and the ROS were collected from the interface between 10 and 18% OptiPrep. The supernatant was diluted three times with Ringer's solution and centrifuged at 30,000g for 20 min. The sedimented material containing the ROS was rinsed once with 200 μl of Ringer's solution. The ROS were osmotically intact. We disrupted the plasma membrane of the ROS by hypotonic shock; 90 μl of water was added directly to the sedimented material, and the ROS were resuspended by intense mixing for ~10 s. The osmolarity was adjusted by adding 10 μl of a 10× intracellular buffer containing 120 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.5), 1 mM dithiothreitol, 10 μM leupeptin, and 100 kallikrein units per 1 ml of aprotinin (final concentrations).

15. Rhodopsin expression in control and W70A retinas was assayed by two different techniques. (i) The absorption of 500-nm light by rhodopsin was measured in retinal flatmount preparations. The fraction of absorbed light was calculated as $(I_2 - I_1)/I_2$, where I_1 and I_2 are the measured intensities of 500-nm light transmitted through the retina before and after bleaching the rhodopsin with bright white light for 10 min. The transmitted intensity of a spot of light (0.015 mm^2) was measured by a photomultiplier that was connected to a digital pulse counter. The mean percent of absorbed light (expressed as minimum, maximum, and n, number of determinations) was 27.6%, (23.6, 34.7, n = 3) in 129/SvJ retinas and 27.5% (21.3, 31.8, n = 4) in W70A retinas. (ii) The amount of rhodopsin in the retinas of four mice was determined through difference spectroscopy [M. D. Bownds, A. Gordon-Walker, A.-C. Gaide-Huguenin, W. Robinson, *J. Gen. Physiol.* **58**, 225 (1971)] after solubilization in 30 mM cetyltrimethylammonium chloride. The rhodopsin content of both control and W70A retinas was 0.3 nmol per retina.
16. J. B. Hurley and L. Stryer, *J. Biol. Chem.* **257**, 11094 (1982).
17. The absolute rates of GTP hydrolysis in control ROS in vitro were slower than the rate of recovery of the photoresponse. This is consistent with many previous reports that show that dilution of cellular components, most likely RGS9, slows the rate of GTP hydrolysis (*6*) [E. A. Dratz, J. W. Lewis, L. E. Schaechter, K. R. Parker, D. S. Kliger, *Biochem. Biophys. Res. Commun.* **146**, 379 (1987); V. Y. Arshavsky, M. P. Antoch, K. A. Lukjanov, P. P. Philippov, *FEBS Lett.* **250**, 353 (1989)]. Similarly, the 2.7-fold difference in GTPase rate between control and W70A ROS in Fig. 2E should be considered only as the lowest estimate for the difference in physiologically intact photoreceptors.
18. D. A. Baylor, T. D. Lamb, K.-W. Yau, *J. Physiol.* **288**, 613 (1979). Mice were adapted to dark conditions for 2 to 18 hours, and the retinas were isolated as described [C.-H. Sung, C. L. Makino, D. A. Baylor, J. Nathans, *J. Neurosci.* **14**, 5818 (1994)]. The retina was chopped, and small pieces were placed into the recording chamber, which was perfused with bicarbonate-buffered Locke's solution [112.5 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM Hepes (pH 7.4), 0.02 mM EDTA, 20 mM $NaHCO_3$, 3 mM Na_2-succinate, 0.5 mM Na-glutamate, 10 mM glucose, and 0.1% vitamin and amino acids supplement solution (Sigma)], bubbled with 95% O_2 and 5% CO_2, and warmed to 34° to 37°C. The outer segments of single rods were drawn into a suction electrode that was connected to a current-measuring amplifier (Axopatch, Axon Instruments, Foster City, CA). The electrode contained 140 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 3 mM Hepes (pH 7.4), 0.02 mM EDTA, 10 mM glucose, and 0.1% vitamin and amino acid supplement (Sigma). The responses were low-pass filtered at 20 Hz with an eight-pole Bessel filter and digitized at 100 Hz with an acquisition program written by F. Rieke for IgorPro (Wave Metrics, Lake Oswego, OR). Brief flashes (10 ms) of 500-nm light were used for stimulation. The intensity of the light source was calibrated with a silicon detector (UDT350, Graseby Optronics, Orlando, FL), and the flash strength was controlled with calibrated neutral density filters. When white light was needed to evoke the maximal response from a W70A rod, its intensity was expressed as the equivalent intensity at 500 nm, using the relative ability of white and 500-nm light to stimulate the rod.
19. T. D. Lamb, P. A. McNaughton, K.-W. Yau, *J. Physiol.* **319**, 463 (1981). The peak amplitude r of the average response at each flash strength was divided by the maximal response amplitude r_{max} of control and W70A rods to produce the normalized amplitude. In control rods, r_{max} is the change in membrane current that results from the closure of all cGMP-gated channels. However, in most W70A rods that were without an exogenous calcium buffer, it was impossible to completely shut off the inward current. Therefore, for Fig. 3B, r_{max} in W70A rods was taken as the maximum amplitude observed.
20. At early times during the rising phase of the flash response, the light-evoked increase in PDE activity as a function of time, PDE*(t), is given by the expression (*24*) $\mathrm{PDE}^*(t) = -(1/n)\ (d\{\ln[1 - r(t)/r_{max}]\}/dt)$, where $r(t)$ is the time course of the flash response and n is the cooperativity of the cGMP-gated channels. We calculated PDE*(t) with this formula, assuming n = 3 [A. L. Zimmerman and D. A. Baylor, *Nature* **321**, 70 (1986); L. W. Haynes, A. R. Kay, K.-W. Yau, *ibid.*, p. 66]. The cell in Fig. 3, E and F, was loaded with BAPTA to determine the saturating maximal amplitude (7.4 pA) and to delay the onset of calcium-dependent negative feedback to the cascade. BAPTA had no effect on the rate of PDE activation in any control or W70A rods examined. To determine PDE*(t) for W70A rods in which the flash response did not saturate, we assumed the dark current (r_{max}) to be 12 pA. For all cells, the mean number of photoisomerizations per flash was calculated by multiplying the flash strength (in photons per square micrometer) by the effective collecting area of the mouse rod (0.23 μm^2).
21. In a mouse rod that is stimulated by an instantaneous flash causing 100 photoisomerizations per disc face, the total complement of activatable PDE in a disc face will be depleted with a time course $f(t) = 1 - \exp(-t/\tau_{act})$, in which the time constant τ_{act} is given by the ratio of the total number of PDE subunits divided by the initial rate of activation (*24*). Assuming 1000 s^{-1} for the initial rate of PDE activation per photoactivated rhodopsin (*24*), one finds that the initial rate of PDE* production will be 1×10^5 s^{-1}. With ~400 PDE subunits per disc face (based on ~1:100 ratio of PDE to rhodopsin) a τ_{act} value of ~4 ms is obtained. Allowing for the finite flash duration of 10 ms and assuming an effective delay of 3 ms (*24*), one would expect activation of the PDE in a normal mouse rod to be completed within a few milliseconds after the end of the flash.
22. M. E. Burns and D. A. Baylor, unpublished observation.
23. Because of the prolonged lifetime of PDE* in the W70A rods, any feedback reaction underlying the fast recovery component should be downstream of PDE*, as any upstream feedback should affect the response amplitude, not the recovery kinetics [for a detailed analysis, see S. Nikonov, N. Engheta, E. N. Pugh, *J. Gen. Physiol.* **111**, 7 (1998)]. Therefore, we propose that the acceleration of guanylate cyclase activity, which is caused by decreased intracellular Ca^{2+}, is mainly responsible for the rapid initial recovery phase of W70A responses to bright flashes.
24. T. D. Lamb and E. N. Pugh, *J. Physiol.* **449**, 719 (1992); E. N. Pugh and T. D. Lamb, *Biochim. Biophys. Acta* **1141**, 111 (1993).
25. V. Y. Arshavsky, C. L. Dumke, M. D. Bownds, *J. Biol. Chem.* **267**, 24501 (1992).
26. B. K. K. Fung, J. B. Hurley, L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 152 (1981).
27. V. Y. Arshavsky, M. P. Gray-Keller, M. D. Bownds, *J. Biol. Chem.* **266**, 18530 (1991).
28. P. D. Calvert, T. W. Ho, Y. M. LeFebvre, V. Y. Arshavsky, *J. Gen. Physiol.* **111**, 39 (1998).

