The Intracellular Granzyme B Inhibitor, Proteinase Inhibitor 9, Is Up-Regulated During Accessory Cell Maturation and Effector Cell Degranulation, and Its Overexpression Enhances CTL Potency

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The Intracellular Granzyme B Inhibitor, Proteinase Inhibitor 9, Is Up-Regulated During Accessory Cell Maturation and Effector Cell Degranulation, and Its Overexpression Enhances CTL Potency

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Granzyme B (grB) is a serine proteinase released by cytotoxic lymphocytes (CLs) to kill abnormal cells. GrB-mediated apoptotic pathways are conserved in nucleated cells; hence, CLs require mechanisms to protect against ectopic or misdirected grB. The nucleocytoplasmic serpin, proteinase inhibitor 9 (PI-9), is a potent inhibitor of grB that protects cells from grB-mediated apoptosis in model systems. Here we show that PI-9 is present in CD4⁺ cells, CD8⁺ T cells, NK cells, and at lower levels in B cells and myeloid cells. PI-9 is up-regulated in response to grB production and degranulation, and associates with grB-containing granules in activated CTLs and NK cells. Intracellular complexes of PI-9 and grB are evident in NK cells, and overexpression of PI-9 enhances CTL potency, suggesting that cytoplasmic grB, which may threaten CL viability, is rapidly inactivated by PI-9. Because dendritic cells (DCs) acquire characteristics similar to those of target cells to activate naïve CD8⁺ T cells and therefore may also require protection against grB, we investigated the expression of PI-9 in DCs. PI-9 is evident in thymic DCs (CD3⁺, CD4⁺, CD8⁺, CD45⁺), tonsillar DCs, and DC subsets purified from peripheral blood (CD16⁺ monocytes and CD123⁺ plasmacytoid DCs). Furthermore, PI-9 is expressed in monocyte-derived DCs and is up-regulated upon TNF-α-induced maturation of monocyte-derived DCs. In conclusion, the presence and subcellular localization of PI-9 in leukocytes and DCs are consistent with a protective role against ectopic or misdirected grB during an immune response. The Journal of Immunology, 2003, 170: 805–815.

Although CL granule contents are efficiently directed into the target cell via the immunological synapse (12), some grB may escape from granules or the synaptic zone into the cytoplasm of the CL or into the extracellular milieu. For example, free grB is evident in the sera of patients with elevated CTL responses (13) or severe Gram-negative bacterial infections (14). Given its cytotoxic potency and ability to degrade extracellular proteins (13, 15–18), it is likely that protective mechanisms exist to counter misdirected or ectopic grB.

GrB is efficiently inhibited by the nucleocytoplasmic serpin, proteinase inhibitor 9 (PI-9) (19, 20), with transfection studies demonstrating that PI-9 protects cells from grB-mediated apoptosis (21). PI-9 is present in cells at immune-privileged sites such as testis and placenta (22, 23), and the expression of PI-9 in endothelial and mesothelial cells suggests that it protects bystander cells from grB released during an immune response (24). Here we show that PI-9 is up-regulated in CTLs in response to grB production and degranulation, that it associates with grB-containing granules in activated CTLs and NK cells, and that overexpression increases CTL potency. We also show that PI-9 is present in several dendritic cell (DC) types. The localization and regulated expression of PI-9 in these leukocyte subsets strongly support the hypothesis that PI-9 protects effector, accessory, and bystander cells from ectopic grB during an immune response.

Materials and Methods

Antibodies

PI-9 was detected with the specific mAb 7D8 (22) or with rabbit 12 or rabbit 15 polyclonal antisera raised against recombinant PI-9 (19). PI-6 was detected with mAb 3A (25). GrB was detected with 2C5 (26) or rabbit anti-grB/gH polyclonal antisera (27), both provided by J. Trapani (The Peter MacCallum Cancer Institute, East Melbourne, Australia), or GrB-7.
Intracellular flow cytometry

Intracellular flow cytometry was performed using peripheral blood drawn from healthy volunteers. Erythrocytes were removed from whole blood by lysis in 167 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA for 5 min at room temperature. Cells, centrifuged at 100 × g for 5 min, were then washing twice in PBS containing 1% FCS and 0.02% sodium azide (repeated for all subsequent washes). Approximately 2.5 × 106 PBLs were fixed in 4% formaldehyde in PBS for 10 min at room temperature, centrifuged, resuspended in FACS Permeabilizing Solution (BD Biosciences) for 10 min, centrifuged, washed, and incubated with 7D8 or isotype control Ab (IgG1; BD Biosciences) for 30 min. Cells were washed twice, then incubated with FITC-conjugated anti-mouse Ig (Chemicon). After two more washes the cells were analyzed using a FACS Calibur and CellQuest software (BD Biosciences).

