Characterization of Bovine Homologues of Granulysin and NK-lysin[1]

Janice J. Endsley,* Jason L. Furrer,† Mark A. Endsley,* Mark A. McIntosh,† Alexander C. Maue,† W. Ray Waters,‡ David R. Lee,† and D. Mark Estes[2]*†

Granulysin and NK-lysin are antimicrobial proteins found in the granules of human and swine cytotoxic lymphocytes. A murine counterpart to granulysin has not been identified to date, indicating the importance of additional models to fully characterize the role of granulysin-like molecules in the immune response to infectious disease. Two partial nucleotide sequences corresponding to the complete functional domain of granulysin and NK-lysin were amplified from bovine PBMC mRNA. Following stimulation with phorbol ester and calcium ionophore, expression of the bovine gene was detected in CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells, WC1$^+$ $\gamma\delta$ T cells, and PBMC depleted of CD3$^+$ T cells, but was absent in CD21$^+$ cells and CD14$^+$ cells. Intracellular flow cytometry and immunoblotting confirmed the presence of protein corresponding to the bovine granulysin homologue in activated T lymphocytes and PBMC. Synthetic human, bovine, and swine peptides corresponding to the C terminus of helix 2 through helix 3 region of granulysin displayed potent antimicrobial activity against *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, and *Mycobacterium bovis* bacillus Calmette-Guérin. Human and bovine peptides corresponding to helix 2 displayed antimycobacterial activity against *M. bovis* bacillus Calmette-Guérin. Expression of the bovine gene was detected in laser microscopy-dissected lymph node lesions from an *M. bovis*-infected animal. The identification of a biologically active bovine homologue to granulysin demonstrates the potential of the bovine model in characterizing the role of granulysin in the immune response to a variety of infectious agents. ***The Journal of Immunology,* 2004, 173: 2607–2614.**

Granulysin and NK-lysin are antimicrobial proteins found in the granules of human and swine T lymphocytes and NK cells. These effector cell lysin molecules colocalize with perforin in cytotoxic granules and are present in NK cells and in T lymphocytes expressing CD4, CD8, and the $\gamma\delta$ TCR (1–3). Killing activity by granulysin and NK-lysin has been reported against tumors and a growing list of microorganisms including Gram-positive and Gram-negative bacteria, fungi, protozoa, parasites, and possibly viruses (1, 4–11). Granulysin expression is induced following mitogenic or antigenic activation of T lymphocytes and increases in NK cells following exposure to IL-2 (8, 12–14). Expression of granulysin by peripheral blood CD8$^+$ T cells requires CD4$^+$ T cells and an accessory cell population (8). In the absence of Th cells or accessory cells, granulysin expression in purified cytotoxic CD8$^+$ T cells can be induced by addition of exogenous IL-15 or in the presence of monocytes activated with a CD40L trimer (8, 15). The regulatory requirements for expression of granulysin in CD4$^+$ and $\gamma\delta$ T cells have not been described to date. Perforin appears to be required for granulysin to enter infected cells and lyse intracellular organisms (1). Despite the cytoplasmic association in granules and cooperative mechanism of action, the levels of protein expressed in infected tissue and mRNA detected after Ag-specific recall indicate that granulysin expression may be regulated separately from perforin upon T cell activation (16, 17).

Currently, one of the most interesting antimicrobial activities associated with granulysin is the ability to directly kill mycobacteria (18). Mycobacteria are particularly resistant to intracellular killing mechanisms and succumb, in vitro, to the effects of granulysin or activation of the infected cell through the $P2X_7$ purinergic receptor (1, 5, 19, 20). *Mycobacterium tuberculosis* can be lysed extracellularly by granulysin or NK-lysin (1, 5, 7), and lysed intracellularly by granulysin following permeation of the cellular membrane by perforin (1). Granulysin-dependent killing of *M. tuberculosis*-infected targets by CD4$^+$, CD8$^+$, and $\gamma\delta$ T cells has been inferred from in vitro studies in which granule-dependent killing occurred despite blocking perforin and/or granzyme activity (1, 3, 16, 21, 22). Cytotoxic activity by T lymphocytes against *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)[3]-infected macrophages correlates to increased expression of granulysin in human T cells isolated from peripheral blood of subjects reactive to purified protein derivative (16). In that study, cytotoxicity against *M. bovis*-infected targets could be exacted by populations enriched for CD4$^+$, CD8$^+$, or $\gamma\delta$ TCR-bearing cells. The correlation of cytotoxicity to granulysin expression levels, however, was only determined for the mixed CD3$^+$ T cell population.

In contrast to other antimicrobial peptides, such as defensins, granulysin is confined to the cytotoxic granules of NK cells and T lymphocytes. Thus, the protein will most likely have access to

*Department of Pediatrics and Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, TX 77555; †Department of Molecular Microbiology and Immunology, University of Missouri, Columbia, MO 65212; and ‡National Animal Disease Center, U.S. Department of Agriculture, Agricultural Research Service, Ames, IA 50010

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[2] Address correspondence and reprint requests to Dr. D. Mark Estes, 2.330 F Children's Hospital, University of Texas Medical Branch, Galveston, TX 77555. E-mail address: dmestes@utmb.edu

[3] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; Bo-lysin, bovine lysin; LB, Luria-Bertani; LCM, laser capture microdissection.

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pathogens as part of a cell-mediated immune response. Once released by the effector cell during degranulation, granulysin molecules appear to directly disrupt microbial membranes by electrostatic charge disruption (23, 24). The biologically active core of the molecule has been attributed to amino acid residues contained within the C terminus of helix 2 through helix 3 (5–7, 9). Antibacterial activity of granulysin against *Salmonella* can be reproduced with peptides from helix 2 and 3, but is greatest when the loop 2 region between helix 2 and 3 is present in combination with either helix (9). Activity of NK-lysin against *M. tuberculosis* also requires the core region provided by the C terminus of helix 2 through helix 3 (6, 7, 9). Maximal killing of tumor cells by granulysin requires the core region, an intact disulfide bridge between the two helices, and the presence of arginine residues in the loop region between helix 2 and 3 (9). Adherence of granulysin to *Escherichia coli* or *M. tuberculosis* membranes is similarly arginine dependent (5). In comparison with bacterial and tumor targets, the direct effect of granulysin against viral targets has not yet been demonstrated. Varicella zoster levels decreased following granulysin exposure (11), but it is unclear whether this occurs via a direct action against the virus or induction of apoptosis of the virally infected cell.

Characterization of the role of granulysin in the T cell arsenal against infectious disease has mostly been inferred from in vitro studies of human PBMC populations. A clinical association between successful chemotherapy in pediatric tuberculosis patients with increased levels of granulysin is consistent with the proposed importance of granulysin in the cytotoxic T cell repertoire used to control myobacterial growth (25). In human leprosy lesions, granulysin protein levels in $CD4^+$ T cells correlate with controlled lesion development compared with the more disseminated form of the disease (17). Determination of tissue-specific expression of granulysin during a cell-mediated immune response, and the effects of therapeutics and vaccination will require a suitable animal model. A murine homologue to granulysin has not been identified to date, precluding the study of clinical effects of gene deletion in a mouse model of disease (26). This series of studies identifies a bovine homologue (Bo-lysin) to granulysin and NK-lysin that is expressed in $CD4^+$, $CD8^+$, and $\gamma\delta$ T lymphocytes. Derived bovine peptides corresponding to the biologically active core region of granulysin are active against *E. coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, and *M. bovis* BCG.