29. We thank E. R. Makino and J. W. Handy for help with biochemical experiments; D. B. Farber for providing *Pdeg* cDNA; J. Xu and M. I. Simon for providing the opsin promoters; T. G. Wensel for providing antibodies against RGS9; F. Costantini, C. Liu, and members of their laboratories for sharing ideas and equipment; V. I. Govardovskii, R. Axel, and E. Kandel for critically reading the manuscript; and M. Mendelsohn, K. Doi, H. Kjeldbye, J. Ma, and D. Wiener for discussion. Supported by NIH grants T32 EY07105, EY05750, EY10336, and EY11510; the Ruth and Milton Steinbach Fund; the McKnight Foundation; and Research to Prevent Blindness (RPB). S.P.G. is an investigator of the Howard Hughes Medical Institute. V.Y.A. is a recipient of a Jules and Doris Stein professorship from RPB.

30 April 1998; accepted 25 August 1998

An Antimicrobial Activity of Cytolytic T Cells Mediated by Granulysin

Steffen Stenger,* Dennis A. Hanson, Rachel Teitelbaum, Puneet Dewan, Kayvan R. Niazi, Christopher J. Froelich, Tomas Ganz, Sybille Thoma-Uszynski, Agustín Melián, Christian Bogdan, Steven A. Porcelli, Barry R. Bloom, Alan M. Krensky, Robert L. Modlin†

Cytolytic T lymphocytes (CTLs) kill intracellular pathogens by a granule-dependent mechanism. Granulysin, a protein found in granules of CTLs, reduced the viability of a broad spectrum of pathogenic bacteria, fungi, and parasites in vitro. Granulysin directly killed extracellular *Mycobacterium tuberculosis*, altering the membrane integrity of the bacillus, and, in combination with perforin, decreased the viability of intracellular *M. tuberculosis*. The ability of CTLs to kill intracellular *M. tuberculosis* was dependent on the presence of granulysin in cytotoxic granules, defining a mechanism by which T cells directly contribute to immunity against intracellular pathogens.

Cytolytic T lymphocytes are required for protective immunity against intracellular pathogens such as *Listeria monocytogenes* and *Trypanosoma cruzi*, pathogens known to escape from the phagocytic vacuoles into the cytoplasm of infected host cells. CTLs have

also been implicated in the control of organisms that are phagocytized by macrophages and remain localized within the phagosomes (for example, *Salmonella typhimurium*, *Escherichia coli*, and *Mycobacterium tuberculosis*). One mechanism by which immunity arises has been postulated to be the lysis of infected cells by the antigen-specific CTLs (*1*). This lysis is thought to be followed by the release of live bacteria, which are subsequently taken up and killed by newly immigrated and freshly activated macrophages (*2*). However, the increasing bacterial burden in the cells will eventually cause spontaneous lysis, which raises the intriguing question of why CTL-mediated lysis of the cell would be beneficial for the host. An explanation for the functional role of CTLs in immunity against intracellular infection was provided by the analysis of CTL in tuberculosis (*2, 3*). These experiments suggested that CTLs that kill infected cells through the granule-exocytosis pathway may release one or more effector molecules with the capacity to directly kill the intracellular microbial pathogen. We now show that granulysin is a critical effector molecule of the antimicrobial activity of CTLs.