Localization of ov-serpins in peripheral blood leukocytes

Highly purified B, CD4+, CD8+, monocyte, and NK cell populations were obtained from whole blood using RosetteSep Enrichment cocktails (StemCell Technologies, Vancouver, Canada) according to the manufacturer’s instructions. Cells were plated on poly-l-lysine-coated slides, fixed in 4% formaldehyde for 20 min, then permeabilized in 0.5% Triton X-100 for 5 min. PI-9 and PI-6 were detected with 7D8 hybridoma supernatant, respectively. Bound Ab was detected with anti-mouse Ig conjugated to FITC. Cells were counterstained with propidium iodide (1 µg/ml), and cross-sections through the nucleus were obtained using laser scanning confocal microscopy (TCS-NT; Leica, Wetzlar, Germany). The purity of each cell population and the presence of any contaminating cell types were assessed using Abs to CD3, CD4, CD8, CD14, CD19, and CD56.

Retroviral transduction of human CTLs

A cDNA encoding PI-9 (19) was subcloned into the MIGR1 retroviral vector in the forward or reverse orientation. PI-9 mRNA was transcribed as a bicistronic message with green fluorescence protein (GFP) (28). Viral supernatant for transduction was obtained by transient transfection of the 293GP packaging cell line (29) using Lipofectamine Plus (Invitrogen, Carlsbad, CA). Supernatant was tested for transduction efficiency on Jurkat cells. PBLs were obtained from normal healthy donors and were cultured in 2 days with PHA and human IL-2 (300 U/ml), then transduced with retrovirus (29). After transduction, PBLs underwent one round of rapid expansion with irradiated feeder cells (BRT Laboratories, Baltimore, MD), and the brightest GFP-expressing PBLs were purified by FACS. Sorted PBLs (72% GFP+, 75–80% CD8+) were then kept in culture by rapid expansion every 3 days. PI-9 mRNA was quantitated by real-time PCR on cDNA (30) using primers and probes specific for PI-9 and GAPDH, respectively (Megabases, Evanston, IL). Retrovirally transduced PBLs were analyzed in 1H release assays (in triplicate wells) with histiocytic targets that express the anti-human CD3 mAb OKT3 (31). The percent specific lysis of JY cells, a human B lymphoblastoid cell line, was determined in parallel (≥6% specific lysis) and subtracted from the lysis of the OKT3 hybridoma to report CTL killing only.

Expression of PI-9 in stimulated cytotoxic lymphocytes

YT cells (32) were cultured as previously described (20). PBMCs were isolated from whole blood using Ficoll-Paque Plus. PBMCs (depleted of monocytes by adherence to plastic) were cultured in IL-2–Sigma-Aldrich, St. Louis, MO) or a combination of Con A and PMA (both from Sigma-Aldrich) as previously described (33). Culture-generated quiescent NK cells were cultured and activated as described previously (34, 35). At the indicated time points, activated cells were harvested, and cell lysates were prepared by lysis in Nonidet P-40 (NP-40) lysis buffer (1% NP-40 in 50 mM Tris–HCl pH 8.0, and 10 mM EDTA (pH 8.0)). To prevent postlysis association of PI-9 and grb, cells were lysed on ice in the presence of protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin). Lysates from 0.5 × 106 cells were resolved by reducing 12.5% SDS-PAGE, transferred to nitrocellulose, immunoblotted for PI-9 with 7D8, and detected with HRP-conjugated anti-mouse Ig (Chemicon) using ECL (NEL, PerkinElmer, Boston, MA). The membranes were stripped (62.5 mM Tris–HCl (pH 6.8), 2% SDS, and 0.1 M 2-mercaptoethanol for 30 min at 50°C), then probed for grb (2C5) and actin (diluted 1/1000). Densitometric analysis of PI-9 levels was determined using MCID Image Analysis software (BD Biosciences).

