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Immunomodulatory Peptide from Cystatin, a Natural Cysteine Protease Inhibitor, against Leishmaniasis as a Model Macrophage Disease▽

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Cystatin, a natural cysteine protease inhibitor, has strong antileishmanial activity, which is due to its potential to induce nitric oxide (NO) generation from macrophages. Cysteine protease-inhibitory activity and NO-up-regulatory activity correspond to different regions, as revealed by the dissection of cystatin cDNA into nonoverlapping fragments. By using synthetic overlapping peptides, the NO-up-regulatory activity was found to be confined to a 10-mer sequence. In addition to having reasonable inhibitory effects on amastigote multiplication within macrophages (50% inhibitory concentration, 5.2 μg/ml), 97 and 93% suppression, respectively, of liver and spleen parasite burdens was achieved with the 10-mer peptide at a dose of 0.5 mg/kg of body weight/day, given consecutively for 4 days along with a suboptimal dose of gamma interferon in a 45-day mouse model of visceral leishmaniasis. Peptide treatment modulated the levels of cytokine secretion by infected splenocytes, with increased levels of interleukin-12 and tumor necrosis factor alpha and increased inducible NO synthase production, and also resulted in resistance to reinfection. The generation of a natural peptide from cystatin with robust immunomodulatory potential may therefore provide a promising therapeutic agent for macrophage-associated diseases.

The key pathogenic event in the fatal disease of visceral leishmaniasis is the harboring of the causative parasite *Leishmania donovani* within the phagolysosomes of the macrophages of the liver, spleen, and bone marrow. Presently, there is no widely available vaccine against leishmaniasis and chemotherapy remains the major medical mode of managing the disease. However, the chemotherapeutic cure of leishmaniasis is largely dependent upon the development of an effective immune response that activates the macrophages to produce toxic nitrogen and oxygen intermediates to kill the parasites. The parasite itself is known to suppress this process by down-regulating the production of such reactive species within the macrophages. Consequently, a potential therapy for leishmaniasis would be to up-regulate such innate immune responses mediated by the parasite infected-macrophages themselves.

Leishmania expresses various molecules believed to contribute to its ability to infect and proliferate in mammals. Cathepsin L-like cysteine protease (CP) is one such molecule, which is predominantly expressed and active in the amastigote form and to a lesser extent in metacyclic promastigotes (20). This observation, together with the fact that *Leishmania* cannot grow within macrophages in the presence of CP inhibitors, suggests that CPs are necessary for successful intracellular parasitism (19). Large amounts of parasite-derived CPs have also been associated with the extracellular milieu of *Leishmania*-infected mouse lesions (17) and are reported to stimulate an increase in the expression of interleukin-4 (IL-4) and IL-1 (9), driving the differentiation of the $CD4^+$ precursors towards the Th2 phenotype and thus favoring the proliferation of the parasite. We have previously demonstrated that cystatin, a natural cysteine protease inhibitor, can synergize with subthreshold concentrations of gamma interferon (IFN-γ) in inducing favorable cytokine responses and the generation of nitric oxide (NO), resulting in the elimination of parasite infection in experimental visceral leishmaniasis (6). Cystatin can stimulate NO production in IFN-γ-activated murine macrophages (27), and this effect may be unrelated to its inhibition of cysteine protease, since the irreversible and structurally unrelated cysteine protease inhibitor E64 did not have any effect on activated macrophages (26).

We therefore characterized the NO-stimulatory domain in cystatin with a view to deciphering the minimal peptide sequence involved in NO generation to develop an immunopotent biopeptide. We used three nonoverlapping recombinant proteins spanning the sequences of the N-terminal region, the intermediate region, and the C-terminal region to demonstrate the presence of the up-regulatory activity in the N-terminal end. Using overlapping synthetic peptides derived from this potent region, we also demonstrated that apart from being able to generate NO with subthreshold amounts of IFN-γ, a 10-mer peptide is able to induce a protective Th1 response to *L. donovani* infection in susceptible mice. Finally, the therapeutic relevance of these data, along with a plausible mechanism for NO stimulation, is discussed.