Granulysin is a protein present in cytotoxic granules of CTL and natural killer (NK) cells (*4*, *5*). Amino acid sequence comparison indicates that granulysin is a member of the saposin-like protein (SAPLIP) family. Granulysin is most similar to NK-lysin (43% identity and 67% similarity), a porcine protein with antibacterial activity (*6*). Granulysin is in the cytotoxic granules of T cells, which are released upon antigen stimulation (*5*). Two subsets of CTLs exist, which differ in phenotype, cytotoxic effector pathway, and antimicrobial activity (*3*). $CD8^+$ CTLs lyse *M. tuberculosis*–infected macrophages by a granule-dependent mechanism that results in killing of the intracellular pathogen. In contrast, the cytotoxicity of $CD4^-CD8^-$ [double negative (DN)] T cells is mediated by Fas-Fas ligand interaction and does not inhibit growth of the mycobacteria. The presence of granulysin in these two populations was therefore investigated (Fig. 1). By protein immunoblot analysis, granulysin was detected in three $CD8^+$ CTLs, two that were CD1-restricted and specific for *M. tuberculosis* and one that was major histocompatibility complex class I–restricted and specific for an influenza virus peptide (Fig. 1B). In contrast, three CD1-restricted, *M. tuberculosis*–specific DN CTLs expressed no granulysin. Confocal microscopy of $CD8^+$ CTLs for granulysin revealed a punctate pattern consistent with granule localization (Fig. 1A). Double staining of the $CD8^+$ T cells with antibodies to granulysin and perforin, a molecule known to be expressed in cytotoxic granules (*7*), showed a substantial colocalization. These data demonstrated the presence of granulysin in cytotoxic granules of $CD8^+$ but not in DN CTLs.

To assess whether the microbicidal effect of CTLs on infected macrophages was mediated by release of cytotoxic granules, we used human CTLs that effectively killed intracellular *M. tuberculosis* residing in macrophages (Fig. 1C). When the CTLs were pretreated with Sr^{++} to induce degranulation and deplete their cytotoxic granules, the ability of CTLs to kill the pathogen was abrogated (Fig. 1C, left). The ability to detect granulysin disappeared in parallel with the loss of microbicidal activity (Fig. 1C, right). In this system, the intracellular killing of mycobacteria was not attributable to a purinergic mechanism (*8*). Thus, the expression of granulysin and the ability to reduce the viability of intracellular *M. tuberculosis* correlated.

We used recombinant granulysin (*9*) to directly test its antimicrobial activity against several microbial pathogens. Culture conditions were adapted for the specific growth requirements of each organism, and growth inhibition induced by recombinant granulysin was initially screened by radial diffusion assay and confirmed by colony-forming unit (CFU) assay (Fig. 2). In these experiments, granulysin showed a dose-dependent growth inhibition of a broad spectrum of pathogens,

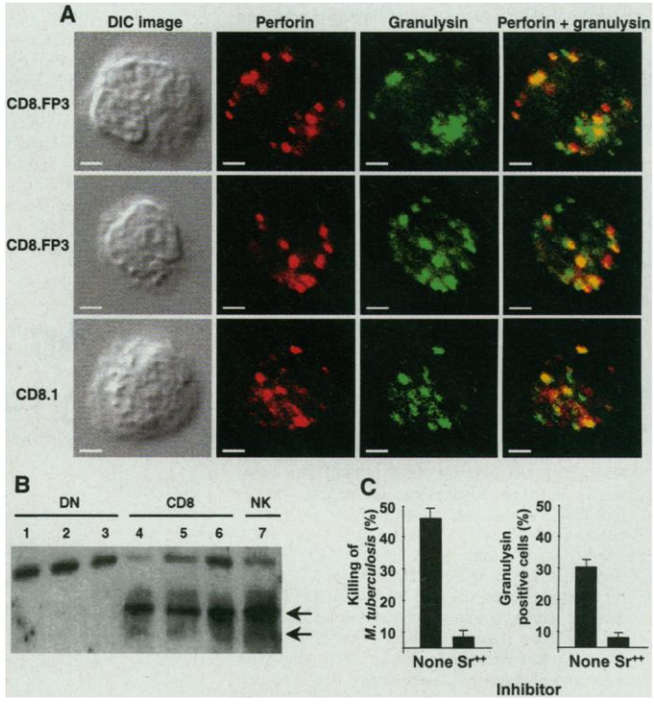

Fig. 1. The presence of granulysin correlates with the antimycobacterial effect of CTLs. (**A**) Detection of perforin and granulysin in CTLs by confocal laser microscopy (*26*). Cell lines recognizing either influenza virus (CD8.FP3) or *M. tuberculosis* (CD8.1) were double-labeled with anti-perforin (red vesicles) and anti-granulysin (green vesicles). Cells were visualized by differential interference contrast (DIC) (first panel of each row). Fluorescent confocal images were obtained for perforin expression (red, second panel of each row) and granulysin expression (green, third panel of each row). The two images were then superimposed (fourth panel of each row) to show vesicles expressing both perforin and granulysin (yellow). (**B**) Protein immunoblot analysis of CTLs for granulysin (*27*). Lysates of three DN (lane 1, DN.PT; lane 2, DN.OR; and lane 3, DN.AJW), three $CD8^+$ (lane 4, CD8.TX; lane 5, CD8.FP3; and lane 6, CD8.2), and an NK cell line (*20*) (lane 7, YT) were separated by SDS–polyacrylamide gel electrophoresis. Granulysin was detected with a rabbit serum and visualized by chemiluminescence. The arrows indicate the two known forms of granulysin, 15 kD and 9 kD (*5*). The blot shown is a representative example of three independent experiments with similar results. (**C**) The ability to reduce the viability of intracellular *M. tuberculosis* correlates with the expression of granulysin. Granules of an *M. tuberculosis*–specific (CD8.TX) or influenza peptide–specific (CD8.FP3) line were released by treatment with strontium (*28*). CTLs were coincubated with macrophages (*25*), which had been infected with *M. tuberculosis* (*29*). After 18 hours, mycobacterial viability was determined (*30*). The number of cells expressing granulysin was determined by immunostaining (*31*). The results shown are representative for two independent experiments, each performed in triplicates. The data are given as CFU ± SEM (left) or percentage of cells expressing granulysin ± SEM (right). Scale bar represents 2 μm in all panels.

