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DOI
10.1086/314629

Publication date
1999

Published in
The Journal of Infectious Diseases

Expression of Granzyme B during Primary Cytomegalovirus Infection after Renal Transplantation

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$CD8^+$ T cells employ granzyme B (GrB) to induce apoptosis in target cells. Increased expression of GrB has been put forward as a diagnostic marker in transplant rejection and viral infection. Three-color flow cytometric analysis revealed that peripheral blood $CD8^+$ T lymphocytosis during primary cytomegalovirus infection after renal transplantation resulted from expansion of a $CD8^+GrB^+CD62L^+$ T cell subset that was almost absent during stable transplant function or acute rejection. This expansion coincided with a temporary increase in systemic soluble GrB (sGrB) levels. No such increase was observed during stable transplant function or acute rejection. Thus, the primary immune response to cytomegalovirus infection is accompanied by appearance of $CD8^+GrB^+CD62L^+$ T cells and increased sGrB levels in the peripheral blood compartment. Determination of the latter may provide a novel approach for monitoring viral infections.

Cytotoxic T lymphocyte (CTL)–mediated killing is, at least in part, induced through delivery of the serine protease granzyme B (GrB) from cytoplasmic granules of activated CTL to the cytosol of the target cell [1]. GrB induces target cell apoptosis through activation of the intracellular cascade of caspases [2]. GrB expression can be used as marker for CTL capable of cytotoxicity in vivo and has been put forward as a potential diagnostic marker of transplant rejection and viral infection [3]. Both acute rejection after solid organ transplantation and cytomegalovirus (CMV) pneumonia complicating lung transplantation have been associated with infiltration of the graft by GrB-expressing CTLs [4–6]. CMV infection is an important infectious complication after transplantation and is characterized by expansion of activated $CD8^+$ T cell subsets [7–9].

Recently, we developed monoclonal antibodies (MAbs) directed against native GrB suitable for flow cytometry and quantitative ELISAs [10, 11]. We questioned whether acute renal allograft rejection or posttransplant primary CMV infection are characterized by increased presence of GrB in peripheral blood. In a prospective cross-sectional study and by three-color flow cytometry, the number of $CD8^+GrB^+$ T cells coexpressing the lymph node–homing receptor CD62L (L-selectin) was analyzed during stable transplant function, acute rejection, or primary CMV infection. Soluble GrB (sGrB) levels were also retrospectively analyzed in longitudinally obtained plasma samples from renal allograft recipients experiencing stable transplant function, acute rejection, or primary CMV infection.

Materials and Methods

Patients. GrB-expressing $CD8^+$ T cells were analyzed in a prospective cross-sectional study of 22 renal allograft recipients (14 males, 8 females; median age, 41 years; range, 11–61). Two patients had experienced an acute rejection episode prior to primary CMV infection and were analyzed separately for either event.

Group 1 comprised 8 patients during stable transplant function as defined by stable plasma creatinine levels and creatinine clearance of $\geq$30 mL/min over at least 5 years (median time after transplantation, 106 months; range, 79–164). Patients did not experience acute rejection at time of analysis as assessed by clinical and routine chemical evaluation, nor did they have viral infections as assessed by routine serologic assays and buffycoat and urine cultures. The second group consisted of 8 patients with acute rejection (median time after transplantation, 22 days; range, 6–152). The diagnosis of acute rejection was based on clinical manifestations and confirmed by histologic examination of a core biopsy. Patients did not experience concurrent viral infections at the time of acute rejection. The third group consisted of 8 patients studied within the first 2

Received 11 June 1998; revised 14 October 1998.

Presented in part: 4th International Symposium on Clinical Immunology, Amsterdam, June 1997; 13th European Immunology Meeting, Amsterdam, June 1997; 34th Congress of the EDTA-ERA, Geneva, September 1997; 5th Basic Sciences Symposium, Chautauqua, New York, September 1997.

Informed consent was obtained from all patients or their parents. The study was approved by the medical ethics review board, Academic Medical Center.

Financial support: Dutch Kidney Foundation (grant C93.1278).