MATERIALS AND METHODS

NO production, parasite killing, and in vivo infection. The origin, in vivo passage, and in vitro propagation of the *L. donovani* isolate (MHOM/IN/1983/AG83) were as reported previously (25). Peritoneal macrophages (BALB/c) were cultured as previously described (25). After the treatment of cultures with various agents, the supernatants of the cell cultures were assayed for nitrite production by using the Greiss assay (6). Adherent macrophages were infected

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with stationary-phase *L. donovani* promastigotes at a 10:1 parasite/cell ratio. Infection was allowed to proceed for 4 h, and the cells were washed to remove excess parasites, as described previously (6). After treatment with various agents at 37°C, the number of parasites per 100 macrophages was determined by staining with Giemsa. For in vivo infection, mice (BALB/c; 20 to 25 g) were injected via the tail vein with 10^7 promastigotes. At day 10 after the injection of parasites, cystatin and cystatin-derived peptides were injected into the tail veins in various doses for 4 consecutive days. Forty-five days after infection, mice were examined for parasite burdens by counting the number of amastigotes in the Giemsa-stained imprints of livers and spleens. Organ parasite burdens, expressed as Leishman-Donovan units (LDU), were calculated as follows: number of amastigotes per 1,000 cell nuclei × organ weight (g) (21).

Cytokine analysis. Splenocyte cultures were prepared from infected mouse spleens every 15 days after infection as described previously (25). After stimulation with 20 μg/ml soluble leishmanial antigen for 48 h, the supernatants of the cell cultures (4×10^6 cells/ml) were assayed for IL-12, tumor necrosis factor alpha (TNF-α), and IL-10 by using an enzyme-linked immunosorbent assay kit (BD Biosciences, San Jose, CA). mRNA profiles for these cytokines along with β-actin as an internal control were analyzed by reverse transcription (RT)-PCR. The reverse transcription of 1 μg of RNA was performed with the Superscript one-step RT-PCR system according to the protocol of the manufacturer (Invitrogen, New Delhi, India). Oligonucleotide primers for these cytokines were selected from published cDNA sequences. After the appropriate number of PCR cycles, the amplified DNA was separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Analysis of iNOS expression by RT-PCR and immunoblotting. RT-PCR was performed to determine the mRNA profile corresponding to inducible NO synthase (iNOS), along with β-actin as an internal control. For immunoblot analysis, 20 μg of whole-cell extracts was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto a nitrocellulose membrane, and probed with murine anti-iNOS antibody (Transduction Laboratories, Lexington, KY) and proteins were detected by using an ECL kit (Amersham Biosciences, Arlington Heights, IL) with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:2,000 dilution).

Saturation of cystatin-inhibitory sites by carboxymethylated papain. For inactivation, 1 mg of papain was kept for 5 min in a solution containing 0.1 M phosphate buffer (pH 6.8), 1 mM EDTA, 0.1% Brij 35, and 2 mM dithiothreitol and incubated for 1 h with 10 mM iodoacetic acid at 37°C in the dark. The excess alkylating reagent was removed by gel filtration through a PD10 column (Amersham Biosciences). The completion of inactivation was indicated by a complete loss of enzymatic activity against the fluorogenic substrate *Z*-Phe-Arg-4-methyl-7-coumarin (*Z*-Phe-Arg-NHMec). Carboxymethylated papain complexes were made by mixing chicken cystatin, stefin, or T-kininogen with a large excess of papain (10:1) at 37°C over a period of 45 min. The saturation of inhibitory sites was checked by measuring the residual activity of native papain on the *Z*-Phe-Arg-NHMec substrate in the presence or absence of the saturated inhibitor.

Effect of cystatin and cystatin-derived peptides on papain. The effect of cystatin and cystatin-derived peptides on papain was determined by measuring the equilibrium constants for dissociation (K_i) of complexes between cystatin and papain, as described by Hall et al. (14). Briefly, continuous-rate assays with *Z*-Phe-Arg-NHMec were carried out in 100 mM sodium phosphate buffer, pH 6.0, containing 1 mM dithiothreitol and 2 mM EDTA (22). K_i were measured directly in equilibrium inhibition experiments according to the method of Henderson (16), and the obtained K_i were corrected for substrate competition by using the K_i of 42 μM for papain (28).