S. Stenger, P. Dewan, K. R. Niazi, T. Ganz, S. Thoma-Uszynski, R. L. Modlin, Divisions of Dermatology and Pulmonary Medicine, Department of Medicine, Department of Microbiology and Immunology and the Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA 90095, USA. D. A. Hanson and A. M. Krensky, Division of Immunology and Transplantation Biology, Department of Pediatrics, Stanford University, Stanford, CA 94305, USA. R. Teitelbaum and B. R. Bloom, Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, NY 10461, USA. C. J. Froelich, Evanston Northwestern Healthcare Research Institute and Evanston Hospital, Department of Medicine, Evanston, IL 60201, USA. A. Melián and S. A. Porcelli, Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital at Harvard Medical School, Boston, MA 02115, USA. C. Bogdan, Institut für Klinische Mikrobiologie, Immunologie und Hygiene, Universität Erlangen, D-91054 Erlangen, Germany.

*Current address: Institut für Klinische Mikrobiologie, Immunologie und Hygiene, Universität Erlangen, D-91054 Erlangen, Germany.

†To whom correspondence should be addressed: rmodlin@medicine.medsch.ucla.edu

including bacteria, fungi, and parasites. We found potent antibacterial activity in the micromolar range against gram-positive and gram-negative bacteria, causing a three orders of magnitude reduction in CFUs of *S. typhimurium, L. monocytogenes, E. coli*, and *Staphylococcus aureus* (Fig. 2, left). Granulysin also killed fungi and parasites, including *Cryptococcus neoformans, Candida albicans*, and *Leishmania major* (Fig. 2, right). The broad antimicrobial spectrum of granulysin is reminiscent of structurally unrelated defensins, which are nonspecifically released from cytoplasmic granules of polymorphonuclear leukocytes to kill phagocytized pathogens, including *M. tuberculosis* (*10*).

Mycobacterium tuberculosis is one of the most resistant pathogens to microbicidal mechanisms of mononuclear phagocytes. The only effector molecules clearly shown to be involved are reactive nitrogen intermediates (*11*). The effect of granulysin on the viability of virulent *M. tuberculosis* was examined by culturing *M. tuberculosis* in 7H9 media in the presence of various concentrations of granulysin (Fig. 3A, left). In five experiments, granulysin killed *M. tuberculosis* in a dose-dependent manner, with up to 90% of the bacteria killed within 72 hours, representing almost a logarithmic reduction in the number of CFUs. However, no antibacterial activity was detected when granulysin was added to *M. tuberculosis*–infected macrophages (Fig. 3A, right). Although the percentage reduction of *M. tuberculosis* CFUs was within an order of magnitude and less than that seen against *E. coli*, *M. tuberculosis* infection in vivo can be produced by as few as 10 to 200 bacilli and is a slow process. In our experiments, the time of in vitro assay was only 72 hours, so that a cumulative antimicrobial effect mediated by these T cells over time could have a profound effect on the number of bacilli during the course of infection.

One explanation for the inability of granulysin to kill intracellular as compared with extracellular *M. tuberculosis* was the possible failure of the purified recombinant protein to gain access to the intracellular compartment in which mycobacteria reside. Such a defect may be overcome by the pore-forming agent perforin, which colocalized with granulysin (Fig. 1A). Experiments were therefore designed to compare relative activities of granu-

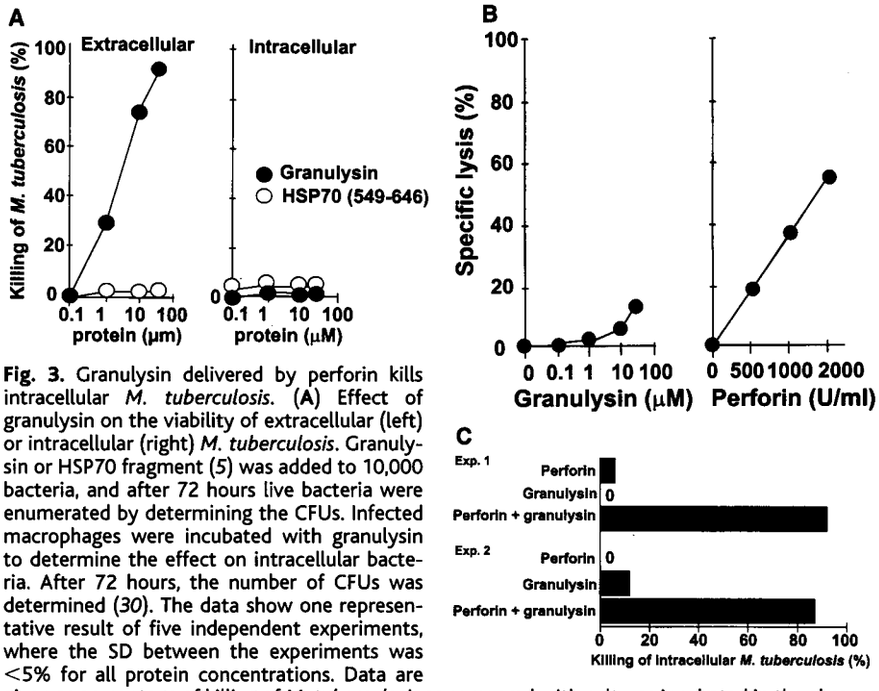

Fig. 3. Granulysin delivered by perforin kills intracellular *M. tuberculosis*. (**A**) Effect of granulysin on the viability of extracellular (left) or intracellular (right) *M. tuberculosis*. Granulysin or HSP70 fragment (*5*) was added to 10,000 bacteria, and after 72 hours live bacteria were enumerated by determining the CFUs. Infected macrophages were incubated with granulysin to determine the effect on intracellular bacteria. After 72 hours, the number of CFUs was determined (*30*). The data show one representative result of five independent experiments, where the SD between the experiments was <5% for all protein concentrations. Data are given as percentage of killing of *M. tuberculosis* as compared with cultures incubated in the absence of protein. (**B**) Lytic activity of granulysin and perforin. Infected macrophages were incubated with granulysin or perforin. After 4 hours, the extent of cell lysis was determined (*33*). Results are given as percentage of specific lysis and show one typical experiment out of six. The SD between the single experiments was <7% for all concentrations. The spontaneous release of LDH by infected macrophages in the absence of protein was <10%. (**C**) Mycobactericidal activity of a combination of perforin and granulysin. Macrophages infected with *M. tuberculosis* were incubated with granulysin (25 μM) and perforin (2000 U/ml). After 72 hours, the number of live bacteria was determined (*30*). The data are given as percentage of killing of *M. tuberculosis* as compared with the bacteria cultured in diluent alone.