Formation of PI-9/grb complexes

Lysate from COS-1 cells transfected with PI-9 (20) was incubated with or without 100 ng of recombinant grb (36) at 37°C for 10 min. Samples were resolved by reducing 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit 12 and rabbit 15 antisera (PI-9) or 2C5 (grb). YT cells (1 × 106) were cultured for 20 h with or without 25 µM calpain inhibitor I (N-acetyl-Leu-Leu-Val-Tyr-CH2Cl, Sigma-Aldrich). Cells were lysed in NP-40 lysis buffer or modified Laemmli sample buffer (60 mM Tris–HCl (pH 6.8), 2% SDS, and 10% glycerol) (37) and passed through a 26-gauge needle to shear the DNA. Twenty micrograms of cell lysate was resolved by reducing 12.5% SDS-PAGE, transferred to nitrocellulose, immunoblotted with rabbit 12 antisera, then stripped and reprobed with rabbit 15 antisera.

Colocalization of PI-9 and grb

Activated CTLs were attached to poly-l-lysine-treated slides, fixed and permeabilized in 1% acetone/methanol for 2 min at room temperature, and stained for PI-9 (7D8), grb (rabbit anti-grb antisera, diluted 1/200), or calpain III (rabbit antisera, diluted 1/500, obtained from A. Pshenikhtsky, University of Montreal, Montreal, Canada). Activated NK cells were cultured on irradiated MM-170 cells for 4 h, fixed in 4% formaldehyde, then permeabilized in 100% methanol and stained for PI-9 (7D8) and grb (rabbit anti-grb antisera, diluted 1/200). YT cells were attached to poly-l-lysine-treated slides, fixed and permeabilized in acetone/methanol, and stained for PI-9 (rabbit anti-PI-9 antisera, diluted 1/2000 and grb (2C5, diluted 1/2000). BeWo cells were cultured and handled for microscopy as previously described (20). Primary Ab was detected with the appropriate secondary Ab conjugated to FITC or rhodamine B isothiocyanate (Chemicon). Two-color images were obtained using laser scanning confocal microscopy.

Tissue localization of PI-9

Human tissues fixed in neutral buffered formalin were obtained from the archive of the Pathology Department of Box Hill Hospital (Melbourne, Australia). Immunohistochemistry was performed as previously described (22). Cryopreserved normal human thymus was provided by R. Boyd (Department of Pathology and Immunology, Monash University, Prahran, Australia). Sections (6 µm) of tissue were air-dried onto silanized slides. Sections were washed and blocked in normal goat serum before incubation with 7D8 or an isotype-matched control Ab (IgG1). Bound Ab was detected with anti-mouse Ig conjugated to FITC. Sections were then stained with anti-CD3, -CD4, or -CD45 mAbs directly conjugated to PE (Diatec, Oslo, Norway). Two-color images were obtained using laser scanning confocal microscopy.

Dendritic cell purification

PBMCs were isolated from normal donors over Ficoll-Paque gradients (Red Cross Blood Transfusion Service, Melbourne, Australia). Monocytes were prepared by elutriation at 2100 rpm using a Beckman J6 M (Palo Alto, CA) with a standard chamber. Monocytes were further purified by sorting using a MoFlo CLS cell sorter (Cytomation, Fort Collins, CO) based on forward and side light scatter and subpopulations sorting for CD16 and CD14 expression. Lineage-negative DCs were purified from the monocyte elutriation fractions by negative selection with Abs to CD3, CD14, CD11b, CD19, and CD16. Lineage-negative DCs were sorted into CD14+ and CD14− monocytes positively selected using MACS columns after labeling for CD14 and goat anti-mouse MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were selected on a MACS column as recommended by the manufacturers. CD14+ monocytes were cultured in IL-4 (20 ng/ml) and GM-CSF (40 ng/ml) for 5–7 days as previously described (40). Immature DCs were matured in the presence of TNF-α (10 ng/ml; R&D Systems, Minneapolis, MN) for 2 days.
Results