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The Journal of Infectious Diseases 1999; 179:693–6

0022-1899/99/7903-0022$02.00

weeks after diagnosis of primary CMV infection (median time after transplantation, 44 days; range, 32–636). These 8 patients were seronegative for anti-CMV IgM and anti-CMV IgG antibodies before transplantation and all had received transplants from CMV-seropositive donors. The diagnosis of primary CMV infection was based on the appearance of anti-CMV IgM antibodies and positive buffycoat cultures. At the time of analysis, patients did not experience acute rejection or viral infections other than CMV. All patients received basic immunosuppressive treatment consisting of prednisolone and cyclosporin. Heparinized blood samples were obtained and peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep density-gradient centrifugation.

The longitudinal course of plasma sGrB levels was studied retrospectively in a separate group of 25 renal allograft recipients (17 males, 8 females; median age 44 years, range 19–63). EDTA plasma samples were obtained just before transplantation, 3 times a week during hospitalization, and once a week after discharge, up to 6 months after transplantation, and stored at −20°C until assayed. Eight patients were characterized by stable transplant function over time, as defined by rapid restoration of renal function after transplantation, subsequent stable renal function, and absence of viral infections. Eleven patients had experienced ⩾1 acute rejection episode after transplantation. Acute rejection was diagnosed as described above. Only first acute rejection episodes were analyzed. Rejection treatment consisted of a 6-day course of 500 mg of methylprednisolone. Concurrent viral infections were absent during all first acute rejection episodes. Seven patients had experienced primary CMV infection after transplantation. Primary CMV infection was diagnosed as described above. At the time of primary CMV infection, no signs of acute rejection or viral infections other than CMV were present. One patient had a first acute rejection episode prior to primary CMV infection and was analyzed separately for either event.

MAbs. MAbs GrB-10 and GrB-11 were raised against native GrB purified from the human NK cell line YT-INDY. In the sGrB ELISA, unlabeled GrB-11 MAb and biotinylated GrB-10 MAb were used as coating and detecting antibody, respectively [11]. Primary MAbs used in flow cytometry were phycoerythrin (PE)-labeled GrB-11 [10], fluorescein isothiocyanate (FITC)-labeled MAb directed against CD62L (Becton Dickinson, San Jose, CA), and R-phycoerythrin-cyanine 5 (RPE-Cy5)–labeled MAb directed against CD8 (Dako, Glostrup, Denmark). FITC-labeled IgG2a and PE-labeled IgG1 MAb directed against keyhole limpet hemocyanin (Becton Dickinson) and RPE-Cy5–labeled IgG1 MAb directed against a nonbiologic hapten (Immunotech, Marseille, France) were used as isotype control.

Flow cytometric analysis. Three-color flow cytometry for identification of GrB-expressing cells among PBMC was performed as described previously [10].

sGrB ELISA. The ELISA for determination of sGrB levels in EDTA plasma samples was done as described previously [11].

Statistical analysis. Data are presented as median (range). Unpaired and paired data were statistically analyzed using the Mann-Whitney *U* test and the Wilcoxon signed rank test, respectively. $P < .05$ was considered significant.

A cutoff level for sGrB was defined as the highest sGrB level measured in 121 posttransplant plasma samples obtained from 8 patients with stable transplant function. For statistical analysis of sGrB levels during first acute rejection episodes, the day of start of rejection treatment (median time after transplantation, 11 days; range, 7–44) was designated day 0. Most patients experienced their first acute rejection episode during hospitalization, when blood samples were obtained 3 times a week. Therefore, sGrB levels preceding start of rejection treatment were analyzed in 3-day periods: days −8/−6, −5/−3, and −2/0. For analysis of the effect of rejection treatment, sGrB levels at days −2/0 were compared with sGrB levels in the first plasma samples obtained after rejection treatment. For statistical analysis of sGrB levels during primary CMV infection, the day that sGrB peak levels were reached (median time after transplantation, 40 days; range, 27–52) was arbitrarily designated day 0. The majority of patients experienced primary CMV infection after discharge from the hospital, when blood samples were obtained once a week. Therefore, sGrB levels preceding peak levels were analyzed in 7-day periods: days −27/−21, −20/−14, −13/−7, and −6/0.