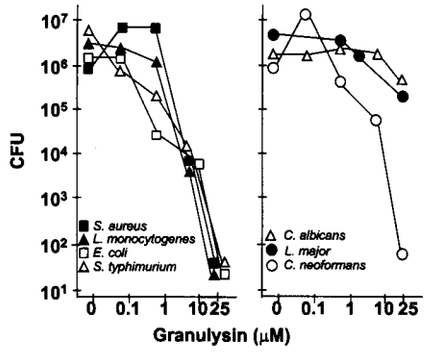

Fig. 2. Dose-dependent cytotoxicity of granulysin against bacteria, parasites, and fungi. Microorganisms and granulysin were mixed and coincubated under appropriate conditions in a volume of 50 μl (bacteria and fungi: 3 hours at 37°C; *L. major*: 72 hours at 26°C). Samples were then diluted and spread on Trypticase soy agar (bacteria) or Sabouraud dextrose agar (fungi). After 24 hours, the number of CFUs was determined (*32*). *Leishmania major* parasites were quantitated by limiting dilution analysis performed on Novy-Nicolle-MacNeal blood agar slants, and the number of positive wells was determined microscopically after 14 days of incubation at 26°C. The results shown are representative for at least three experiments with similar results and are given as CFUs.

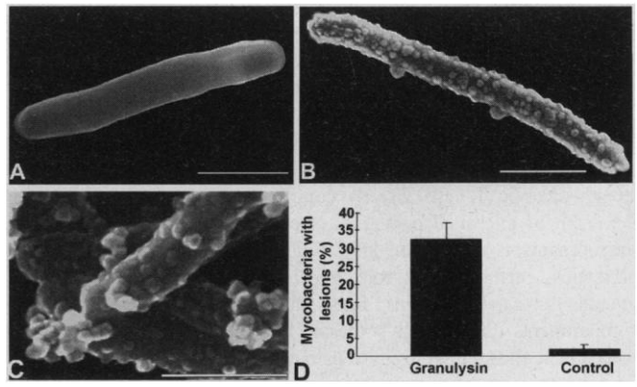

Fig. 4. Granulysin induces lesions in the mycobacterial cell surface. *Mycobacterium tuberculosis* was incubated with or without granulysin (30 μM) for 80 hours. 1×10^6 bacteria were fixed and processed for scanning electron microscopy (*34*). (**A**) *Mycobacterium tuberculosis* incubated for 80 hours in medium alone (×23,000; scale bar represents 1 μm in all panels). (**B**) *Mycobacterium tuberculosis* coincubated for 80 hours with granulysin results in the formation of marked lesions in the bacterial surface (×23,000). (**C**) Granulysin-treated bacteria singly and in clusters reveal surface lesions (×40,000). (**D**) Percentage of granulysin-treated *M. tuberculosis* with lesions. Results shown represent the mean ± SEM of more than 400 bacilli per group enumerated per treatment.

lysin and purified perforin (*12*) in lysing human macrophages infected with virulent *M. tuberculosis* (Fig. 3B). Granulysin, when added in a concentration range that efficiently killed extracellular bacteria, showed little lytic activity against infected macrophages. In contrast, purified perforin, which is known to lyse various hematopoietic targets (*13*), exhibited substantial lytic activity against identically infected macrophages but was ineffective in reducing the viability of *M. tuberculosis* either in culture or intracellularly in macrophages. Thus, granulysin is a powerful mycobactericidal agent but may not be effectively delivered to the phagolysosomal compartment. In contrast, perforin is an ineffective antimicrobial agent, although it has the capacity to lyse infected target cells. A combination of perforin and granulysin resulted in macrophage lysis and decreased viability of intracellular mycobacteria (Fig. 3C). Thus, granulysin could kill intracellular *M. tuberculosis* if perforin, or possibly other pore-forming molecules of T cell granules, provided access to the intracellular compartment. The bacteria may be killed intracellullarly or extracellularly in tuberculosis, but this issue may not be of great biological importance. We know that *M. tuberculosis* grows both intracellularly or extracellularly, such that a CTL granule protein that can reduce the viability of the pathogen within infected cells or in the vicinity of infected or dying cells could have profound protective activity.

Both perforin and members of the amoebapore family are thought to mediate their biological effects by formation of microscopic pores leading to damage of target cell membranes (*14*). Consequently, we sought to ascertain whether granulysin killed *M. tuberculosis* by altering the structure or integrity of the bacteria. Scanning electron microscopy revealed that granulysin induces discrete lesions and distortions in the bacterial surface of *M. tuberculosis* (Fig. 4). Mycobacteria, singly and in clusters, were found to contain multiple small protrusions that were almost entirely absent from control samples. The reduction in viable CFUs in conjunction with evidence of bacterial surface alteration and distortion indicates that granulysin is directly cytotoxic to *M. tuberculosis* and other bacteria.