Distribution of PI-9 in peripheral blood leukocytes

To extend our previous observations that PI-9 is expressed in lymphoid (T, B, and NK-like) cell lines, but not myeloid cell lines (19), we examined PI-9 in PBLs by intracellular flow cytometry using a specific mAb (22). PI-9 was evident in the majority of PBLs in both mononuclear cells (R1) and granulocytes (R2; Fig. 1A). Most mononuclear cells were clearly positive (>90%). Dual-color analysis showed that >95% of CD3+ cells are PI-9 positive, 100% of CD4+ and CD8+ cells are PI-9 positive, and ~75% of CD19+ cells are PI-9 positive (data not shown). Many granulocytes (>70%) were also positive, but appeared to have lower amounts of PI-9, with only a slight positive shift in staining apparent over the isotype control (Fig. 1A). Comparison of the mean fluorescence intensities indicated that mononuclear cells express at least 5-fold more PI-9 than granulocytes.

To further identify the populations of mononuclear cells that produce PI-9, B cells, CD4+ and CD8+ T cells, NK cells, monocytes, and granulocytes were enriched from PBLs using established immunodepletion procedures. These were analyzed for PI-9 by indirect immunofluorescence so that its intracellular distribution could be simultaneously assessed (Fig. 1B). The efficiency of immunodepletion was monitored by staining for CD3, CD4, CD8, CD14, CD19, or CD56 (Fig. 1C). PI-9 was evident in all populations, with the highest levels in NK and T cells (CD4+ and CD8+ T cells expressed equivalent levels). B cells, monocytes, and granulocytes had much lower levels. In all cell types PI-9 exhibited a nucleocytoplasmic localization, consistent with previous reports (19, 20). The expression pattern of PI-9 differed from that of the closely related serpin, PI-6, which is a cathepsin G inhibitor present in myeloid cells (41).

Intracellular localization of PI-9 in grB-expressing cells

The observation of high PI-9 expression in NK and CTLs suggests that it protects grB-producing cells against endogenous grB. Using indirect immunofluorescence and laser scanning confocal microscopy we examined the intracellular localization of PI-9 in primary NK cells and the NK-like cell line, YT. This showed that in addition to its previously reported cytoplasmic and nuclear distribution (Fig. 2A) (20), PI-9 is associated with vesicles in the CL cytoplasm that also contain grB (Fig. 2, B and C). These vesicles were positive for the lysosomal/granule marker lysosome-associated membrane protein-1 and not with markers for the endoplasmic reticulum or Golgi apparatus (data not shown), indicating that they are secretory lysosomes (cytotoxic granules). To determine whether the association of PI-9 with lysosomes is unique to CLs, the distribution of endogenous PI-9 in the epithelial cell line BeWo was also examined (Fig. 2D). Here PI-9 was evident only in the cytoplasm and nucleus, and no colocalization between PI-9 and lysosomal markers was observed.

In a previous study we separated membrane and cytosolic fractions of CLs and showed that PI-9 is present in the cytosol and does not copurify with grB in the granule fractions (19). Given that PI-9 is not present within granules, the results shown in Fig. 2 suggested that it is associated with the cytoplasmic surface of the

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![FIGURE 1.](http://www.jimmunol.org/) Distribution of PI-9 in peripheral blood leukocytes. A, Flow cytometric analysis of PI-9 in PBLs. PBLs were stained with a PI-9-specific mAb, 7D8 (filled histogram), compared with an IgG1 isotype control (open histogram). This figure is representative of four different donors. B, Distribution and subcellular localization of PI-9 in PBL subsets. Leukocyte subsets were enriched and assessed as described in Materials and Methods. Indirect immunofluorescence was performed with a mAb specific to PI-9 (7D8) or PI-6 (3A) and was detected with FITC-conjugated anti-mouse Ig (green). The nuclei were counterstained with propidium iodide (red), and images were obtained using laser scanning confocal microscopy. Relative levels of PI-9 can be compared as all images were captured using the same settings. This figure is representative of three separate experiments. C, Proportion of PI-9- and PI-6-positive cells in enriched leukocyte subsets. Subsets were assessed for marker expression as described in Materials and Methods.
granule and is poised to rapidly inactivate grB entering the cytoplasm from leaking granules. To test this idea we examined YT cells for evidence of extragranular grB in complex with PI-9. We took advantage of the fact that grB-PI-9 complexes are stable under SDS-PAGE and immunoblotting conditions (19, 21), and that we have two high affinity PI-9 Abs that detect PI-9 in complex with grB very efficiently. As shown in Fig. 3A, these Abs (rabbit 12 and 15) detect the grB-PI-9 complex better than they detect free PI-9, and they also detect the degradation products commonly observed following serpin-proteinase complex formation. Degradation of the complex occurs because distortion of the protease (in particular) renders it highly susceptible to proteolysis (42, 43). The Abs do not bind grB (Fig. 3A), and their corresponding preimmune sera do not recognize PI-9, complex, or degradation products (data not shown). None of the four grB Abs we have tested detected the complex or degradation products with similar sensitivity to the PI-9 Abs. An immunoblot using the most sensitive of these grB Abs (2C5) is shown in Fig. 3A.

