A key function of human granzyme B (GrB) is to induce apoptosis of target cells in conjunction with perforin. The RAH allele is the first documented variant of the human GrB gene, occurs at a frequency of 25–30%, and encodes three amino acid substitutions (Q48R, P88A, and Y245H). It was initially reported that RAH GrB is incapable of inducing apoptosis, but here we show that it has essentially identical proteolytic and cytotoxic properties to wild type GrB. Recombinant RAH and wild type GrB cleave peptide substrates with similar kinetics, are both capable of cleaving Bid and procaspase 3, and are equally inhibited by proteinase inhibitor 9, an endogenous regulator of GrB. Furthermore, cytotoxic lymphocytes from RAH heterozygotes and homozygotes have no defect in target cell killing, and in vitro, RAH GrB and wild type GrB kill cells equally well in the presence of perforin. We conclude that the RAH allele represents a neutral polymorphism in the GrB gene.

Cytotoxic lymphocytes (CLs) play a central role in cell-mediated immunity by destroying virus-infected, foreign, or tumor cells. They also contribute to the pathology of autoimmune diseases such as diabetes and to graft versus host disease following bone marrow transplantation. On contact with a target cell, the CL releases cytosotins such as perforin, proteases (granzymes), and death ligands from secretory granules into the intercellular space. Target cell death then follows one of two pathways: activation of death receptors on the target cell surface coupled to caspase activation within the cell or uptake of granzymes in a perforin-dependent manner that leads to caspase activation and intracellular proteolysis. In the latter pathway, perforin and granzymes are endocytosed by the target cell, and perforin mediates release of granzymes into the cytoplasm. Caspase activation, loss of mitochondrial membrane potential, proteolysis of key housekeeping proteins, DNA degradation, and the disintegration of cellular structures follow rapidly.

Human CLs produce granzyme A (GrA) and granzyme B (GrB), two cytotoxic granule serine proteases that activate different death pathways (2). GrA cleaves after basic residues, appears to cause single-stranded DNA breaks, and disrupts repair mechanisms in the target cell, leading to caspase-independent death (4). By contrast, GrB has an unusual preference for cleaving after Asp and is thought to act as an apical caspase in triggering classical apoptosis (2). The role of GrB in cell-mediated immunity is supported by the phenotype of GrB-deficient mice, which produce CLs that cannot induce rapid DNA degradation in target cells, although killing still occurs, possibly via other granzymes (5). Furthermore, apoptosis of transplanted allogeneic hepatocytes in rats is reduced by treatment with GrB inhibitors (6), elevated GrB serum levels occur in several diseases including rheumatoid arthritis, and GrB is implicated in the generation of autoimmune antigens (7).

The importance of the granule pathway to CL function is illustrated by familial hemophagocytic lymphohistiocytosis, a rare congenital immune deficiency syndrome that is fatal unless treated by bone marrow transplantation. Approximately 20% of familial hemophagocytic lymphohistiocytosis patients have severely reduced CL activity because of mutation of the perforin gene (8). Although granzyme deficiency has not been associated with familial hemophagocytic lymphohistiocytosis (9), it was recently reported that 25–30% of the population carries a triple-mutated (Q48R, P88A, Y245H (RAH)) allele of GrB and that the product of this allele is incapable of inducing apoptosis (10). Cytotoxic lymphocytes from RAH homozygotes produce normal amounts of GrB and have normal lytic activity. By contrast, expression of the RAH mutant in transfected tumor cell lines does not induce apoptosis (10). To explain these results, it was proposed that CLs from RAH homozygotes use GrB-independent pathways to kill targets and that either the RAH variant is catalytically inactive against key proapoptotic substrates such as Bid and procaspase 3 or it is sequestered within the cell and cannot access these substrates.

However, the RAH variant was not biochemically characterized in this study, nor was its ability to kill cells examined by delivering it exogenously with perforin. The latter point is important because the physiologically relevant pathway for GrB cytotoxicity involves internalization of GrB by the target cell and perforin-mediated release of GrB into the cytoplasm (3, 11). Furthermore, the GrB and RAH forms expressed in the tumor cell line were engineered to be synthesized in active conformations, which do not reflect the situation in vivo because GrB is usually produced as an inactive zymogen that is activated only when it reaches a dedicated storage compartment (12, 13). Because reduced granzyme B activity in RAH carriers would be predicted to lower their antiviral immunity, it is important...
to carefully examine the cytotoxic potential of the RAH variant. We show here that GrB activity in RAH carriers is indistinguishable from wild type GrB, both biochemically and in perforin-mediated cytotoxicity.

**EXPERIMENTAL PROCEDURES**

**Single Strand Conformation Polymorphism Analysis—**DNA was isolated from 50 ml of whole blood using a PUREGENE kit from Gentra Systems. PCR-single strand conformation polymorphism analysis, described previously (14), was carried out using human GrB exon-specific primers.