These findings define a pathway by which antigen-specific T cells directly contribute to the death of microbial pathogens, specifically microorganisms residing in intracellular compartments. Perforin has a role in delivery of granule-associated proteins into subcellular compartments (*15*). We hypothesize that this mechanism allows granulysin to gain access to intracellular pathogens and destroy them. Although initial studies with perforin gene knockout mice had suggested that perforin is not required for the early control of tuberculosis (*16*), more recent studies indicate that perforin is in fact required for long-term protection (*17*). The perforin-granulysin microbicidal pathway is likely to complement non–antigen-specific mechanisms by which monocytes kill pathogens, including the generation of nitric oxide and oxygen free radicals.

A number of biologic functions have been proposed for the SAPLIP family, which includes granulysin, NK-lysin, saposins, surfactant-associated protein B, amoebapores, and plant aspartic proteinases. The present results provide evidence for the antimicrobial activity of granulysin. The presence of granulysin in NK cells (*18*) and of related peptides in cytoplasmic granules of *Entamoeba histolytica*, the amoebapores (*19*), suggests that the SAPLIP family represents an ancient yet highly conserved form of antimicrobial host defense, likely contributing to innate immune responses. The importance of this pathway is reflected by the presence of granulysin and other family members in CTLs, indicating that the adaptive immune response has evolved to include antimicrobial peptides for effective immunity. We propose that the role of CTLs in protection against intracellular pathogens is not merely to lyse target cells and disperse the intracellular pathogens but in addition to deliver granulysin, a lethal weapon by which CTLs can directly reduce the viability of a variety of bacteria, fungi, and parasites genetically selected to evade host defense.

References and Notes

1. S. H. E. Kaufmann, E. Hug, G. de Libero, *J. Exp. Med.* **164**, 363 (1986); E. G. Pamer, J. T. Harty, M. J. Bevan, *Nature* **353**, 852 (1991); J. T. Harty, R. D. Schreiber, M. J. Bevan, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11612 (1992).
2. G. De Libero, I. Flesch, S. H. E. Kaufmann, *Eur. J. Immunol.* **18**, 59 (1988); S. H. E. Kaufmann, *Immunol. Today* **9**, 168 (1988).
3. S. Stenger *et al.*, *Science* **276**, 1684 (1997).
4. J. Jongstra *et al.*, *J. Exp. Med.* **165**, 601 (1987).
5. S. V. Pena, D. A. Hanson, B. A. Carr, T. J. Goralski, A. M. Krensky, *J. Immunol.* **158**, 2680 (1997).
6. M. Andersson *et al.*, *EMBO J.* **14**, 1615 (1995); S. V. Pena and A. M. Krensky, *Semin. Immunol.* **9**, 117 (1997).
7. D. Masson and J. Tschopp, *J. Biol. Chem.* **260**, 9069 (1985); E. R. Podack, J. D. Young, Z. A. Cohn, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8629 (1985).
8. D. A. Lammas *et al.*, *Immunity* **7**, 433 (1997); A. Molloy, P. Laochumroonvorapong, G. Kaplan, *J. Exp. Med.* **180**, 1499 (1994).
9. Granulysin was produced in *E. coli* BL21 (DE3) with the kanamycin-selective pET28a vector (Novagen). The protein-coding region of granulysin cDNA corresponding to the 9-kD form, starting at the glycine residue 48 of the 519 message and continuing through the arginine residue 121, was fused to the pET28a-encoded NH_2-terminal polyhistidine sequence at the Nde 1 site. The recombinant proteins were expressed and purified under denaturing conditions as described (*5*). Preparations of recombinant granulysin were separated on SDS gel, and silver staining of proteins showed a single band. Purity of the preparation was additionally confirmed by ion mass spectroscopy, which revealed the presence of a single peptide of 9.071 kD, correlating with the predicted molecular mass of granulysin.
10. R. I. Lehrer, A. K. Lichtenstein, T. Ganz, *Annu. Rev. Immunol.* **11**, 105 (1993); Y. Miyakawa *et al.*, *Infect. Immun.* **64**, 926 (1996).
11. M. Denis, *Cell. Immunol.* **132**, 150 (1991); J. Chan, Y. Xing, R. S. Magliozzo, B. R. Bloom, *J. Exp. Med.* **175**, 1111 (1992); S. Nicholson *et al.*, *ibid.* **183**, 2293 (1996); J. D. MacMicking *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5243 (1997); M. G. Bonecini-Almeida *et al.*, *J. Immunol.* **160**, 4490 (1998).
12. Human perforin was extracted in 1 M NaCl from the granules of the NK-like cell line YT (*20*) and purified by ion metal affinity chromatography (*21*). The cells were resuspended in ice-cold relaxation buffer and disrupted by gradual decompression after pressurization in a nitrogen cavitation bomb (model 4639; Parr Instruments, Moline, IL) at 175.75 kg/cm^2 at 0°C for 9 min. High-density granules were prepared for salt extraction from the disrupted cells by spinning at 400*g* for 7 min to remove nuclei and unbroken cells. The postnuclear supernatant was then spun at 14,500*g* for 15 min to yield the granule pellet and the cytosolic supernatant. The granule pellet was then extracted and spun at 8500*g* for 10 min. The supernatant was filtered through a 0.8-μm filter apparatus, applied to PD-10 columns containing P-6 matrix (Bio-Rad), and eluted with 1 M NaCl, 20 mM Hepes, and 10% betain. The granule extract was then exchanged into Hepes buffer and immediately injected onto an HR 5/5 column (Pharmacia) loaded with Poros 20 MC metal chelate affinity media (PerSeptive Biosystems, Cambridge, MA) to perform ion metal affinity chromatography. Eluted fractions were all individually assayed for hemolytic activity. The fractions with the highest hemolytic activity were pooled and concentrated for use.
13. R. C. Duke *et al.*, *J. Exp. Med.* **170**, 1451 (1989).
14. G. Berke, *Annu. Rev. Immunol.* **12**, 735 (1994); M. Leippe, *Cell* **83**, 17 (1995).
15. C. J. Froelich, V. M. Dixit, X. Yang, *Immunol. Today* **19**, 30 (1998).
16. P. Laochumroonvorapong *et al.*, *Infect. Immun.* **65**, 127 (1997); A. M. Cooper, C. D'Souza, A. A. Frank, I. M. Orme, *ibid.*, p. 1317.
17. A. De Souza and B. Bloom, personal communication; G. Kaplan, personal communication; I. Orme, personal communication.
18. T. Yabe, C. McSherry, F. H. Bach, J. P. Houchins, *J. Exp. Med.* **172**, 1159 (1990).
19. M. Leippe, J. Andra, R. Nickel, E. Tannich, H. J. Muller-Eberhard, *Mol. Microbiol.* **14**, 895 (1994).
20. J. Yodoi *et al.*, *J. Immunol.* **134**, 1623 (1985).
21. C. J. Froelich, J. Turbov, W. Hanna, *Biochem. Biophys. Res. Commun.* **229**, 44 (1996).
22. M. Sugita *et al.*, *Science* **273**, 349 (1996); R. M. Jackman *et al.*, *Immunity* **8**, 341 (1998); R. A. Rogers, R. M. Jack, S. T. Furlong, *J. Cell Sci.* **106** (part 2), 485 (1993).
23. P. A. Neighbour, H. S. Huberman, Y. Kress, *Eur. J. Immunol.* **12**, 588 (1982); P. C. Quan, T. Ishizaka, B. R. Bloom, *J. Immunol.* **128**, 1786 (1982); P. A. Neighbour and H. S. Huberman, *ibid.*, p. 1236.
24. J. C. Foreman, *J. Physiol.* **271**, 215 (1977).
25. Peripheral blood mononuclear cells from healthy donors were treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) (200 U/ml; Genetics Institute, Cambridge, MA) and interleukin-4 (IL-4) (100 U/ml; Schering-Plough, Kenilworth, NJ) for 72 hours. Nonadherent cells were discarded, and the adherent fraction, which was enriched for CD1$^+$ cells, was detached by treatment with 1 mM EDTA (Sigma) and replated in six-well plates at a density of 3×10^6 cells per well. We used adherent CD1$^+$ antigen-presenting cells (APCs), because these cells fulfilled the requirements for all our experiments: (i) CD1$^+$ APCs are adherent and therefore allow removal of nonphagocytized mycobacteria, (ii) they can be infected with virulent *M. tuberculosis* with high efficiency (3.9 bacteria per infected cell, 85% of the cells infected), and (iii) they present antigen to CD1-restricted CTLs.
26. Cultured CD8$^+$ T cell lines, CD8.FP3 and CD8.1, were fixed and permeabilized as previously described (*22*). Cells were incubated at 4°C for 1 hour in suspension with dG9 (3 μg/ml) (Kamija Biomedical, Tukwila, WA), antibody to perforin (anti-perforin) in phosphate-buffered saline (PBS) with 5% goat serum, or MPC11 (3 μg/ml), a nonbinding isotype-matched