When YT cells are lysed in buffers containing NP-40, active grB is released from granules and binds cytoplasmic PI-9 postlysis (20, 21). By contrast, lysis of CTLs in buffers containing SDS prevents postlysis interactions, presumably by rapidly denaturing cellular proteins (37). Thus, lysis of YT cells in SDS will denature free PI-9 and free grB, and a complex evident in SDS-treated YT cell extracts will have formed before lysis. As shown in Fig. 3B, lysis of YT cells in NP40 generated a postlysis complex of ~70 kDa as well as smaller products resulting from complex degradation that were detected by both PI-9 antisera, but more efficiently by rabbit 15. When cells were lysed in SDS buffer, small, but reproducible, amounts of complex and degradation products were detected by rabbit 15 (Fig. 3B). This suggests that there is a pool of extragranular grB in the cytoplasm of YT cells that is bound to PI-9 and undergoing proteolysis.

Because serpin/proteinase complexes are irreversible and involve distortion of both serpin and proteinase (42, 43), we predicted that cytoplasmic PI-9/grB complexes would be recognized and degraded by the ubiquitin-proteosomal machinery. Cells cultured in the presence of a proteosome inhibitor should therefore accumulate complexes. Indeed, greater amounts of complexes were detected by both PI-9 antisera when cells were incubated with the proteosomal inhibitor, calpain inhibitor I, and lysed in SDS (Fig. 3B). This also supports the idea that grB enters the cytoplasm of CLs during normal cellular function and is rapidly inactivated by PI-9.
PI-9 enhances the potency of human CTLs

Since PI-9 potentially protects CLs from death induced by misdirected grB, we wanted to determine the effect on CTL potency of overexpressing PI-9 in activated PBLs. Normal donor PBLs were activated with PHA and transduced with retroviral vectors containing the PI-9 gene in either the forward (coding) or the reverse (noncoding) orientation. These vectors allow transcription of the inserted cDNA as a bicistronic mRNA that also encodes GFP (28, 44). Cultures transduced with either the coding or noncoding vectors were enriched to equivalent amounts of GFP-expressing cells (~72%) by FACS (data not shown). Those transduced with the PI-9 gene in the coding orientation expressed 4-fold higher levels of PI-9 mRNA ($p < 0.02$) compared with those in which the PI-9 gene was in the noncoding orientation (Fig. 4A). Both cultures contained an equal proportion of CD8 cells, which was 75–80% of the total PBLs (data not shown).

The ability of uncloned populations of transduced CTLs to lyse target cells expressing an anti-CD3 mAb (OKT3) (31) was evaluated. In parallel, we also determined the lysis of JY target cells, which do not express anti-CD3 mAbs, so we were able to account for NK cell activity in the cultures. In each experiment the specific lysis of JY targets (NK cell killing) was subtracted from that of OKT3 targets (total killing) to give the level of CTL activity. At every E:T cell ratio tested we observed increased potency of CTLs that expressed elevated levels of PI-9 (Fig. 4B). For example, at an E:T cell ratio of 0.5:1 we observed a 3-fold increase in potency of CTLs that expressed elevated levels of PI-9 (Fig. 4B). While activated NK cells killed MM-170 cells (data not shown) and up-regulated grB, which peaked on day 3, no significant increase in PI-9 was detected over endogenous levels (Fig. 5B). This treatment also generated the higher $M_i$ form of grB (35 kDa) that is only observed following T cell degranulation (45). Thus, in vitro, PI-9 up-regulation is associated with the release of grB from CLs.