Materials and Methods

Detection of a bovine granulysin homologue

PBMC were isolated from a healthy bovine donor and cultured at 10^6 cells/ml complete RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and penicillin-streptomycin (Life Technologies, Grand Island, NY). Total RNA was extracted (RNeasy; Qiagen, Valencia, CA) following 5 days of stimulation with 10 ng/ml PMA and 1 μg/ml ionomycin. The extracted RNA was treated with DNase (Ambion, Austin, TX) to remove potentially contaminating DNA. RT-PCR (Titan One Tube; Roche Biomedical Laboratories, Indianapolis, IN) was performed, according to manufacturer's instructions, using degenerate primers designed from conserved sequences of NK-lysin and granulysin, as follows: forward, 5′-GAGGRCCCCC AGGGTGAC-3′, and reverse, 5′-ATTCYCSTGAGAGGGGAGG-3′. The resulting Bo-lysin amplicons were cloned into the pCR2.1 TA sequencing vector (Invitrogen Life Technologies, Carlsbad, CA) and sequenced by the dye termination method (DNA Core Facility, University of Missouri). Bovine-specific primers, forward, 5′- CTGCTGCTC CAAGGAGAAGA-3′, and reverse, 5′-GCAGTGGAGGGAGTTTGGT-3′, were generated to confirm the nucleotide sequence in three additional bovine donors. Nucleotide identity, amino acid identity, and amino acid residue alignment were determined using Clustal W (European Bioinformatics Institute, Cambridge, U.K.; http://www.ebi.ac.uk/services).

Expression of Bo-lysin in purified cell populations

Purified leukocyte populations were obtained by magnetic separation from PBMC of healthy bovine donors. Primary Abs used to label leukocyte subpopulations for magnetic separation were as follows: CD3 (MM1A), CD4 (CACT138A), CD8 (BAQ111A), $\gamma\delta$ TCR (GB21A), B cells (GB25A, CD21), and monocytes (MM61A, CD14) (Veterinary Medical Research Development (VMRD), Pullman, WA). Magnetically labeled rat Abs to mouse IgG1, IgM, and IgG2b isotypes were used to positively sort labeled cells using an AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the positively selected population was assessed by flow cytometry using additional Abs to cell surface markers: CD4 FITC (MCA 1653; Serotec, Oxford, U.K.), CD8 (MCA1654; Serotec), and WC1 (ILA-29; VMRD), and isotype-matched control mouse Abs (Sigma-Aldrich, St. Louis, MO). Primary Ab was detected on the CD8 and WC1 populations with FITC-labeled rat Ab to mouse IgG1 (BD Pharmingen, San Diego, CA). Purity of CD3 and CD14 populations was determined using biotinylated MM1A and MM61A, respectively. Primary Abs (VMRD) were affinity purified using immobilized protein G (Pierce, Rockford, IL) and biotinylated using sulfo-NH-biotin EZ link (Pierce). Biotinylated Ab was detected using a streptavidin-PE conjugate (BD Immunocytometry Systems, San Jose, CA). Nonspecific binding was assessed using a biotinylated, isotype-matched, mouse Ab (VMRD), as described above. Purity of CD21-sorted cells was assessed using FITC-conjugated Ab to bovine IgG (H + L) and bovine IgM (Jackson ImmunoResearch Laboratories, West Grove, PA).

The PBMC population was depleted of T lymphocytes using Ab to bovine CD3 (MM1A; VMRD) and magnetic bead-conjugated rat anti-mouse IgG1 by AutoMACS sorting (Miltenyi Biotec). After depletion of the magnetically labeled cells, the remaining negative fraction was further depleted with a mixture of primary Abs (VMRD) to known T lymphocyte surface markers: CD3 (MM1A), CD4 (CACT138A), CD8 (CACT80C), and WC1 (ILA29), and magnetic bead-conjugated rat Ab to mouse IgG1 (Miltenyi Biotec). Purity of the doubly depleted population was assessed using primary Abs MM1A (CD3), 1653F (CD4), BAQ111A (CD8), and GB21A ($\gamma\delta$ TCR), as described above. Primary Ab was detected using FITC-labeled rat Ab to mouse IgG2b, IgG1, and IgM (BD Pharmingen).

Purified cell populations were cultured at 10^6 cells/ml complete RPMI 1640 for 48 h in the presence of 10 ng/ml PMA and 1 μg/ml ionomycin. Monocytes were also stimulated with 100 ng/ml LPS from *E. coli* (serotype 055:B5; Sigma-Aldrich). Following 48 h of stimulation, total RNA was extracted (RNeasy; Qiagen) and treated with DNase (Ambion, Austin, TX) to remove potentially contaminating DNA. RT-PCR (Titan One Tube; Roche Biomedical Laboratories) was performed on the extracted RNA using bovine-specific primers: forward, 5′-CTGCTGCTCCAAG GAGAAGA-3′, and reverse, 5′-GCAGTGGAGGGAGTTTGGT-3′. Equivalent RNA template was assessed by amplification of the G3PDH gene using bovine-specific primers: forward, 5′-GGAGAAACCT GCCA-3′, and reverse, 5′-GTGTCGCGTGTTGA-3′. Bovine perforin was amplified from purified $CD3^+$ cells and PBMC depleted of $CD3^+$ cells using bovine-specific primers: forward, 5′-GATGCCAACTT CGCCGCCCA-3′, and reverse, 5′-TGTCAGTCACGTACTTGCTC-3′. The resulting perforin amplicons were cloned into pCR2.1-Topo cloning vector (Invitrogen Life Technologies) and sequenced by the dye termination method (DNA Core Facility, University of Missouri).

Ab production

Bo-lysin 62 was cloned into the expression vector pcDNA3.1 (Invitrogen Life Technologies). Four C57BL6 mice (The Jackson Laboratory) were immunized i.m. with three doses of 100 μg of Bo-lysin pcDNA3.1 plasmid DNA and boosted i.p. with one dose of peptide mixture (peptides corresponding to residues 3–21, 19–38, 34–55, and 52–71) mixed equally with IFA. Sera from immunized mice were obtained by ocular bleed and screened for specificity to Bo-lysin peptides by ELISA. Splenocytes from sacrificed mice were fused with FO myeloma cells (CRL-1646; American Type Culture Collection, Manassas, VA) using polyethylene glycol and fusions selected by growth in hypoxanthine/aminopterin/thymidine medium (Sigma-Aldrich). Hybridoma populations were screened for Ig production by ELISA. Supernatant from polyclonal hybridomas was purified by column chromatography using protein G (Pierce). Purified Ig was analyzed for Bo-lysin specificity by ELISA and immunoblot using lysates from PBMC.