immunoglobulin G_{2b} (IgG_{2b}) control. Cells were then washed two times with PBS and 5% goat serum and incubated with Texas Red–conjugated goat anti-mouse IgG_{H+L} (2.5 μg/ml) (Molecular Probes) for 1 hour at 4°C. Subsequently, incubation with 10% mouse serum in PBS for 10 min and two washes with PBS and 5% goat serum were performed to block nonspecific binding. Cells were then stained with either anti–granulysin DH2 (4 μg/ml) (D. Hanson and A. Krensky, unpublished data) or P3, an IgG1 control, followed by two additional wash steps with PBS and 5% goat serum. For confocal microscopy, cells were mounted on glass slides in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) as an antifading agent. Cells were then examined with a Leica TCS-NT confocal laser scanning microscope fitted with krypton and argon lasers. Cells were illuminated with 488 and 568 nm of light after filtering through an acoustic optical device. Images of cells decorated with fluorescein isothiocyanate (FITC) and Texas Red were recorded simultaneously through separate optical detectors with a 530-nm band-pass filter and a 590-nm long-pass filter, respectively. Pairs of images were superimposed for colocalization analysis (*22*).

27. M. tuberculosis–specific, CD1-restricted DN or $CD8^+$ CTLs and influenza peptide–specific $CD8^+$ CTLs were generated and cultured as described previously (*3*). Granulysin protein was detected by pelleting 1×10^6 CTLs 1 week after the last restimulation with antigen, lysing the cells in SDS sample buffer, and separating proteins on 15% SDS gels. Proteins were then transferred to nitrocellulose membranes. To ensure equal loading, we initially determined the protein concentration spectrophotometrically and confirmed it by staining the nitrocellulose membranes with Ponceau red. Granulysin protein was detected with 519/GST rabbit serum (1/1000) (*5*) followed by horseradish peroxidase–conjugated secondary antibodies. Immunodetection was performed with enhanced chemiluminescence following the manufacturer's guidelines.
28. CTLs were degranulated by pretreatment with 25 mM strontium (Aldrich, Milwaukee, WI) for 18 hours. Previous studies by Bloom and colleagues (*23*), based on many studies on degranulation of mast cells (*24*) by strontium, as well as the previous study of CTLs (*3*), have demonstrated that strontium induces degranulation of lymphocytes without causing cellular toxicity. Strontium inhibits the cytotoxicity of $CD8^+$ CTLs by inducing the release of cytotoxic granules, reflected by the release of granzyme A and the marked reduction of perforin and granulysin-containing granules (Fig. 1C) (S. Stenger *et al.*, data not shown). The capacity of $CD8^+$ CTLs to proliferate and release interferon γ upon antigen-specific activation was not affected by treatment with strontium. In addition, strontium did not affect the ability of DN, CD1-restricted CTLs to lyse infected target cells. Strontium-treated lymphocytes were thoroughly washed before coculture with *M. tuberculosis*–infected cells. Therefore, the inhibition of the antimicrobial activity of $CD8^+$ CTLs by strontium is not due to a nonspecific or toxic effect.
29. *Mycobacterium. tuberculosis* (virulent strain H37Rv) was grown in suspension with gentle, constant rotation in roller bottles containing Middlebrook 7H9 medium (Difco, Detroit, MI) supplemented with 0.05% Tween 80 (Sigma), 1% glycerol (ICN, Costa Mesa, CA), and 10% OADC (oleic acid, albumin, dextrose, catalase) (Becton-Dickinson). Portions from logarithmically growing cultures were frozen in PBS containing 10% glycerol. Comparison of microscope counts of mycobacteria and their growth on Middlebrook 7H11 agar plates revealed a viability of the bacteria above 90%. Adherent monolayers (*25*) were infected with live *M. tuberculosis* for 4 hours at a multiplicity of infection of 5:1. After extensive washing, macrophages were detached, and the efficiency of infection was determined by staining a portion with auramine-rhodamine acid-fast stain.
30. To determine the viability of intracellular mycobacteria, we lysed the macrophages with 0.1% saponin and plated fivefold dilutions of the lysates in duplicates on 7H11 agar plates. The number of colonies was counted after 3 weeks of incubation. The percentage of killing of intracellular *M. tuberculosis* mediated by CD8.TX (Fig. 1C) was calculated according to the following formula: [(CFUs in macrophages cultured with CD8.FP3 − CFUs in macrophages cultured with CD8.TX)/CFUs in macrophages cultured with CD8.FP3] × 100, where CD8.FP3 is an influenza peptide–specific CTL without lytic activity against mycobacteria-infected macrophages and was therefore included as a negative control. Killing of *M. tuberculosis* by granulysin and perforin (Fig. 3C) was calculated as follows: {[CFUs in macrophages cultured in diluent alone − CFUs in macrophages cultured in the presence of perforin or granulysin (or both)]/CFUs in macrophages cultured in diluent alone} × 100.