Anti-cystatin and anti-peptide antibodies. The peptide was first conjugated to keyhole limpet hemocyanin according to the method of Gullick (12), which yielded a conjugate of 0.24 μmol/mg of keyhole limpet hemocyanin. Antibodies to cystatin-peptide conjugates were raised in New Zealand rabbits as described previously (2). Anti-cystatin antibodies were separated from sera according to the method of Hall et al. (15), whereas anti-peptide antibodies were affinity purified by the method of Edwards et al. (7).

Molecular cloning, expression, and purification of cystatin-derived recombinant peptides. The nonoverlapping fragments of cystatin cDNA encoding amino acid sequences corresponding to the NH_2-terminal region (amino acid [aa] residues 1 to 28), the intermediate region (aa residues 29 to 72), and the COOH-terminal part of the molecule (aa residues 73 to 116) were amplified as oligonucleotides containing BamHI and XhoI overhangs by using sequence-specific primers from chicken cystatin cDNA (a kind gift from Rita Collela, Bureau of Biological Research, NJ). These amplified fragments were cloned into the BamHI-XhoI site of a prokaryotic expression vector, pGEX-5X-2 (Amersham Biosciences), and *Escherichia coli* strain BL21(DE3) (Novagen, San Diego, CA) was transformed with the pGEX-5X-2-GST constructs. Transformed *E. coli* was grown in Luria-Bertani and induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and recombinant glutathione *S*-transferase (GST) fusion proteins were purified by a standard procedure using reduced glutathione beads.

Electrophoresis and immunoblotting. The cystatin-derived recombinant proteins were analyzed on a 10% SDS-PAGE gel under reducing conditions and immunoblotted with anti-cystatin or anti-peptide antibody according to the method described above.

Statistical analysis. The statistical significance of differences between any two groups was analyzed by using two-tailed Student's *t* test.

RESULTS

Resolution of visceral leishmaniasis by cystatin through NO up-regulation. Although a suboptimal dose of IFN-γ was required for cystatin to induce NO production in mouse peritoneal macrophages (Fig. 1A), in an in vivo situation, IFN-γ was not a prerequisite. Thus, peritoneal macrophages isolated from BALB/c mice given intravenous (i.v.) injections of cystatin produced significantly higher levels of NO_2^- than those from mice that did not receive cystatin (Fig. 1B). In a mouse model of visceral leishmaniasis, the administration of cystatin at a dose of 20 mg/kg of body weight/day for 4 consecutive days beginning 10 days after infection could cause a marked suppression of spleen parasite burdens (mean ± standard deviation [SD] $\log_{10}$ number of LDU, 1.17 ± 0.17, compared to 2.47 ± 0.05 for the untreated controls; $P < 0.001$). However, when a suboptimal dose of IFN-γ (5×10^5 U/kg/day) was coadministered with the cystatin, a much more pronounced effect (complete suppression of the spleen parasite burdens) was obtained at a much lower dose of cystatin of 5 mg/kg/day (Fig. 1C). The coadministration of 0.1 mg/kg/day of 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT), an inhibitor of iNOS, caused a reversal of the parasite-suppressive effect, suggesting the involvement of NO in cystatin-mediated antileishmanial activity. In the in vitro situation of amastigote multiplication within macrophages also, the inhibitory effect of cystatin in the presence of 100 U/ml of IFN-γ (50% inhibitory concentration, 4.3 μg/ml) was abolished by treatment with 10 μM AMT (Fig. 1D). The in vivo and in vitro effects of cystatin in the presence of AMT were also reflected in the iNOS mRNA expression pattern analyzed by RT-PCR and the iNOS levels analyzed by Western blotting, as shown in Fig. 1E and F, respectively. It may be mentioned that cystatin plus IFN-γ did not have any influence on the in vitro proliferation of *L. donovani* promastigotes.