Immunoblot and immunoprecipitation

Lysates were prepared from PBMC using 10^7 cells/200 μl M-Per mammalian lysis reagent (Pierce). Proteins were separated by SDS-PAGE with

10 and 15% acrylamide (10% perforin, 15% Bo-lysin), transferred to nitrocellulose (Bio-Rad, Hercules, CA), and blocked overnight in 5% nonfat milk. Perforin protein was detected using Ab to human perforin (BD Pharmingen) that is cross-reactive to the bovine homologue. Bo-lysin protein was detected using protein G-purified Ig from polyclonal hybridoma supernatant. Bound Ab was detected using human-adsorbed, HRP-conjugated, goat Ab to mouse Ig (1/5000; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots were developed using ECL plus chemiluminescence reagent (Amersham Biosciences, Uppsala, Sweden) and visualized on autoradiography film (BioMax XAR; Eastman Kodak, Rochester, NY). Relative protein size was determined by reference to Kaliedescope Prestained Standards (Bio-Rad). Bo-lysin protein was also immunoprecipitated from PBMC lysates using the Seize Classic Mammalian Immunoprecipitation kit (Pierce), according to the protocol supplied by the manufacturer. The precipitated immune complex was separated by SDS-PAGE with 15% acrylamide and visualized by silver stain (Silver Stain PLUS; Bio-Rad).

Intracellular staining

$CD3^+$ T lymphocytes were positively selected from PBMC by AutoMACS with magnetic particle-conjugated Abs and stimulated for 6 days with 10 ng/ml PMA and 1 μg/ml ionomycin. Lymphocytes were restimulated with PMA and ionomycin in the presence of brefeldin A (BD Pharmingen) during the last 5 h of culture before intracellular staining. Protein G-purified Ab to Bo-lysin was conjugated to FITC (Sigma-Aldrich). Staining was performed using Cytofix/Cytoperm intracellular staining reagents (BD Pharmingen), according to the protocol supplied by the manufacturer. Intracellular proteins were detected using PE-conjugated mAb to human perforin (BD Pharmingen), and FITC-conjugated polyclonal Ab to Bo-lysin. Samples were fixed using 2% buffered paraformaldehyde before analysis by flow cytometry.

Laser capture microdissection

Tracheobronchial lymph node tissue from *M. bovis* strain 95–1315 (virulent *M. bovis*, white-tailed deer isolate)-infected animals was collected 100 days postinfection and flash frozen with dry ice in a 2-ml cryovial and stored at −80°C. Tissue was embedded in OCT freezing compound, and 5 μM sections were cut for laser capture analysis. Tissue sections were stained with H&E and dehydrated, as described (27). Approximately 1.5×10^4 cells were captured on high sensitivity caps from the periphery of a granulomatous area using a Pixcell II laser capture microscope system (Arcturus, Mountain View, CA). Total RNA was isolated using the PicoPure RNA isolation kit (Arcturus) and treated with DNase (Ambion) to remove any contaminating DNA. Additional sections from the tracheobronchial lymph node tissue were used to capture tissue adjacent (center of the granulomatous area) to the tissue analyzed for Bo-lysin expression. Approximately 2×10^4 cells captured by laser capture microdissection (LCM) on regular nylon polymer caps were used to extract DNA, as previously described (28), and confirm the presence of mycobacteria in tissue adjacent to the captured cells analyzed for Bo-lysin expression. *M. tuberculosis* complex IS6110 sequences were amplified from DNA using previously described primers: forward, 5′-GCTGAGGGCATCGAGGTGGC-3′, and reverse, 5′-GCGTAGGCGTCGGTGACAAA-3′ (29).

Peptide synthesis

Peptides were synthesized by standard fluorenylmethyloxycarbonyl chemistry on an Applied Biosystems 432A peptide synthesizer (Foster City, CA). Lyophilized peptide was stored in desiccant at −20°C and dissolved at 5 mM concentration in sterile Hanks' buffered saline solution before use. The predicted amino acid sequence from common base usage in the polymorphic bovine sequences was used to design peptides corresponding to residues 3–21, 19–38, 34–55, and 52–71 of the predicted bovine protein homologous to 9-kDa granulysin, as shown in Table I. After initial screening for antimicrobial activity, peptide equivalents in human granulysin and NK-lysin were synthesized for comparison with their bovine counterparts that had significant levels of antimicrobial activity. Peptides corresponding to aa residues 19–38 were synthesized for human (VDKPTQRSVS NAATRVCRTG) and bovine (GDQPDENTVIEEASKVCSKM). The corresponding peptides for aa residues 34–55 were also synthesized for human (CRTGRSRWRDVCRNFMRRYQSR), swine (CDKMKILRGVCKK IMRTFLRR), and bovine (CSKMRLLKGLCKSIMKKFLRR). A longer bovine peptide corresponding to aa residues 26–55 (TVIEEAASKVCSK MRLLKGLCKSIMKKFLRR) was also synthesized.

Antimicrobial activity

Log phase cultures of human clinical isolates (School of Medicine Clinical Bacteriology Laboratory, University of Missouri) of urinary tract *E. coli*, *E. coli* 0157:H7, *S. enteritidis*, and *S. aureus* were diluted 1/1000 in Luria-Bertani (LB) medium. Average CFU of bacterial organisms diluted 1/1000 after overnight growth were 2×10^6 CFU/ml. Cultures of urinary tract *E. coli*, *E. coli* 0157:H7, and *S. enteritidis* were grown to log phase in LB medium, and *S. aureus* was grown to log phase in brain-heart infusion medium (Difco, Detroit, MI). A 50-μl aliquot of diluted microorganism was preincubated with 50 μl of LB medium and PBS or 50 μl of individual granulysin peptides serially diluted in LB medium to final concentrations of 0.1, 1, and 10 μM. After 3 h of incubation at 37°C, 100-μl aliquots of peptide and urinary tract *E. coli*, *E. coli* 0157:H7, and *S. enteritidis* were plated on LB agar plates, and 100-μl aliquots of peptide and *S. aureus* were plated on Mueller-Hinton agar plates (REMEL, Lenexa, KS). CFU were counted after overnight incubation at 37°C. Results were verified by three independent experiments, each plated in triplicate. Antimycobacterial activity against *M. bovis* BCG Pasteur was assessed using a frozen stock of 1.2×10^7 CFU/ml obtained from the U.S. Department of Agriculture National Animal Disease Center (Ames, IA). The antimycobacterial protocol was optimized in the absence of peptides before onset of the trial to ensure that clumping of mycobacterial organisms was controlled via vortexing and addition of Tween to the dilution medium. Frozen stocks of mycobacteria were thawed, vortexed for 30 min at high speed, and diluted to 2×10^4 CFU/100 μl Middlebrook 7H9 (REMEL) liquid medium containing 0.1% Tween in 0.5-ml polypropylene microcentrifuge tubes. Peptides were diluted in Middlebrook 7H9 liquid medium and added to mycobacterial cultures at a final concentration of 0.1, 1, 10, and 100 μM in 100 μl final volume. A mock culture of *M. bovis* and 7H9 medium with PBS in the absence of peptide was included as a negative control. Following 72 h of incubation at 37°C, samples were vortexed, 10-fold dilutions in PBS-Tween (0.1% Tween 20) were plated on Middlebrook 7H11 (REMEL) agar plates, and CFU were determined at 3 wk. Antimycobacterial activity was assessed for each experiment as the percentage of *M. bovis* BCG colonies following peptide exposure compared with *M. bovis* BCG exposed to PBS control. Antimycobacterial results were verified by three (bovine peptide 26–55 and human 19–38) to four (bovine peptide 19–38, and bovine, human, and swine peptide 34–55) independent experiments.