**Preparation of Native and Recombinant GrB—**Native GrB was prepared from isolated cytotoxic lymphocyte granules as described (15). Recombinant wild type and RAH GrB were produced as zymogens in *P. pastoris*. Native GrB was prepared as described (20) was used to mediate the delivery of GrB into human cells. Jurkat cells were washed twice in a modified Hanks’ balanced salt solution. 50 µl aliquots of the cells in modified Hanks’ balanced salt solution, 50 µl of the appropriate dilution of perforin and adding it to an equal volume of the cells in modified Hanks’ balanced salt solution. After 30 min at 37 °C, the number of cells permeabilized by the perforin was determined by counting propidium iodide-positive cells. A suitable dilution of perforin in HE buffer (150 mM NaCl, 10 mM Hepes, pH 7.2) containing 4 mM CaCl₂ and adding it to an equal volume of the cells in modified Hanks’ balanced salt solution. After 4 h at 37 °C, the proportion of surviving cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results (10) comprehensively screened various racial groups for GrB RAH heterozygotes and homozygotes and found an allelic frequency of 25–30%. The wild type QPY allele was

**RESULTS**

**Frequency of the RAH Allele and Function of RAH CLs—**McIlroy et al. (10) comprehensively screened various racial groups for GrB RAH heterozygotes and homozygotes and found an allelic frequency of 25–30%. The wild type QPY allele was
found at frequencies of 54–71%. We screened 28 individuals for these alleles by single strand conformation polymorphism analysis and found 18 QPY/QPY homozygotes, 8 QPY/RAH heterozygotes, and 1 RAH/RAH homozygote (data not shown). These frequencies are similar to those reported by McIlroy et al. (10).

To assess the cytolytic potential of CLs from these individuals, freshly isolated, unstimulated peripheral blood mononuclear cells were used as effectors in conventional cytotoxicity assays to measure natural killer cell activity. Using K562 cells as targets, no difference in cytotoxicity was evident in peripheral blood mononuclear cells from QPY homozygous, QPY heterozygous, or RAH homozygous individuals, indicating that natural killer cell function is unaffected by the RAH mutations (data not shown).

We also cultured peripheral blood mononuclear cells for 5 days in the presence of interleukin-2, which promotes the development of LAK cells. These cells kill targets via the calcium-dependent perforin/granzyme pathway and do not use the death receptor pathway. This is illustrated in Fig. 1A, where killing of K562 cells by LAKs in a cytotoxicity assay is completely abrogated by EGTA. As shown in Fig. 1B, there was no significant difference in cytotoxicity of LAKs from QPY homozygous, QPY heterozygous, or RAH homozygous individuals when K562 cells were used as targets. Similar results were obtained when Daudi and Raji cells were used as targets (data not shown). Thus the RAH GrB mutations have no appreciable effect on perforin/granzyme-mediated killing by CLs.

Production of Recombinant GrB Containing the RAH Mutations—To study the impact of the RAH mutations on GrB function, we produced recombinant RAH GrB in a P. pastoris system. As described previously (16), the protease was produced as a His-tagged zymogen and purified by immobilized metal affinity chromatography from the culture medium. Activation of the zymogen was achieved by incubation with enterokinase, which removes an amino-terminal sequence com-

### Table I

|                 | Abz-IEPDSSMESK-DNP | PI-9 |
|-----------------|---------------------|------|
|                 | $K_{m}$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_{m}$ ($s^{-1} \mu M^{-1}$) | SI | $k_{o}$ ($M^{-1} s^{-1}$) |
| GrB wild type   | 5.8 ± 0.2          | 4.4 ± 0.2         | 750,000                               | 1.0 | $1.7 \times 10^6$ (17) |
| GrB RAH         | 5.7 ± 0.6          | 4.6 ± 0.3         | 807,000                               | 1.0 | $1.7 \pm 0.15 \times 10^6$ |

* Abz-IEPDSSMESK-dinitrophenyl (dnp) is a GrB substrate described in Ref. 17.

* SI, stoichiometry of inhibition.
prising the yeast α-factor propeptide, His tag, and enterokinase recognition site, thus exposing the amino terminus of mature GrB (Fig. 2A). Similar expression levels of wild type and RAH GrB were achieved using this system (~1 mg of purified propeptide per liter of culture supernatant).

Activity of the RAH Variant on Peptide and Protein Substrates—Activity of the enterokinase-activated GrB was assessed by cleavage of the conventional tripeptide substrate, Boc-AAD-thiobenzyl ester (18). As shown in Fig. 2B, both enzymes cleaved the substrate efficiently, suggesting that the RAH mutations have little or no impact on activity. Similar activities were seen on the newer substrate IETD-p-nitroanilide (data not shown). To compare the activities of the enzymes in more detail, cleavage of the substrate Abz-IETDSSMESK-2,4-dinitrophenol (17) was measured kinetically. As shown in Table I, the binding ($K_{\text{on}}$) and catalytic constants ($k_{\text{cat}}$) were indistinguishable, confirming that the wild type and RAH forms of GrB have essentially identical catalytic properties.