Regions for NO up-regulation and cysteine protease inhibition. In order to determine whether the cysteine proteinase-inhibitory region overlaps with the NO-stimulatory region, cystatin was saturated with inactivated papain. When examined, complexed cystatin in IFN-γ-activated macrophages was found to have NO-generating abilities comparable to those of the free form (Fig. 2). Other members of the cystatin superfamily, human stefin B and T-kininogen, when complexed with reduced papain also generated nitrite levels which were comparable to those generated by the free inhibitors after 48 h of incubation, while aprotinin, an unrelated protease inhibitor, did not show any induction of NO. These results indicate the presence of a distinct NO-stimulatory domain unrelated to protease-inhibitory activity in cystatin and related compounds.

Domain of cystatin involved in NO up-regulation. To gain insight into the region of cystatin responsible for NO up-reg-

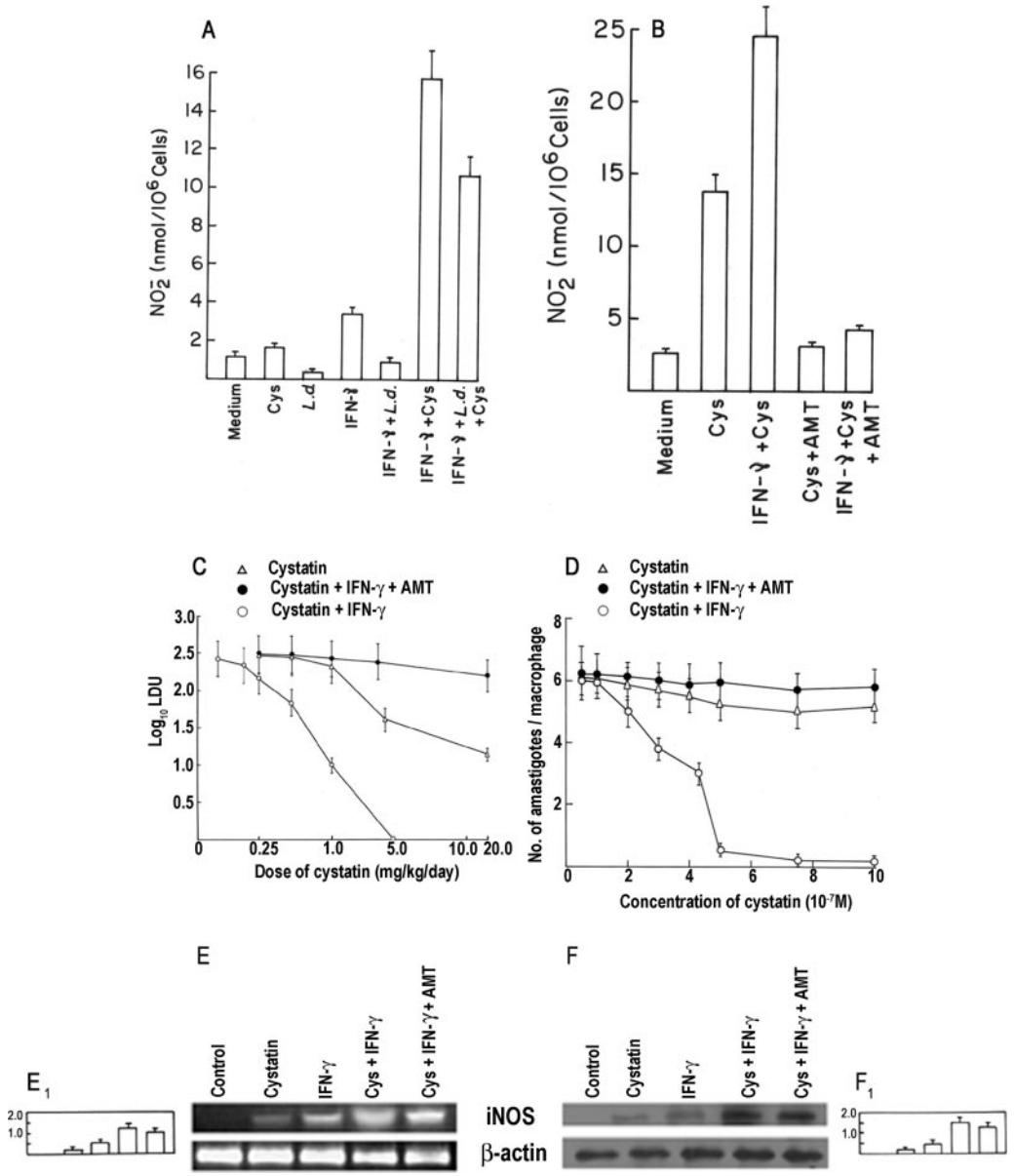

FIG. 1. Antileishmanial activity of cystatin through the generation of NO. (A) NO production by peritoneal macrophages (10^6/ml) incubated for 48 h in culture medium with cystatin (Cys; 5×10^{-7} M), *L. donovani* (*L.d.*; macrophage/parasite ratio, 1:10), IFN-γ (100 U/ml), IFN-γ plus *L. donovani*, IFN-γ plus cystatin, and IFN-γ plus cystatin plus *L. donovani*. (B) Generation of NO in peritoneal macrophages isolated from mice which received i.v. injections of either cystatin (5 mg/kg/day) or IFN-γ (10^4 U/mouse) or both for 4 consecutive days. Macrophages were isolated 10 h after the last injection. Data represent the means ± SD of results from three experiments. AMT (5 mg/kg/day) was used along with cystatin and IFN-γ in a separate experiment. (C) In vivo antileishmanial activity of cystatin and cystatin plus IFN-γ. Mice were challenged with 10^7 promastigotes, and after 10 days of infection, they were treated with various i.v. doses of cystatin with or without IFN-γ (10^4 U/mouse) daily for 4 consecutive days. Spleen parasite