Table I. *Comparison of the predicted bovine gene product and synthetic peptides with granulysin and NK-lysin in the biologically active region of effector cell lysin molecules*[a]

	↓ Helix 1 — Helix 2 — Helix 3 — Helix 4 — Helix5
Granulysin	**E**G**PQ**GDL**L**TKTQ**EL**GRDYRT**C**LT**I**V**Q**K**L**KKMV-DK**P**TQRS**V**SNA**A**TR**VC**RTGRSRWRDV**C**RNF**M**RRYQS**R**VIQGLVA**G**ETAQQI**CED**LRL**C**IPSTGPL
NK-lysin 1	**E**D**PQ**DGQ**L**LQRE**EL**GLECES**C**RK**I**I**Q**K**L**EDMVGPQ**P**NEDT**V**TQA**A**SR**VC**DKMK-ILRGV**C**KKI**M**RTFLR**R**ISKDILT**G**KKPQAI**C**V**D**IKI**C**KEK-TGLI
NK-lysin 2	GYFCES**C**RK**I**I**Q**K**L**EDMVGPQ**P**NEDT**V**TQA**A**SQ**VC**DKLK-ILRGL**C**KKI**M**RSFLR**R**ISWDILT**G**KKPQAI**C**V**D**IKI**C**KE
Bo-lysin 89	**E**G**PQ**GDL**L**LQGE**EL**GLLCGS**C**QR**I**I**Q**H**L**MDKLGDQ**P**DENT**V**IEA**A**SK**VC**GKMG-PLKGL**C**KSITKRFLR**R**IAADITA**G**KTSRVV**CED**IKMCKSKPVGFI
Bo-lysin 62	**E**D**PQ**GDL**L**LQGE**EL**SLRCGS**C**RR**I**I**Q**H**L**MDKLGDQ**P**DENT**V**IEE**A**SK**VC**SKMR-LLKGL**C**KSI**M**KKFLRTIAEDIVA**G**KTSQVI**C**V**D**IKMCKSKPVGFI
	Peptides 19–38
	Peptides 34–55
	Bovine peptides 26–55

[a] Alignment was determined by Clustal W analysis, (European Bioinformatics Institute, http://www ebi.ac.uk/services). Amino acid residue locations conserved among human, porcine, and bovine are shown in bold. Predicted helical regions are based on protein modeling of granulysin and NK-lysin. NK-lysin 2 is an amino acid sequence of a protein isolated from porcine small intestine and spleen. Arrow indicates the amino terminus of posttranslationally modified 9-kDa human granulysin. Peptides are numbered starting with 1 from GRDYR of granulysin.

GenBank submission of bovine nucleotide sequences

The nucleotide sequence for Bo-lysin clones 62 and 89 corresponds to GenBank accession numbers AY245799 and AY245798, respectively. A bovine perforin sequence of 929 nucleotides has been submitted to GenBank, with an accession number of AY313172.

Results

Detection of a polymorphic gene homologue of granulysin and NK-lysin

Using degenerate primers, a polymorphic bovine nucleotide sequence 441 bases in length was cloned from stimulated PBMC isolated from an individual blood donor (Table II). Bovine-specific primers were used to confirm the expression of the gene in PBMC of the original donor and determine expression of the gene in PBMC of three additional donors. Ten clones were sequenced from each blood donor to determine the expression profile of the polymorphic genes. Bo-lysin clones 89 and 62 were detected in all four donors. These amplicons were 405 bases in length and shared 94% nucleotide identity. Predicted amino acid sequence of the amplified bovine genes shared 84% identity (Table I). Nucleotide identity of the bovine sequence to human granulysin and porcine NK-lysin was 56.6–57.3% and 74.6–74.8%, respectively. The identity of the bovine amino acid sequences to granulysin and NK-lysin was 36.4–36.5% and 56.1–59.2%, respectively.

Expression of Bo-lysin in leukocyte populations

Bo-lysin transcription in PBMC increased for 24 h following exposure to PMA and calcium ionophore, and thereafter remained constant through 72 h (Fig. 1*A*). Stimulation of PBMC with PMA and calcium ionophore for 96 and 120 h did not result in a detectable increase in Bo-lysin expression compared with levels observed at 72 h (data not shown). Expression of the Bo-lysin gene was detected in $CD3^+$, $CD4^+$, $CD8^+$, and $WC1^+$ $\gamma\delta$ T cells, but was absent in $CD21^+$ cells and $CD14^+$ cells (Fig. 1*B*) following 48 h of stimulation with PMA and calcium ionophore. Bo-lysin expression was also absent in monocytes stimulated with LPS (data not shown). AutoMACS-sorted peripheral blood leukocyte populations were >98% pure by flow cytometric analysis. Expression of the bovine granulysin molecule was detected by intracellular flow cytometry in purified $CD3^+$ T lymphocytes following 6 days of culture with PMA and calcium ionophore (Fig. 2*A*). Intracellular perforin was also detected in activated T lymphocytes after 6 days of culture (Fig. 2*A*). Preliminary investigation in our

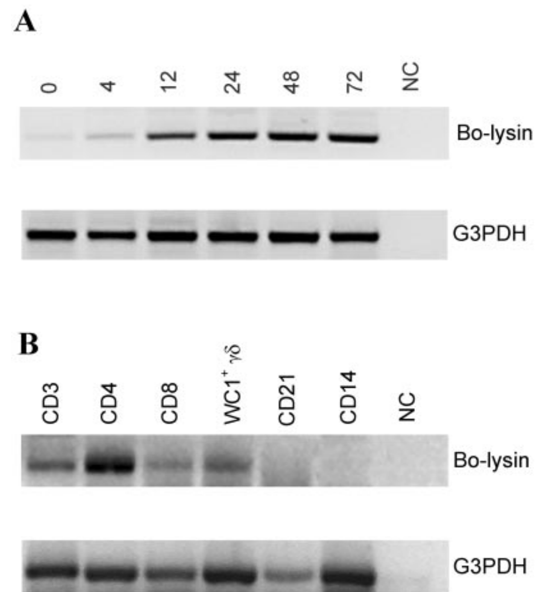

FIGURE 1. Bovine granulysin gene homologue transcription in peripheral blood $CD4^+$, $CD8^+$, and $\gamma\delta$ T lymphocyte populations. RT-PCR with bovine-specific primers was performed on RNA from *A*, PBMC following 0, 4, 12, 24, 48, and 72 h of stimulation with 10 ng/ml PMA and 1 μg/ml ionomycin, and *B*, purified leukocyte populations following 48 h of stimulation with 10 ng/ml PMA and 1 μg/ml ionomycin. A negative control (NC) consisting of RT-PCR reagents and primers in the absence of template was included with each reaction. Equivalent RNA template was assessed by amplification of the G3PDH gene using bovine-specific primers.