31. The number of cells expressing granulysin was determined by immunostaining of a portion of the same cell suspension used for the determination of mycobacterial growth. Sr^{++}-treated or untreated CTLs were immobilized on poly-L-lysine–coated slides, fixed with 4% paraformaldehyde, and incubated with permeabilization/blocking solution (5% human serum, 5% goat serum, 0.1% Triton X-100, 0.01% saponin, and 1% nonfat dry milk). Cells were stained with a monoclonal mouse anti-human (DH2, 5 μg/ml) and detected with a FITC-conjugated goat anti-mouse.
32. Bacteria or fungi were incubated with the indicated concentrations of granulysin at 37°C for 3 hours in 10 mM sodium phosphate (pH 7.4) supplemented with 0.03% Trypticase soy broth (Becton-Dickinson) for bacteria or 0.03% Sabouraud dextrose broth (Difco) for fungi in a volume of 50 μl. After incubation, the samples were diluted 1:100 in ice-cold 10 mM PO_4 and spread on Trypticase soy agar or Sabouraud dextrose agar plates (Clinical Standard Laboratories, Rancho Domingez, CA) with a spiral plater (Spiral Systems, Cincinnati, OH), which delivers a defined volume per area and thus allows precise counts of microbial colonies. For quantification of *L. major*, 20,000 stationary phase promastigotes were incubated with various concentrations of granulysin or diluent. After 72 hours, limiting dilution was performed, and the number of live parasites was estimated by applying Poisson statistics and x^2-minimization. The principal protein used to control the activity of granulysin was a cloned fragment of HSP70, residues 549 to 646, previously used as a control for granulysin (*5*). It has a molecular weight of 10.8 kD, quite comparable to the size of the 9-kD form of granulysin. The HSP70(549-646) fragment was cloned into the same expression vector with the same cloning sites as granulysin, and both proteins were purified exactly the same way: nickel column under denaturing conditions, refolded, dialyzed, and finally purified by reversed-phase high-performance liquid chromatography. The endotoxin content of both preparations was <0.05 ng/ml. Granulysin, the HSP70(549-646) fragment, and all cell lines used in this study were of human origin. The HSP70(549-646) fragment was included in our initial screening of the antimicrobial activity of granulysin by radial diffusion assay and did not show any activity. We also used purified perforin as an additional control on the antimicrobial killing of several species of bacteria in vitro and found it to be without effect.
33. The percentage of cell lysis was determined by measuring the enzymatic activity of lactate dehydrogenase (LDH) in the supernatant (CytoTox 96; Promega, Madison, WI). Specific lysis was calculated according to the formula {[absorbance at 490 nm (A_{490}) from experimental − A_{490} spontaneous release)/(maximal release − A_{490} spontaneous release × 100]}.
34. *Mycobacterium tuberculosis* (2×10^6) was cultured in 7H9 media supplemented with OADC with or without the addition of purified granulysin (30 μg/ml). After incubation at 37°C with gentle shaking for 80 hours, portions were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 4 hours. Bacteria were washed twice in PBS, placed on cover slips coated with poly-L-lysine or fibronectin-coated tissue culture inserts (BIOCOAT; Becton-Dickinson) for 45 min, and then processed for scanning electron microscopy. Specimens were viewed on a JEOL 6400 scanning electron microscope.
35. Supported by the AIDS Stipendium, Deutsches Krebsforschungszentrum, Heidelberg (S.S.), NIH (R.L.M., S.A.P., A.M.K., and B.R.B), Deutsche Forschungsgemeinschaft (C.B. and S.T.-U.), the Arthritis Foundation (S.A.P.), the American Cancer Society (S.A.P), the Howard Hughes Medical Institute (B.R.B.), the UNDP/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases (IMMLEP), and the Dermatology Research Foundation of California. A.M.K. is the Shelagh Galligan Professor of Pediatrics and a Burroughs Wellcome Scholar in Experimental Therapeutics. We thank M. Horwitz for allowing us to use his P3 laboratory, W. Ernst and M. Röllinghoff for critical reading of the manuscript, and R. A. Rogers, J. Lai, and J. Mervis for assistance with confocal microscopy. We thank the Genetics Institute for the gift of GM-CSF and Schering-Plough for the gift of IL-4.

6 July 1998; accepted 24 August 1998