic granule proteins were seen. Similar results in granzyme B immunopositivity in renal tissue with allograft rejection obtained Kummer et al.[13]. In study of Grimm et al.[14] immunohistochemical quantification of perforin expression showed no significant difference between normal biopsies, subclinical rejection and clinical rejection, although there was a trend toward an increase in perforin detection in both forms of rejection compared with normal biopsies. Since granzymes and perforin constitute the primary effector cells of the granule egzocytosis pathway, identification of these proteins by immunohistochemical or molecular biologic techniques has been used to assess the involvement of activated CTLs or NK cells in pathologic processes[15-17]. Molecular analyses of human renal allografts revealed that intragraft display of mRNA encoding granzyme B and perforin correlate with acute rejection[18,19]. The expression of perforin mRNA, when used in combination with other cytotoxic markers such as, granzyme B and Fas ligand, has been shown to discriminate between normal biopsies and those with early postransplantation clinical rejection[20,21]. Lipman et al.[22] pointed that normal biopsies have the lowest, clinical rejection the highest, and sublinical rejections an intermediate amount of perforin mRNA

Table II. The correlations between cells with immunopositivity of perforin and granzyme B and T lymphocytes in renal biopsies with acute allograft rejection (AAR) Banff IA and IB

Correlation between	AAR Banff IA	AAR Banf IB
Perforin+ cells and CD8+ T lymphocytes	r = 0.56, P = 0.07 ,(NS)	r = 0.69, P < 0.03
Perforin + cells and CD3+ T lymphocytes	r = 0.12, P = 0.12, (NS)	r = 0.23, P = 0.52, (NS)
Granzyme B + cells and CD 8+ T lymphocytes	r = 0.48, P = 0.13, (NS)	r = 0.73, P < 0.002
Granzyme B+ cells and CD3 + T lymphocytes	r = 0.03, P = 0.9, (NS)	r = 0.31, P = 0.38, (NS)

expression. In our study, the perforin positive cell count was similar in Banff IA and Banff II group, meanwhile the number of granzyme B positive cells was significantly higher in Banff IB group than in renal allograft tissues classified as Banff IA. According to Banff 97 criteria acute renal allograft rejection type Banff IB is characterized by severe tubulitis (t3-foci with > 10 cells/cellular cross section) and the presence of mononuclear cell interstitial inflammation (at least i2- 26-50% of parenchyma inflammed). In renal acute allograft rejection type Banff IA the interstitial infiltrates are less abundant and only foci of moderate tubulitis are detected. Showed in our study high immunopositivity for granzyme B in AAR Banff IB may suggest that cytotoxic cells contained granzyme B are responsible for more severe graft damage. There is no such relationship between perforin immunopositivity and the intensity of graft destruction, although in renal tissue in acute tubulointerstitial allograft rejection the immunopositivity for perforin on infiltrating cells was significantly higher in comparison with normal controls. The presence of perforin-associated cytotoxic cells in allograft rejection suggests their role in the disease process. Sharma and et al.[23] revealed a direct correlation between the histological severity of acute rejection and intrarenal coexpression of mRNA encoding granzyme B and perforin Fas ligand, and Fas, however in several studies no correlation between the severity of allograft damage and the number of granzyme A positive cells or mRNA expression of granzyme B and perforin was found[13, 24, 25]. The differences in tissue processing, counting of perforin and granzyme B immunopositive cells, as well antibodies used may reflect differences between our results and others. The present study demonstrated that the number of granzyme B positive cells and perforin positive cells correlated significantly with the CD8 + T lymphocytes count in AAR Banff IB, but not in mild tubulointerstitial damage classified as AAR Banff IA. It is worthy of note that in AAR Banff IA these correlations were also positive, but they did not reach statistical significance. Our results did not reveal the correlations between immunopositivity of perforin or granzyme B and CD3 + T lymhocytes number. These findings may suggest that a majority of cells containing cytotoxic granules reflect CD8 T lymphocytes, although double immunostaining method may clarify this phenomenon. Robertson et al.[26] revealed cells expressing perforin mRNA in severely damage tubular areas in renal allograft rejection and considerable proportion of infiltrating cells was CD8+. In the study of Kummer et al.[13] phenotypic analysis of renal transplant tissues showed that granzymes A and B were expressed by CD3+ cells, representing cytotoxic lymphocytes and by CD56+ NK cells. Unfortunately, these authors did not study the CD8+ cells count. It was revealed that the diffuse cortical CD8+ infiltrate is associated with graft loss within the 10 weeks[27]. CD8+ cells are relatively resistant to the immunosuppressive drugs, or mediate more severe injury. It was shown that cyclosporine treatment did not prevent graft infiltration, but lowered the frequency of CD8+ cells expressing perforin and granzyme[28]. Our study revealed that in AAR Banff IB renal tissues the mean number of CD3 + T, CD8 + T lymphocytes and granzyme B+ cells was higher than in AAR Banff IA group. What is more, in this group statistical analysis revealed correlation between granzyme B+ cells, perforin+ cells and CD8 + T cells. All these findings point that in acute tubulointerstitial allograft rejection with severe destruction of renal tissue activated cytotoxic T lymphocytes play a major role.

In summary, the present study revealed enhanced immunopositivity of perforin and granzyme B in infiltrating cells in acute renal allograft rejection as compared with normal renal tissue. In acute renal tubulointerstitial allograft rejection the detection of strong immunopositivity of granzyme B in activated infiltrating cells is suggested as marker of severity of graft damage.

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