Table II. *Polymorphic nucleotide sequences of a bovine effector lysin molecule homologous to the biologically active site of granulysin and NK-lysin*[a]

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Granulysin  ctgttgacca aaacacagga gctgggccgt gactacagga cctgtctgac gatagtccaa aaactgaaga agatggtgg-
NK-lysin    cagctgctcc aaagagagga gctgggcctc atctgtgagt cttgtcggaa gataatccag aagctggagg acatggtggg
Bo-lysin 89 ctgctgctcc aaggagaaga gctgggccta ctctgtggtt cttgtcagag gataatacaa catctgatgg acaagttggg
Bo-lysin 62                           a           g                g    a
Granulysin  -ataagccc  acccagagaa gtgtttccaa tgctgcgacc cgggtgtgta ggacggggag gtcacgatgg cgcgacgtct
NK-lysin    accacaaccc aacgaggaca ctgtcaccca ggcagcctcc cgggtgtgtg acaagatgaa gatactgaga ---ggtgtgt
Bo-lysin 89 agatcagccc gatgagaata ccgtcatcga ggcagcctcc aaggtgtgcg gcaagatggg gccgctgaaa ---ggtctgt
Bo-lysin 62                           t            ag                  a         t
Granulysin  gcagaaattt catgaggagg tatcagtcta gagttatcca aggcctcgtg gccggagaaa ctgcccagca gatctgtgag
NK-lysin    gcaagaagat catgaggacc tttctccgtc gcatctccaa ggacatcctg actgggaaga aaccccaggc tatctgtgtg
Bo-lysin 89 gcaagtcaat cacgaagaga tttctccgtc gcatcgctgc agacatcaca gctggaaaaa cctctcgggt tgtctgtgag
Bo-lysin 62               t     a            a c        a g      gt                    a     a      t
Granulysin  gacctcaggt tgtgtatacc ttctac---a ggtcccctct gagccctctc accttgtcct gtggaagaag cacaggctcc
NK-lysin    gacatcaaga tctgtaaaga gaagac---a ggtctcatct gagccc---- -ccgcgtccc ctg-----ac ccca---cca
Bo-lysin 89 gacatcaaga tgtgcaaaag caagccagta ggtttcattt gattcc---- -ctgggtcct ctt-----ac ccca---tcc
Bo-lysin 62
Granulysin  tgtcctcaga tcccgggaac gtcagcaacc tctgccggct cctcgcttcc tcgatccaga -atccactct ccagtctccc
NK-lysin    tggagaagaa gcacagaaac tccagcaccc tcggccggtt ccttgcctcc taaatccagg -gtctgccct cttgtttctc
Bo-lysin 89 tggggaaaaa gcacagaaac tccagcatcc tcggccggct ccttccttcc tgaatccagg agtcttctct ccagtttctc
Bo-lysin 62
Granulysin  tcccctgactcc-------------
NK-lysin    tcacctaactcctgccaccgccctc
Bo-lysin 89 tcaccaaactccctccactgc----
Bo-lysin 62
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[a] Nucleotide sequence is shown in frame starting with ctg and corresponds to nt 395–799 of granulysin 519 mRNA and nt 335–740 of NK-lysin mRNA. Nucleotides shown for Bo-lysin 62 indicate positions in which the sequence differed from Bo-lysin 89. Alignment was determined by Clustal W analysis (European Bioinformatics Institute, http://www.ebi.ac.uk/services). A stop codon (tga) conserved among the three species is shown in bold.

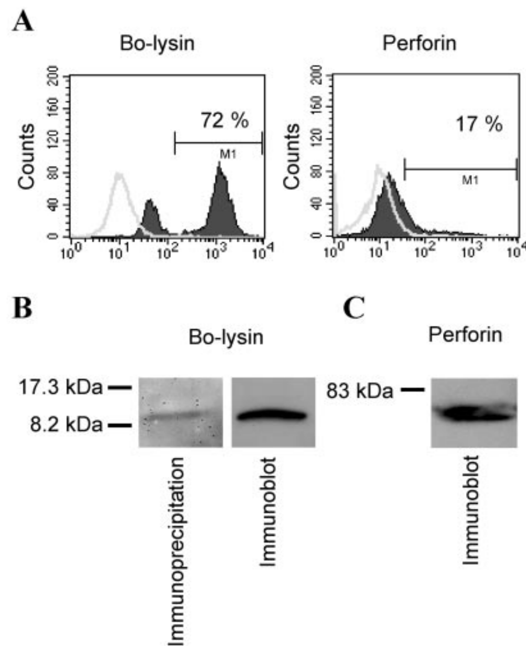

FIGURE 2. Expression of Bo-lysin and perforin protein in T lymphocytes and PBMC. *A*, Intracellular expression of Bo-lysin and perforin by AutoMACS-purified CD3$^+$ T lymphocytes following 6 days of stimulation with PMA and ionomycin. *B*, Detection of Bo-lysin in PMBC lysates by silver stain following immunoprecipitation with specific Ab and by immunoblot of lysates with specific Ab. *C*, Detection of perforin by immunoblot of PBMC lysates with specific Ab. Lysates from PBMC stimulated for 5 days with 10 ng/ml PMA and 1 μg/ml ionomycin were used for immunoprecipitation. Immunoblots were performed using lysates from nonstimulated PBMC.