The regulation of PI-9 was also investigated in NK cells. Culture-generated quiescent NK cells were activated by incubation with targets (irradiated MM-170 cells) in the presence of IL-2. These NK cells constitutively express both PI-9 and grB, as evident on day 1 of stimulation (Fig. 5C). While activated NK cells killed MM-170 cells (data not shown) and up-regulated grB, which peaked on day 3, no significant increase in PI-9 was detected over time. Taken together, these results suggest that the level of PI-9 in proliferating, nondegranulating T cells and activated NK cells is sufficient to protect against grB. However, the increase in PI-9 expression during T cell degranulation suggests that granule exocytosis is associated with increased entry of grB into the effector cell cytoplasm, and that degranulating cells consequently require higher levels of PI-9.

Since PI-9 is up-regulated in response to T cell degranulation in vitro, its expression was also investigated in activated T cells in vivo by immunohistochemical analysis of normal and inflamed human tissues. When normal spleen was examined using the specific PI-9 mAb, 7D8, in standard immunohistochemical procedures, no PI-9-positive lymphocytes were detected (Fig. 6, b and c). However, when lymphocytes were extracted from freshly isolated spleen and stained for PI-9 using the more sensitive technique of indirect immunofluorescence, PI-9-positive cells were apparent (Fig. 6a). This indicated that the level of PI-9 expressed in

**FIGURE 3.** Detection of preformed complexes in CLs. A. Rabbit anti-PI-9 polyclonal antisera preferentially recognize complexes of PI-9 and grB as well as complex degradation products. Lysates of COS cells expressing PI-9 were incubated in the presence (+) or the absence (−) of excess grB, and samples were detected by immunoblotting with rabbit anti-PI-9 antisera R12 or R15, or the anti-grB mAb 2C5. Note that the 2C5 blot was overexposed to demonstrate that the Ab can detect grB in complex. B. Detection of preformed complexes in cytolytic cells. YT cells were incubated in the presence (+) or the absence (−) of a proteasome inhibitor (Prot. Inhib.), then lysed in NP40 or SDS lysis buffer. Cell lysates were immunoblotted with rabbit anti-CD3 mAb R15, then stripped and reprobed with rabbit 12 antiserum. The position of uncomplexed PI-9 is arrowed, and the positions of the complex and primary degradation products are bracketed. Minor complex degradation products (+) are indicated on the right.
quiescent lymphocytes is below the level of immunohistochemical detection using this Ab. By contrast, when we examined in situ, a role for PI-9 in protecting grB-expressing cells is supported by its unusual intracellular distribution, described in the first part of this study. In addition to its cytoplasmic and nuclear localization (49), cellular FLIP blocks Fas-mediated apoptosis (50), and Bcl-2 family members regulate apoptosis at the mitochondria (reviewed in Refs. 51 and 52). We and others believe that PI-9 and other intracellular serpins are part of the anti-apoptotic machinery of cells involved in or exposed to the cellular immune response (23, 41, 53, 54). In particular, we have proposed that PI-9 protects cells against misdirected grB (19, 21, 22, 24).

**Expression, regulation, and function of PI-9 in CLs**

A role for PI-9 in protecting grB-expressing cells is supported by its unusual intracellular distribution, described in the first part of this study. In addition to its cytoplasmic and nuclear localization (20), PI-9 in CLs is clearly associated with grB-containing cytotoxic granules. Fractionation studies indicate that PI-9 is not present within granules (19). Other studies show that, like PI-6 (55), when PI-9 is provided with an efficient signal peptide, it is retained in the endoplasmic reticulum and cannot move through the secretory pathway (A. Gillard and P. Bird, unpublished observations). Therefore, PI-9 must associate with the external, cytoplasmic face of the granule through interaction with a specific granule component. Our demonstration that PI-9 does not associate with lysosomes in epithelial cells suggests that it interacts with a protein or other component not found on nonsecretory lysosomes or on other membrane-bound organelles. For example, it may bind a specific lipid, as the lipid content of the granule membrane differs from that of the plasma membrane and other organelles (56).
GrB is stored in granules in an active form (57); thus, leakage from granules or from the synapse would allow it to access nucleocytoplasmic substrates and kill the CL. The positioning of PI-9 at the granule surface and of the PI-9/grB complexes evident within CLs suggests that granules do leak and that PI-9 is present to rapidly inactivate extragranular grB. Once granule exocytosis is triggered, the granules are refilled with newly synthesized grB; however, some of the newly synthesized grB is constitutively secreted as a 35-kDa form (45). The 35-kDa form of grB is specifically associated with degranulating cells, so the correlation we report with a 3-fold increase in PI-9 strongly suggests that grB misdirection occurs during T cell degranulation. While this in vitro system probably does not fully recapitulate the situation in vivo, our demonstration of an increase in CTL killing efficiency on 3- to