Results

Flow cytometric analysis. During posttransplant primary CMV infection, there was a significant increase compared with both stable transplant function and acute rejection in $CD8^+$ T cells (shown as cells per cubic millimeter and range): stable transplant function, 368 (124–647); acute rejection 210, (77–

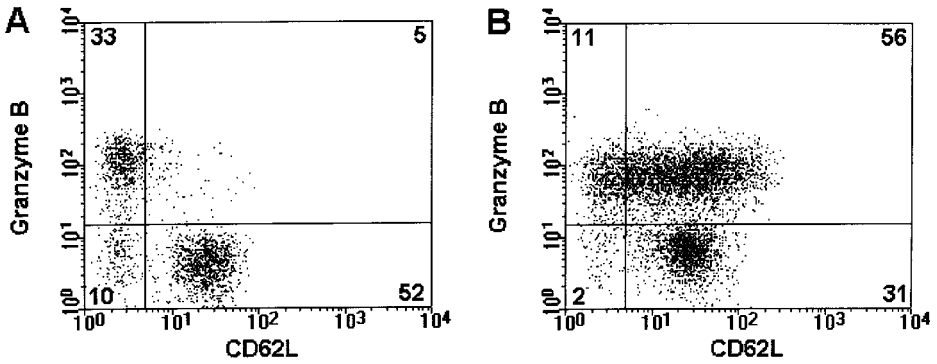

Figure 1. Representative expression patterns of granzyme B (GrB) and CD62L in peripheral blood $CD8^+$ T cells from renal allograft recipients during *(A)* acute rejection and *(B)* posttransplant primary CMV infection.

1059); and primary CMV infection, 694 (226–2458). Figure 1 shows representative expression patterns of GrB and CD62L in the $CD8^+$ T cell subset. CD62L was nearly absent on $CD8^+GrB^+$ T cells during acute rejection (figure 1A) but was present on most $CD8^+GrB^+$ T cells during posttransplant primary CMV infection (figure 1B). No differences in distribution of CD62L on $CD8^+GrB^+$ T cells were observed between patients with stable transplant function or acute rejection. During posttransplant primary CMV infection, a significant increase compared with both stable transplant function and acute rejection was observed in the number of $CD8^+GrB^+CD62L^+$ T cells/mm^3: stable transplant function, 34 (12–155); acute rejection, 23 (6–159); and primary CMV infection, 420 (138–2026). $CD8^+GrB^+CD62L^-$ T cell counts did not differ among the 3 groups: stable transplant function, 78 cells/mm^3 (26–272); acute rejection, 41 (6–318); and primary CMV infection, 74 (14–243); indicating that expansion of the $CD8^+$ T cell subset during posttransplant primary CMV infection resulted from generation of $CD8^+GrB^+CD62L^+$ T cells.

sGrB levels. In 121 posttransplant plasma samples from 8 patients with stable transplant function, the sGrB level was 1 pg/mL (1–23). Thus, a cutoff level for sGrB was set at 23 pg/mL.

Figure 2 shows sGrB levels in patients experiencing either acute rejection or primary CMV infection. No increase in sGrB level was observed during acute rejection (figure 2A). The sGrB