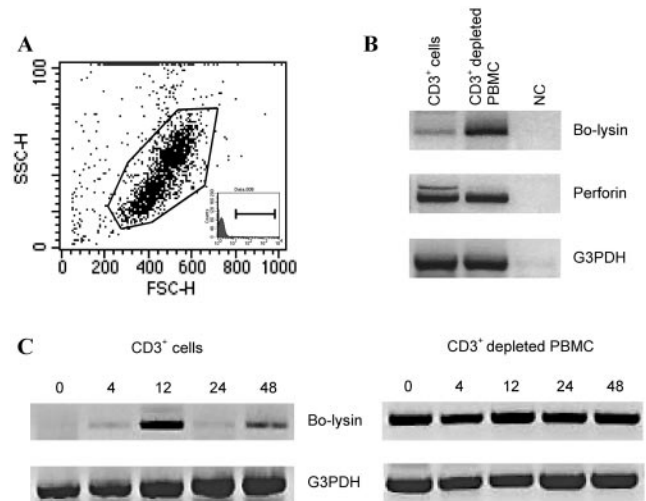

FIGURE 3. Transcription of Bo-lysin and perforin in T lymphocytes and PBMC depleted of T lymphocytes. *A*, Forward scatter and side scatter characteristics of the PBMC depleted of CD3$^+$, CD4$^+$, CD8$^+$, and $\gamma\delta$ T cells. *Inset*, Shows lack of surface expression of T lymphocyte phenotype markers CD3, CD4, CD8, and $\gamma\delta$ TCR on T lymphocyte-depleted PBMC. *B*, Expression of Bo-lysin and perforin in T lymphocyte-depleted PBMC by RT-PCR following 48 h of stimulation with 10 ng/ml PMA and 1 μg/ml ionomycin. *C*, Kinetics of Bo-lysin expression in CD3$^+$ T lymphocytes and PBMC depleted of T lymphocytes following 0, 4, 12, 24, and 48 h of stimulation with 10 ng/ml PMA and 1 μg/ml ionomycin.

laboratory indicates that peak perforin expression in bovine T lymphocytes occurs at ~3 days following activation (data not shown). A molecule ~9–15 kDa in size was immunoprecipitated from lysates of activated PBMC by polyclonal Ab to Bo-lysin (Fig. 2*B*). Immunoblotting of PBMC lysates demonstrated the presence of a 9- to 15-kDa molecule detected with Ab to Bo-lysin (Fig. 2*B*) and a molecule ~70–75 kDa detected with Ab to perforin (Fig. 2*C*). The Bo-lysin gene was also expressed in PBMC rigorously depleted of CD3$^+$, CD4$^+$, CD8$^+$, and WC1$^+$ $\gamma\delta$ T cells (Fig. 3*B*). Forward scatter and side scatter patterns assessed by flow cytometry indicated that the T lymphocyte-depleted population was comprised of both lymphoid and myeloid lineage cells (Fig. 3*A*). The T lymphocyte-depleted PBMC population and CD3$^+$ lymphocytes also expressed a bovine homologue of perforin (Fig. 3*B*) with 72% nucleotide identity to murine and 80% nucleotide identity to human perforin. Transcription of Bo-lyin was induced in CD3$^+$ lymphocytes following stimulation with PMA and calcium ionophore (Fig. 3*C*). The decrease in expression of Bo-lysin by CD3$^+$ lymphocytes between 12 and 48 h was consistently observed in three independent experiments using PBMC from two blood donors (data not shown). Constitutive expression of Bo-lysin was evident in PBMC depleted of CD3$^+$ lymphocytes (Fig. 3*C*). An additional amplicon of ~500 bases was occasionally amplified with the Bo-lysin primers. Sequencing revealed this product to be the result of nonspecific PCR amplification and not amplification of splice variants of Bo-lysin.

Antibacterial activity against representative Gram-positive and Gram-negative bacteria

Bovine peptides corresponding to aa residues 3–21 (helix 1) and 52–71 (helices 4 and 5) did not display any antimicrobial activity against urinary tract *E. coli*, *E. coli* 0157:H7, *S. aureus*, *S. enteritidis*, or *M. bovis* BCG, under the conditions described (data not shown). A bovine peptide corresponding to the C terminus of helix 2 through helix 3 (peptide 34–55) displayed potent killing activity against *E. coli*, *E. coli* 0157:H7, *S. aureus*, *S. enteritidis*, and *M. bovis* BCG (Figs. 4 and 5). Human and swine counterparts to bovine peptide 34–55 were synthesized to compare antimicrobial activity of these peptides among species. Complete reduction of urinary tract *E. coli*, *E. coli* 0157:H7, *S. aureus*, and *S. enteritidis* growth was achieved with 10 μM concentration of peptide 34–55 derived from human, bovine, and swine amino acid sequence (Fig. 4). The human 34–55 residue peptide appeared to have reduced activity against a human urinary tract *E. coli* isolate compared with bovine and swine, requiring a log increase in peptide concentration to achieve a similar level of antimicrobial activity (Fig. 4*B*). The swine peptide 34–55 was very active against *S. aureus*, reducing CFU by half compared with human and bovine counterparts at 0.1 μM concentration (Fig. 4*D*). Control experiments demonstrated that there was no detectable change in the number of organisms due to growth effects during the 0–3 h of incubation (data not shown). Synthetic peptides corresponding to human, bovine, and swine residues 34–55 inhibited growth of *M. bovis* in a dose-dependent manner (Fig. 5*B*), reducing bacterial numbers by 74–89% at 100 μM concentration. Surprisingly, a bovine peptide corresponding to helix 2 (residues 19–38) also reduced growth of *M. bovis* BCG (Fig. 5*A*), but was not active against *E. coli*, *E. coli* 0157:H7, *S. aureus*, or *S. enteritidis* (data not shown). A human granulysin counterpart to bovine peptide 19–38 was synthesized to determine conservation of antimycobacterial activity by this region of granulysin-like molecules. Peptides derived from human and bovine aa residues 19–38 were not effective against urinary tract *E. coli*, *E. coli* 0157:H7, *S. aureus*, or *S. enteritidis* under the described conditions (data not shown). Human and bovine peptides corresponding to aa 19–38 reduced numbers of *M. bovis* by 60–70% (Fig. 5*A*), although a log increase in peptide concentration