burdens were determined 45 days after infection and are expressed as the mean $\log_{10}$ number of LDU ± SD for six animals. In another set of experiments, AMT (5 mg/kg/day) was used along with cystatin and IFN-γ. The $\log_{10}$ number of LDU in the infected control was 2.8 ± 0.08. (D) In vitro antileishmanial activity. Macrophages were infected with *L. donovani* promastigotes, excess parasites were washed off, and cells were treated with graded concentrations of cystatin with or without IFN-γ (100 U/ml) for 48 h at 37°C. AMT (10 μM) was given along with cystatin plus IFN-γ in a separate set of experiments. The parasites inside each macrophage were counted. The infected controls had 7.22 ± 0.65 amastigotes/macrophage. The nature of iNOS was determined by RT-PCR analysis of the mRNA transcript (E) and by Western blot analysis of the protein levels (F) corresponding to various treatment regimens. Band intensities were analyzed by densitometry (E_1 and F_1).

ulation, the cDNA of cystatin was dissected into three nonoverlapping fragments corresponding to Cst I, Cst II, and Cst III and representing the NH_2-terminal region (aa residues 1 to 28); the intermediate region (aa residues 29 to 72) comprising the conserved QLSVG segment, known to play a central role in the cysteine proteinase-cystatin interaction; and the COOH-terminal part of the molecule (aa residues 73 to 116), respectively. By using sequence-specific PCR primers, the respective

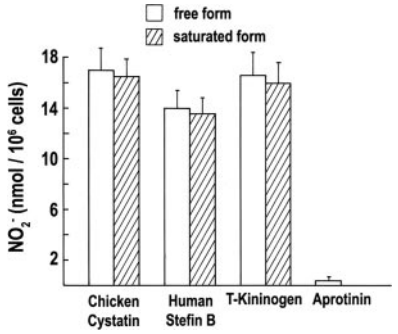

FIG. 2. Up-regulation of nitrite release by members of the cystatin superfamily complexed with papain. Papain was inactivated by reduction and alkylation. Inactivation was indicated by the complete loss of enzyme activity. The carboxymethylated papain (10^{-5} M) was then mixed at 37°C for 45 min with 10^{-6} M chicken cystatin, human stefin B, or T-kininogen. The free form or saturated forms were then introduced into the culture medium for 48 h before the nitrite measurement. Data represent the means ± SD of results from three experiments.