**FIGURE 5.** Regulation of PI-9 expression in CLs. PBMCs were activated in the presence of 100 U/ml IL-2 (A) or a combination of Con A and PMA (10 μg/ml and 10 ng/ml, respectively; B), or culture-generated quiescent NK cells (C) were activated in the presence of irradiated MM-170 cells and 200 U/ml IL-2. On the indicated days cells were harvested, and lysates were prepared. Cells (0.5 × 10⁶ loaded/lane) were resolved by reducing SDS-PAGE and sequentially immunoblotted with mAbs to PI-9 (7D8, hybridoma supernatant diluted 1/10), grB (2C5, diluted 1/2000), and an antiserum to actin (diluted 1/1000). FACS analysis indicated that 40% of IL-2-stimulated T cells and 64% of Con A/PMA-stimulated T cells were CD8⁺. Presence of the 35-kDa form of grB. Densitometric analysis was performed on immunoblots from four separate stimulations, and the relative levels of PI-9 are plotted.

**FIGURE 6.** Expression of PI-9 in activated lymphocytes and DCs. a, Indirect immunofluorescence of PI-9 (green) in lymphocytes isolated from normal spleen; b, PI-9 is undetectable by immunohistochemistry in formalin-fixed, paraffin-embedded normal spleen; c, isotype control of a serial section of the same normal spleen; d, PI-9 in activated lymphocytes in ductal breast carcinoma; e, coexpression of grB in activated lymphocytes; f, isotype control; g, localization of PI-9 to DCs within a tonsillar germinal center; h, higher magnification illustrating the dendritic morphology of the PI-9-positive cells; i, isotype control; j–l, PI-9-positive cells within the thymus. PI-9-positive cells (green) are located within the medulla identified by the presence of Hassall corpuscles (HC). PI-9 does not colocalize with CD3⁺ thymocytes (j, red), but slight colocalization is noted where cytosolic PI-9 overlaps with membrane CD45 (k, red) and CD4 (l, red) expressed on DCs.
5-fold overexpression of PI-9 suggests that the similar fold up-regulation seen in Con A/PMA-treated cells is physiologically relevant. We therefore conclude that CTL viability is enhanced by a PI-9-mediated reduction in suicide or fratricide induced by misdirected grB.

What might cause granule leakage? Granules are secretory lysosomes containing lysosomal hydrolases in addition to granzymes and perforin (58–60). While there is no direct evidence that granules leak, it is clear that lysosomal rupture can be induced by stressors such as oxidation and UV irradiation, leading to apoptosis and necrosis (61, 62). Increased levels of reactive oxygen species have been observed in CLs, which may lead to apoptosis (63). This suggests that granule leakage occurs during effector cell activation and/or function.

Release of grB into the effector cell cytoplasm need not only occur from leaking granules. It is possible that secreted grB is endocytosed by effectors or bystanders. The uptake of grB into target cells is thought to be mediated by the 300-kDa M6PR (2). The M6PR is expressed in all nucleated cells, with up to 20% of the receptor present at the cell surface (64–68). Interestingly, the M6PR is up-regulated on activated T cells (69), which might increase their susceptibility to secreted grB.

**Role of PI-9 in accessory cells**

The cellular immune response involves a complex interplay between many cell types. APC or accessory cells (DCs, macrophages, and B cells) induce differentiation of naive T cells into cytotoxic or Th lymphocytes by secretion of cytokines and expression of costimulatory molecules. These accessory cells are closely associated with CLs and are likely to be exposed to collateral damage mediated by grB and perforin during the immune response. The presence of PI-9 would provide protection against inadvertent killing of these cells.