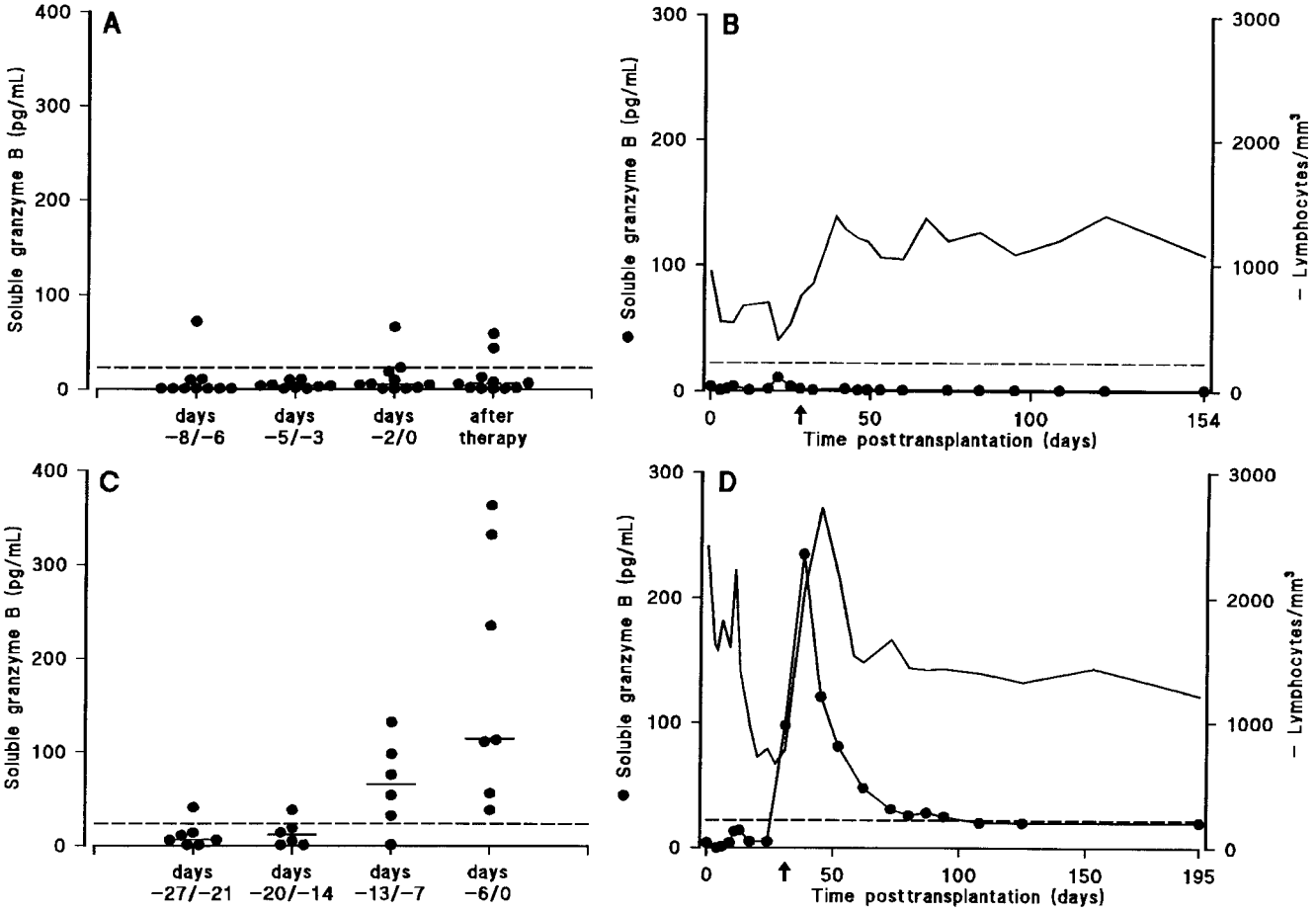

Figure 2. Peripheral blood soluble granzyme B (sGrB) levels and lymphocyte counts in renal allograft recipients experiencing rejection *(A, B)* or posttransplant primary CMV infection *(C, D)*. All panels, broken line represents sGrB cutoff level (23 pg/mL). Horizontal bars show medians. *A,* Peripheral blood sGrB levels in 11 renal allograft recipients. Rejection was treated with 6-day course of 500 mg of methylprednisolone; day 0 is start of treatment. sGrB levels preceding start of rejection treatment were analyzed in 3-day periods. sGrB levels after therapy are from first samples after rejection treatment. No. of observations at days −8/−6, −5/−3, −2/0, and after rejection treatment were 10/11, 9/11, 11/11, and 11/11, respectively. sGrB levels at days −2/0 did not differ significantly from those at days −5/−3, −8/−6, and after rejection treatment (Wilcoxon signed rank test). *B,* Representative renal allograft recipient experiencing acute rejection. Arrow indicates start of rejection treatment. *C,* Peripheral blood sGrB levels in 7 renal allograft recipients. Day 0 is day sGrB peak levels were reached. sGrB levels preceding peak levels were analyzed in 7-day periods. No. of observations at days −27/−21, −20/−14, −13/−7, and −6/0 were 7/7, 6/7, 6/7, and 7/7, respectively. Compared with days −27/−21, sGrB levels were significantly increased at days −13/−7 and −6/0 (Wilcoxon signed rank test). *D,* Representative renal allograft recipient with primary CMV infection. Arrow indicates infection diagnosis.