fragments were picked up from the cDNA and ligated into a prokaryotic expression vector, which allowed the production of large amounts of the respective polypeptides as GST fusion proteins in *E. coli*. Following IPTG induction of the bacteria transformed with the respective constructs, an additional band of ~30 kDa (27 kDa from GST and ~3 kDa from the respective nonoverlapping polypeptides of cystatin) was obtained upon SDS-PAGE (Fig. 3A, lanes 2, 4, and 6), whereas the lysate of induced bacteria harboring the vector alone showed a GST band of 27 kDa (Fig. 3A, lanes 1, 3, and 5). That GST fusion did not interfere with the native forms of the respective polypeptides was evidenced by Western blot analysis of the fusion constructs with anti-cystatin antibody (Fig. 3B). Analysis by PAGE showed that the various recombinant polypeptides purified from bacterial lysates by affinity chromatography using GST purification columns produced single bands corresponding to a molecular mass of ~30 kDa (Fig. 3C), and anti-cystatin antibody was found to react with single bands from these recombinant polypeptides (Fig. 3D, lanes 2 to 4). When these recombinant polypeptides were analyzed for their NO-generating capabilities, Cst I showed the maximum level of NO induction (12 nmol/10^6 cells at 0.1 nM concentration) while Cst II and Cst III had no significant effect (Fig. 3E). This result was also reflected in the iNOS mRNA expression pattern (Fig. 3F) and protein level (Fig. 3G). When these recombinant peptides were assessed for their cysteine protease-inhibitory activity with papain, only the intermediate fragment Cst II inhibited papain, with a K_i of 900 nM. Both Cst I and Cst III exhibited >1,000-fold-weaker inhibition than the whole molecule, indicating thereby that the N-terminal region of cystatin is not involved in cysteine protease inhibition but has a role to play in NO up-regulation.

Cystatin-derived immunomodulatory peptide exhibiting antileishmanial activity. To determine whether the region of the N-terminal domain of cystatin with NO-generating capability could be narrowed down to a minimal amino acid sequence, a set of six synthetic overlapping peptides (compounds 1 to 6) of 8 amino acids each, spanning the entire 28 aa of Cst I, were assessed. Maximum NO-generating ability was found to be associated with compound 4 (Fig. 4A). Further, by using four overlapping peptides of 10 aa (compounds A to D) covering the 8 amino acids of compound 4, a 10-mer region (compound B) from positions 11 to 20 of Cst I was found to be the domain responsible for NO-stimulatory activity (Fig. 4B), and the NO-stimulatory activity of this region was comparable to that of the whole cystatin molecule. The level of NO_2^- release by macrophages progressively increased with increasing concentrations of compound B up to 1 μM, when the level of release reached a maximum (Fig. 4C). In order to ascertain the specificity of this compound, a rabbit polyclonal antibody against this 10-mer region was raised. The presence of 5 μg/ml of the anti-peptide antibody could almost totally abolish the NO generation at concentrations of compound B as high as 5 μM (Fig. 4C). We then examined the efficacy of compound B against the intracellular growth of amastigotes within macrophages. *L. donovani*-infected cultures were treated with graded concentrations of compound B, ranging from 0.1 to 5 μM, in the presence of subthreshold concentrations of IFN-γ. As shown in Fig. 4D, compound B exhibited profound antileishmanial activity, with a 50% inhibitory concentration of 0.52 μg/ml. Both AMT, a specific iNOS inhibitor, and anti-peptide antibody could cause a reversal of the antileishmanial effect, suggesting thereby that the antileishmanial effect of compound B may be correlated with an increased production of NO.