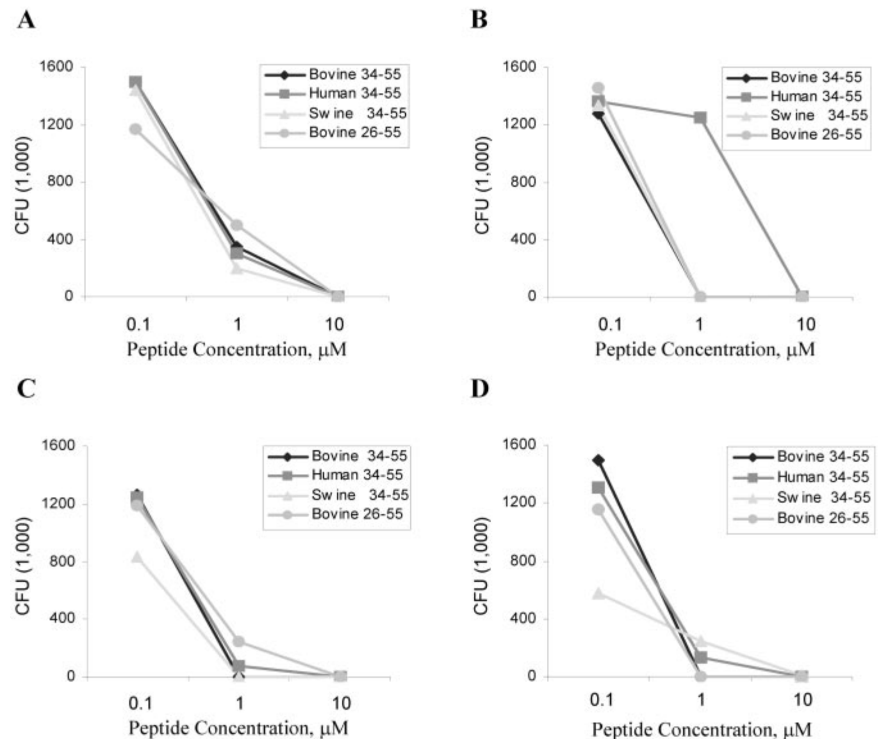

FIGURE 4. Bovine, human, and swine peptides corresponding to the C terminus of helix 2 through helix 3 of granulysin inhibit growth of representative Gram-positive and Gram-negative bacteria. Antimicrobial activity of bovine, human, and swine peptide 34–55 and bovine peptide 26–55 against *A*, *E. coli* 0157:H7; *B*, *E. coli* isolate from human urinary tract; *C*, *S. enteritidis*; and *D*, *S. aureus*. Bovine peptides corresponding to aa residues 3–21 or 52–71 did not display any antimicrobial activity against *E. coli*, *E. coli* 0157:H7, *S. aureus*, or *S. enteritidis* when tested at 100 μM concentration under the conditions described (data not shown). Activity of peptides 34–55 was similar to negative control at 0.1 μM concentration. Average CFU of bacterial organisms diluted 1/1000 after overnight growth were 2×10^6 CFU/ml. Peptides (0.1, 1, 10 μM) were preincubated with bacteria for 3 h at 37°C, and antimicrobial activity was evaluated after overnight growth on LB agar. Data are presented as mean CFU of a representative experiment performed in triplicate.

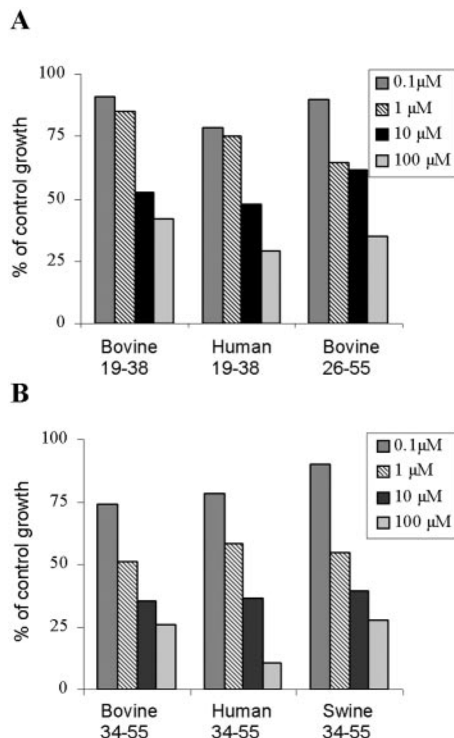

FIGURE 5. Bovine, human, and swine granulysin-like peptides inhibit growth of *M. bovis* BCG. Antimycobacterial activity of 0.1, 1, 10, and 100 μM concentrations of *A*, peptides corresponding to bovine and human aa residues 19–38 and bovine aa residues 26–55, and *B*, peptides corresponding to bovine, human, and swine aa residues 34–55, against 2×10^4 CFU *M. bovis* BCG following 72-h incubation and 3-wk growth on 7H11 Middlebrook agar plates. Bovine peptides corresponding to aa residues 3–21 or 52–71 did not reduce growth of *M. bovis* BCG when tested at 0.1, 1, 10, or 100 μM concentration under the conditions described (data not shown). Data are presented as the mean percentage reduction from *M. bovis* BCG incubated with PBS alone.

was required to achieve antimicrobial activity similar to peptides 34–55. A longer bovine peptide spanning the helix 2 and 3 region (peptide 26–55) inhibited growth of *E. coli*, *E. coli* 0157:H7, *S. aureus*, and *S. enteritidis* at very similar levels to the bovine peptide 34–55 (Fig. 4). The antimycobacterial activity of bovine peptide 26–55 against *M. bovis* BCG was similar to the activity demonstrated for bovine peptide 19–38 (Fig. 5, *A* and *B*).

Expression of granulysin homologue in granulomatous tissue

Expression of the Bo-lysin gene was detected in LCM-enriched granulomatous tissue from a lymph node of a *M. bovis*-infect cow (Fig. 6). DNA sequencing of the PCR product confirmed that the nucleotide sequence was consistent with Bo-lysin clone 89. A consecutive tissue section (Fig. 6*A*), fixed and stained with H&E, demonstrated the presence of two large granulomas in the tracheobronchial lymph node tissue sections used for LCM. Amplification of insertion sequences from *M. tuberculosis* complex indicated mycobacterial infection in the tissue analyzed by LCM (Fig. 6*E*).

Discussion

The broad spectrum antimicrobial activity of granulysin-like molecules suggests an important mechanism by which granule exocytosis of T lymphocytes and NK cells contributes to pathogen control beyond killing cellular hosts. Antimicrobial activity of granulysin-like molecules is particularly important against pathogens such as mycobacteria, in which killing of the cell does not necessarily result in killing of the pathogenic organism (21). Mycobacteria are particularly resistant to intracellular killing mechanisms, succumbing predominantly to the effects of granulysin or activation of the infected cell through the $P2X_7$ purinergic receptor (1, 5, 19–21). In vitro experiments have allowed for structural characterization, evidence for lytic mechanisms, cellular sources, and basic killing activity of granulysin-like molecules. Full characterization of the role of these molecules in the immune response to infectious disease, however, requires an animal model. The absence of a homologue to granulysin in the mouse indicates that additional animal models should be explored to characterize the in