To assess cleavage of authentic GrB protein substrates, Bid and procaspase 3 were produced by in vitro transcription/translation and incubated with varying concentrations of wild type and RAH GrB. As shown in Fig. 3A, both enzymes cleaved these substrates with similar efficiency. This contrasts with the results of McIlroy et al. (10) who suggested that RAH GrB is incapable of cleaving Bid and procaspase 3.

Inhibition of the RAH Variant by PI-9—GrB is regulated in vivo by the intracellular serpin, PI-9, which protects CLs, dendritic cells, and bystanders against ectopic or misdirected GrB (19, 21, 22). The interaction between GrB and PI-9 results in the formation of a stable complex that can be visualized by SDS-PAGE (17, 23), and the rate of inhibition of GrB by PI-9 can be assessed by standard kinetic analysis (17, 23). As shown in Fig. 3B, the RAH variant retains the ability to form a complex with PI-9, and the rate of RAH GrB inhibition by PI-9 is indistinguishable from the rate of inhibition of wild type GrB by PI-9 (Table I). Thus, the RAH mutations do not affect the ability of GrB to interact with PI-9.

Perforin-mediated Cytotoxicity of the RAH Variant—The generally accepted model for GrB-mediated cytotoxicity involves release of GrB and perforin into the intermembrane space between the CL and target cell (1). Endocytosis of GrB and perforin by the target cell is followed by disruption of an endocytic compartment by perforin, which releases GrB into the cytoplasm and triggers apoptosis (3), (11). Incubation of cultured cells with exogenous, purified GrB and perforin alone is sufficient to rapidly induce death (24).

It has been suggested that the mannose 6-phosphate receptor is involved in the uptake of GrB into target cells (25, 26). To ensure that potential glycosylation differences between recombinant (from yeast) and native GrB do not result in differences in potency, we first compared the ability of these two forms of GrB to kill Jurkat cells in the presence of sublytic amounts of perforin. As shown in Fig. 4A, recombinant wild type GrB was indistinguishable from native GrB, both in the shape of the dose-response curve and EC50.

We then compared recombinant wild type GrB to the RAH variant. As shown in Fig. 4B, the cytotoxicity of these two forms was identical. We conclude that the RAH mutations do not affect perforin-mediated cytotoxicity of GrB.

**DISCUSSION**

The physiological function and significance of a particular protein is often gauged by the study of naturally occurring mutants or by reverse genetics involving the generation of knockout mice. For example, the importance of perforin has been amply illustrated by the fatal consequences of its lack or dysfunction in familial hemophagocytic lymphohistiocytosis disease (8) and by the immune-compromised phenotype of knockout mice (27).

Unlike perforin, the importance of GrB to humans remains unclear, despite the wealth of in vitro data suggesting it has a pivotal role in CL-mediated cytotoxicity (for reviews see Refs. 1 and 2). There are no human GrB mutants, and the in vitro data are supported by the wild type phenotype of cGrB knockout mice (5), which are defective in CL-mediated DNA degradation in target cells but otherwise are not significantly immune-compromised. Granzyme A and/or other mouse paralogs probably compensate for the loss of GrB in this context, so it is difficult to draw conclusions from this mouse model about the function of human GrB in humans.

Before the description of the RAH allele there had been no report of naturally occurring human GrB variants, and the initial conclusion that this allele encodes a common, non-cytotoxic form of GrB suggested that a fundamental shift in thinking about the role(s) of this protease was required (10). However, purified RAH and wild type enzymes were not compared in this study, and the assay used to assess GrB cytotoxicity was unconventional, involving the production of active GrB within target cells and coupled with its perforin-independent release from the secretory pathway into the nucleocytoplasm via an unknown mechanism (10). This contrasts with the usual mode of GrB delivery (following zymogen activation in the CL granule), which involves release of GrB from the CL, endocytosis by the target cell, and perforin-mediated entry into the cytoplasm (reviewed in Ref. 1). Furthermore, although the RAH mutations are clearly in linkage disequilibrium (10), it is unlikely that a dysfunctional GrB allele occurring at this frequency would have arisen through natural selection.

Using purified, recombinant enzymes and conventional assays, the work we report here clearly shows that RAH GrB retains the biochemical and cytotoxic functions of wild type GrB. We conclude that in the context of the documented functions of GrB the RAH allele represents a neutral polymorphism. Thus, the physiological significance of human GrB remains to be determined.

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