Effect of the cystatin-derived 10-mer peptide on experimental leishmaniasis. The efficacy of the 10-mer peptide for the treatment of visceral leishmaniasis in vivo was determined with a mouse model. BALB/c mice were infected i.v. with *L. donovani* AG83. Animals were given i.v. injections of graded doses (0.01 to 1.0 mg/kg/day) of the peptide along with a constant dose of 10^4 U of IFN-γ daily for 4 consecutive days beginning 10 days after infection, and infection was allowed to proceed for 45 days. The degree of leishmanicidal potency of the peptide was assessed in terms of liver and spleen parasite burdens. Reductions of 93 and 95% in liver and spleen parasite burdens, respectively, were obtained at a dose of 0.5 mg/kg/day (Fig. 5A). In the placebo-treated infected controls, high parasite burdens were present in livers ($\log_{10}$ number of LDU, 3.41 ± 0.32) and spleens ($\log_{10}$ number of LDU, 2.32 ± 0.21). This was also the case in animals receiving anti-peptide antibody in conjunction with compound B treatment. The reinfection of cured animals after 45 days resulted in only a slight and transient increase in organ parasite burdens, suggesting the possible development of protective immunity (Fig. 5B). To ascertain whether compound B-treated mice also controlled the infection by a NO-dependent mechanism, AMT (5 mg/kg/day) was administered 1 week after reinfection. Within 3 to 4 days of AMT administration, the organ parasite burdens started increasing. The infections were under control again after AMT was withdrawn (Fig. 5B).

Effect of cystatin on cytokine production. To gain insight into the type of immunological response in *L. donovani*-infected mice after peptide therapy, levels of transcription of IL-10, IL-12p40, and TNF-α mRNA in isolated splenocytes were determined 45 days after infection. There was a high level of IL-10 in infected, untreated controls and a low level of expression of transcripts for IL-12 and TNF-α (Fig. 5C). In contrast, high levels of IL-12 and TNF-α expression and a very low level of IL-10 expression in infected, cystatin-treated cells