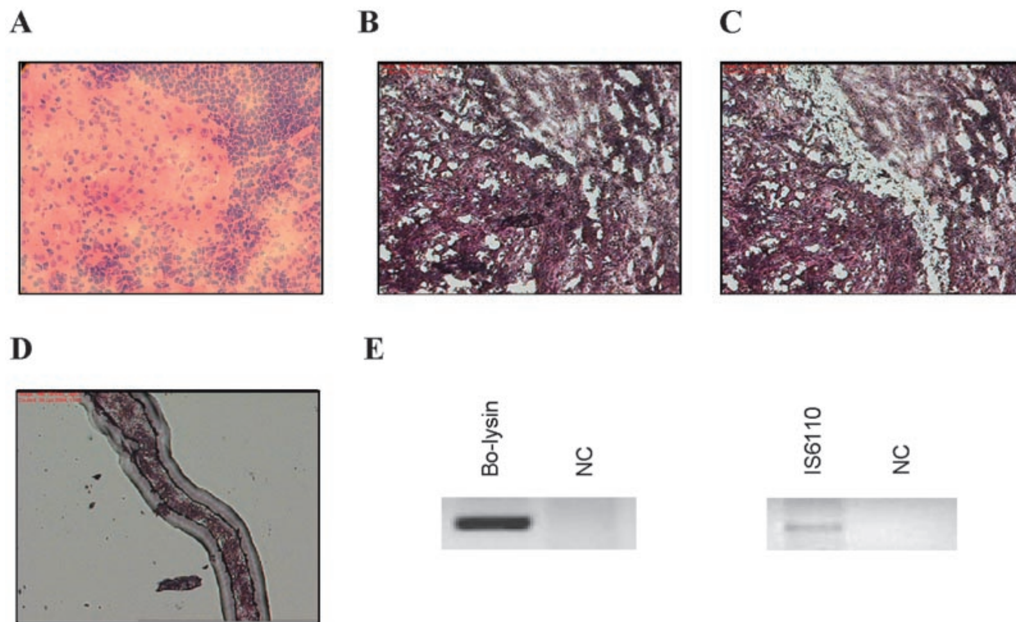

FIGURE 6. Expression of Bo-lysin in granulomatous area of a lymph node from an *M. bovis*-challenged animal at 100 days postinfection. *A*, H&E-stained section showing lymphocytic cuffing at the periphery of a granuloma in the tracheobronchial lymph node, original magnification ×40. *B*, Frozen section of tracheobronchial lymph node before LCM, and *C*, following LCM capture, and *D*, captured cells on nylon polymer cap, original magnification ×20. *E*, RT-PCR expression of Bo-lysin mRNA and PCR detection of *M. tuberculosis* complex insertion 6110 sequences in tracheobronchial lymph node tissue captured by LCM. A negative control (NC) consisting of RT-PCR reagents and primers in the absence of template was included with each reaction.

vivo role of granulysin-like molecules in human disease. The results of the current trial identify and characterize a granulysin homologue in the cow, an important animal model for tuberculosis. The nucleotide and amino acid identity of the bovine gene homologous to granulysin is similar to the identity between NK-lysin and granulysin (2). Consistent with human and porcine effector cell lysin proteins, the bovine effector cell lysin was expressed in the major subsets of T lymphocytes and a potential NK cell population (1, 2). Antimicrobial activity of peptides derived from the predicted bovine sequence identifies the helix 2 through helix 3 region as the lytic site, also consistent with previous observations in granulysin and NK-lysin (1, 5, 7, 9, 10). The bovine gene and the predicted gene product are polymorphic in nature. Variation in the porcine sequence is also evident from discrepancy between the reported gene product and amino acid sequence of protein isolated from porcine small intestine and spleen (2, 30). Sequence analysis of different human donors, to date, indicates a single polymorphic site in the biologically active portion of the granulysin gene, resulting in a change from an isoleucine to a threonine (31).

Granulysin and NK-lysin are expressed by NK cells as well as T lymphocytes. In the current trial, a bovine homologue was expressed in all subsets of T lymphocytes and constitutively expressed in a population of cells rigorously depleted of T lymphocytes. This depleted population also expressed bovine perforin at levels comparable to stimulated T lymphocytes. Among the mononuclear cells remaining after T lymphocyte depletion, our results indicate that B lymphocytes and monocytes would not contribute to expression of the bovine effector cell lysin. Speculatively, the T lymphocyte-depleted population expressing perforin and the bovine granulysin homologue may represent a bovine cell population with NK cell characteristics. The reagents necessary to identify bovine NK cell surface markers are not currently available, and reagents developed for other species have not been shown to be cross-reactive to the bovine, to our knowledge. Definitive characterization will require the development of reagents to detect NK cell surface markers and subsequent demonstration of killing activity.

Comparisons of the relative microbicidal activity among the different granulysin-like molecules may provide insight into the functional significance of residues within the biologically active site of effector cell lysin molecules. A mechanism for the antimicrobial activity of granulysin was recently proposed following elucidation of the crystal structure (24). Anderson et al. (24) propose that disruption of the microbial membrane results when several molecules accumulate at the microbial surface, with the lytic face of molecule orientated toward the targeted membrane. Alignment of amino acid residues among the human, porcine, and bovine lysin molecules demonstrates structural and lytic motifs are conserved among species. The placement of cysteine residues within the predicted helices 1, 2, 3, and 5 indicates that the bovine molecule is saposin-like and would adopt a tertiary structure very similar to granulysin and NK-lysin.

The cationic properties of granulysin, NK-lysin, and the predicted bovine effector lysin molecules result from an abundance of arginine and lysine residues. Arginine is the predominant positively charged residue in granulysin, in contrast with a predominance of lysine in NK-lysin and the bovine molecule. The individual contribution of arginine and lysine to lytic activity is not well established. Substitutions of arginine with glutamine residues in the loop 2 region eliminated antitumor activity of synthesized granulysin peptides, while only moderately inhibiting activity against *Salmonella* (9). Adherence of granulysin to *E. coli* or *M. tuberculosis* membranes and subsequent killing of these organisms are completely abrogated by general modification of arginine residues in recombinant granulysin (5). In the same experiment, lysine residues in recombinant granulysin were resistant to chemical modification, a result interpreted to indicate lack of involvement of lysine residues in lytic activity. Despite differences in arginine and lysine representation, peptides derived from the C terminus of helix 2 through helix 3 regions of granulysin, NK-lysin, and the

predicted bovine gene product all display potent killing activity against several bacterial organisms and mycobacteria. The bovine peptide corresponding to helix 2 has no arginine residues and yet demonstrated similar activity against *M. bovis* BCG as the corresponding human peptide that contains three arginine residues. Comparisons among human, porcine, and bovine sequences indicate the general location of positively charged residues is frequently conserved, while substitutions of arginine with lysine at these conserved sites are common. The functional effect of arginine to lysine substitutions will need to be further explored through residue substitution.

This is the first study to demonstrate antimycobacterial activity by amino acid residues of the loop 1 through helix 2 region of granulysin. A longer granulysin peptide spanning helix 1 through helix 2 was previously demonstrated to be active against *M. tuberculosis*, and had activity comparable to a peptide corresponding to the C terminus of helix 2 through helix 3 (5). In the same trial, the antimycobacterial activity of a shorter peptide corresponding to loop 1 through helix 2 apparently was not assessed after initial experiments indicated lack of activity against *E. coli*. The results of the current trial are consistent with the identification of residues within the C terminus of helix 2 through helix 3 as the important lytic site for general antimicrobial activity of granulysin-like molecules. The additional activity against *M. bovis* BCG observed for peptides derived from human and bovine helix 2 may indicate the importance of the residues contained within the overlapping segment (C terminus of helix 2) of the compared peptides for mycobacterial killing. Alternately, lytic activity by residues in loop 1 through helix 2 may indicate redundancy of granulysin's antimycobacterial mechanisms in species susceptible to tuberculosis.

The identification of the bovine gene homologue to granulysin and the demonstration of antimycobacterial activity support the use of the bovine model for characterizing the role of granulysin in the immune response to tuberculosis and other diseases in which granulysin may have a critical protective role. Experimental infection of cattle with mycobacteria will allow for a detailed in vivo characterization of important cellular sources, kinetics of expression, and colocalization with mycobacteria, of effector cell lysin molecules at sites of infection. The enhancement of protective memory to mycobacteria via strategies that augment expression of granulysin-like molecules can then be fully explored.

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