How likely is such inadvertent killing? DCs have established roles in presenting Ag to CD4+ Th cells and eliciting Th1 (CTL) or Th2 (B cell) responses. However, distinct DC subpopulations also directly interact with B cells or CD8+ CTLs. For example, follicular DCs in the germinal center directly contribute to B cell proliferation and differentiation (70), while virally infected DCs and DCs purified from blood can directly present to CD8+ T cells in the absence of CD4+ Th cells (71–74).

This close association of activated CTLs and DCs may result in elimination of DCs by effector CTLs (33, 75). Elimination of DCs in normal mice is unusual, presumably due to protective mechanisms, but elimination of Ag-specific DCs by cognate CTLs has been observed in transgenic mouse models (76, 77). Although this demonstrates that DCs are potentially susceptible to CTLs, the mechanism of CTL-dependent clearance of DCs is unclear. One study found DC elimination to be independent of Fas and perforin (77), while another reports that it is mediated partly by the perforin pathway (78). Consistent with the latter results, we and others have suggested that expression of PI-9 in DCs prevents grB-mediated apoptosis during Ag presentation to CTLs (23, 53). This is further supported by the recent report that SP16, one of seven murine PI-9 homologues (79), protects murine DCs from CTL-induced apoptosis (54).

Our results show that PI-9 is expressed in specific DC subsets. Thymic medullary DCs comprise three different subsets: a major...
CD11b− subset of lymphoid origin, a minor CD11b+ subset of myeloid origin (46), and a population of plasmacytoid DCs (80). The CD11b+ thymic DCs resemble tonsillar germinal center DCs (81) and are thought to be phenotypically and morphologically related (46). Considering this relationship, it is likely that the PI-9-positive DCs observed in the thymus are related to the PI-9-positive DCs located in the tonsil. Both these DC populations are probably derived from CD16+ myeloid precursors (47, 48, 82). PI-9 is highly expressed in CD16+ monocytes, which is consistent with PI-9 expression in the CD11b+ thymic DCs and tonsillar germinal center DCs.

The differential expression of PI-9 in DCs suggests differing requirements for protection from grB-mediated apoptosis in DC subsets. Thymic medullary DCs are essential in the positive and negative selection of thymocytes. GrB-positive cells are present in the thymic medulla, with grB transcripts detected in double-positive (CD4+ CD8+) thymocytes (83) and in both CD4+ or CD8+ single-positive thymocytes (84). This suggests that thymocytes undergoing selection express grB and have cytotoxic potential. Thus, the presence of PI-9 in thymic medulla DCs is consistent with a role in protecting these DCs from grB-mediated apoptosis.

Some subsets of germinal center DCs are involved in the presentation and activation of T cells directly (81), while plasmacytoid DCs are IFN-producing cells (85–87) that initiate potent Th1 (CTL) responses (88). The expression of PI-9 in these cells suggests that DC populations that present to and activate CD8+ precursor T cells are at risk from the effector functions of the T cells they activate. This is also supported by the up-regulation of PI-9 in MDDCs upon TNF-α-induced maturation, suggesting that mature APC require protection from inadvertent apoptosis.

Role of PI-9 in other cells

It would be advantageous for other cell types to express PI-9 to protect against misdirected grB during the immune response. For example, PI-9 is expressed in endothelial and mesothelial cells likely to be exposed to CLs and is up-regulated by inflammatory stimuli (24, 89). The expression of grB and perforin has also been demonstrated in human CD4+ CTLS (90, 91), suggesting that PI-9 has a cytoprotective role in some CD4+ T cells. The level of PI-9 expressed in B cells, monocytes, and granulocytes is lower than in CLs or A. Kummer, A. J. Swaak, J. M. Middeldorp, H. G. Huisman, C. J. Froelich, and A. J. Kummer. 2000. Human granzyme B induces caspase-mediated apoptosis of Jurkat cells resulting in cleavage of poly(ADP-ribose) polymerase to the 89-kDa apoptotic fragment and less abundant 64-kDa fragment. Biochem. Biophys. Res. Commun. 227:658.