level at days −2/0 exceeded the sGrB cutoff level in only 1/11 patients. This particular patient exhibited continuously increased but gradually declining sGrB levels after transplantation: The sGrB level on posttransplantation day 1 was 113 pg/mL, after which it decreased to 66 pg/mL at the start of rejection treatment. Figure 2B shows the longitudinal course of sGrB levels in a representative patient experiencing an acute rejection episode.

The sGrB level exceeded the sGrB cutoff level in 7/7 patients experiencing primary CMV infection (figure 2C). The increase in sGrB closely paralleled the increase in total lymphocytes. After peak levels were reached, sGrB levels gradually declined and had returned to normal in 7/7 patients at the end of follow-up. Figure 2D shows the longitudinal course of sGrB levels in a representative patient experiencing posttransplant primary CMV infection.

Discussion

Our findings show that $CD8^+$ T lymphocytosis during posttransplant primary CMV infection results from expansion of a $CD8^+$ T cell subset expressing both GrB and the lymph node homing receptor CD62L. Yet, antigen-specific $CD8^+$ T cells in the primary immune response to Epstein-Barr virus down-regulate expression of CD62L [12]. This suggests that generation of the $CD8^+GrB^+CD62L^+$ T cell subset during posttransplant primary CMV infection results from bystander activation rather than antigen-specific activation. The presence of CD62L on this T cell subset might enable these cells to directly interact with the luminal surface of high endothelial venules and survey peripheral lymph nodes for the presence of specific antigen.

Generation of $CD8^+GrB^+CD62L^+$ T cells during posttransplant primary CMV infection appeared to be associated with a temporary increase in systemic sGrB levels. Presumably, this increase results from direct secretion of newly synthesized GrB. During biogenesis of cytoplasmic granules, most newly synthesized cytoplasmic granule constituents are directly secreted via a constitutive secretory pathway rather than stored within the CTL [13].

Analysis of GrB levels in the peripheral blood compartment seems useful for differentiating posttransplant primary CMV infection from acute rejection. This seems surprising, since we previously showed that acute rejection is associated with infiltration of the renal allograft by GrB-expressing T cells [4]. However, a comparable observation was seen during posttransplant follow-up of lung allograft recipients where no relation was detected between presence of acute rejection and number of perforin-expressing T cells in peripheral blood [14]. In our opinion, the lack of increased GrB levels in peripheral blood during acute rejection can be explained by rapid trapping of GrB-expressing $CD8^+$ T cells into the antigenic site (that is, the renal allograft) after entry into the circulation. Indeed, skin grafting in a mouse model induced in the draining lymph nodes a subpopulation of allospecific $CD8^+$ granzyme A^+CD62L^- T cells expressing high levels of adhesion molecules [15]. This indicates that once in the circulation, these cells are highly susceptible to extravasation into sites containing antigens. Likewise, trapping of allospecific GrB-expressing $CD8^+$ T cells in the renal allograft presumably explains the lack of increased GrB levels during acute rejection.

We conclude that posttransplant primary CMV infection results in the expansion of an otherwise almost absent population of $CD8^+GrB^+CD62L^+$ T cells, coincident with a temporary increase in systemic sGrB levels. Analysis of GrB levels in the peripheral blood compartment seems useful for differentiating primary CMV infection from acute rejection. Prospective follow-up of sGrB levels may provide an approach for monitoring the onset of primary CMV infection and possibly of other viral infections in immunocompromised renal transplant recipients.

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