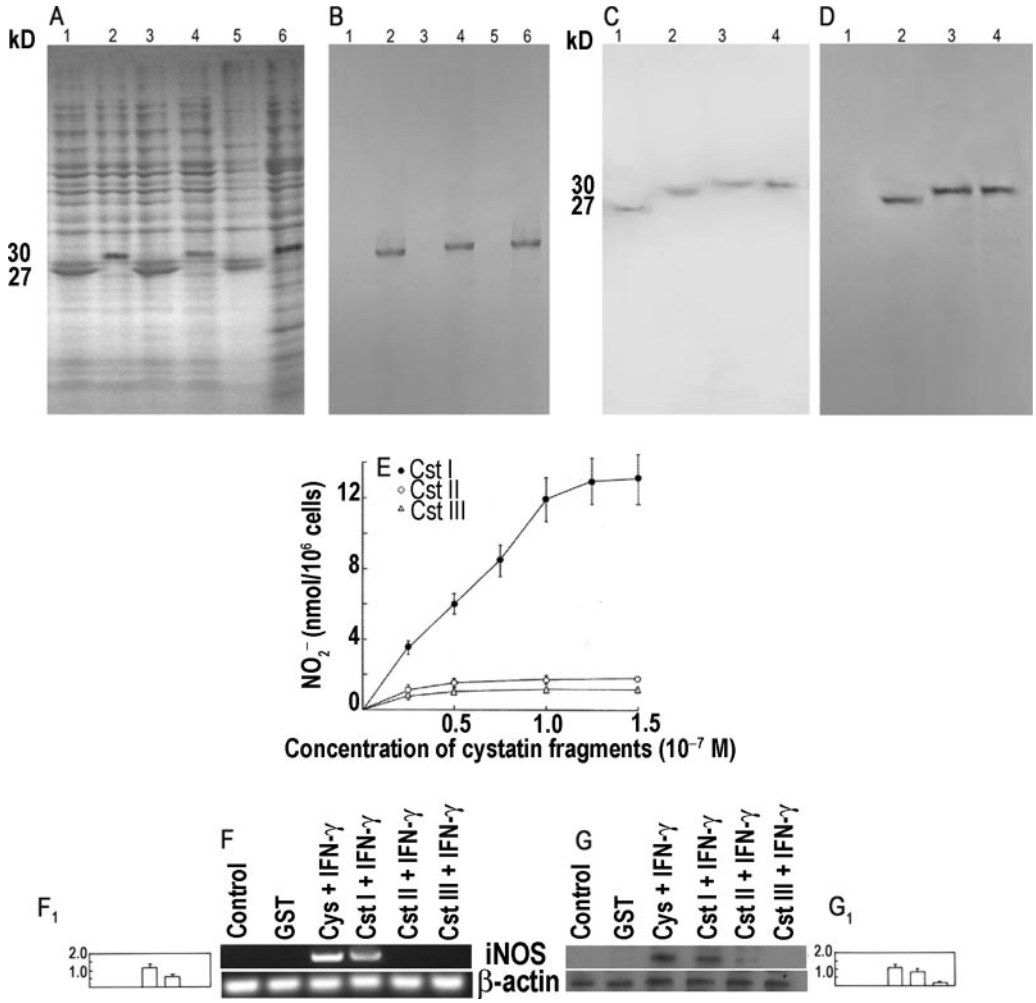

FIG. 3. Identification of the NO-stimulatory domain of cystatin. (A) Three nonoverlapping fragments representing the N-terminal (Cst I, aa 1 to 28), the intermediate (Cst II, aa 29 to 72), and the C-terminal (Cst III, aa 79 to 116) domains were cloned into a prokaryotic expression vector (pGEX-5X-2) and expressed as GST fusion proteins. Lanes 2, 4, and 6 show the Coomassie-stained SDS-PAGE gel of GST fusion proteins (~30 kDa) with Cst I, Cst II, and Cst III, respectively, whereas lanes 1, 3, and 5 show the induced bacterial lysate. (B) Corresponding Western blot analysis using polyclonal anti-cystatin antibody. The purified recombinants as GST fusion proteins (lane 1, recombinant GST; lane 2, GST-Cst I; lane 3, GST-Cst II; and lane 4, GST-Cst III) were separated on a 10% SDS-PAGE gel (C) and immunoblotted with polyclonal anti-cystatin antibody (D). (E) Macrophages were incubated with graded concentrations of recombinant GST fusion products of Cst I, Cst II, and Cst III along with a suboptimal dose of IFN-γ (100 U/ml) for 48 h, and the release of NO_2^- was quantified. Data are means ± SD of results from three experiments. The nature of iNOS expression was also determined by RT-PCR analysis of the mRNA transcript levels (F) and by Western blotting for analysis of protein levels (G) in response to 1×10^{-7} M cystatin (Cys) and various recombinants as GST fusion peptides, along with 100 U of IFN-γ/ml. Band intensities were analyzed by densitometry (F_1 and G_1). Cys II and Cys III, Cst II and Cst III.

as well as cystatin–IFN-γ-treated cells were detected (Fig. 5C). iNOS was also found to be up-regulated in infected, cystatin-treated cells and cystatin–IFN-γ-treated cells (Fig. 5D). Levels of transcripts for various cytokines and iNOS after peptide therapy and after peptide–IFN-γ therapy were found to be comparable to those in infected, cystatin-treated cells and those in cystatin–IFN-γ-treated cells, respectively (Fig. 5C and D). To determine the specificity of peptide therapy, we administered 500 μg of anti-peptide antibody along with peptide–IFN-γ to *L. donovani*-infected mice. The anti-peptide antibodies greatly reduced the level of peptide-mediated iNOS in splenocytes as well as Th1 cytokine induction at the mRNA level (Fig. 5C and D). The therapeutic effect of the 10-mer cystatin-derived peptide may be attributed to the up-regulation of iNOS resulting from the up-regulation of Th1 cytokines.

DISCUSSION

Cystatins represent a widely distributed superfamily of cysteine protease-inhibitory proteins. Chicken egg white cystatin has a molecular weight of approximately 13,000 and binds tightly to members of the papain family of cysteine proteases. The human analog is cystatin C, and like the other members of the cystatin family, it is comprised of one nonglycosylated 120-residue polypeptide chain (11). It is ubiquitous in human tissue and body fluid (1) and efficiently inhibits endogenous

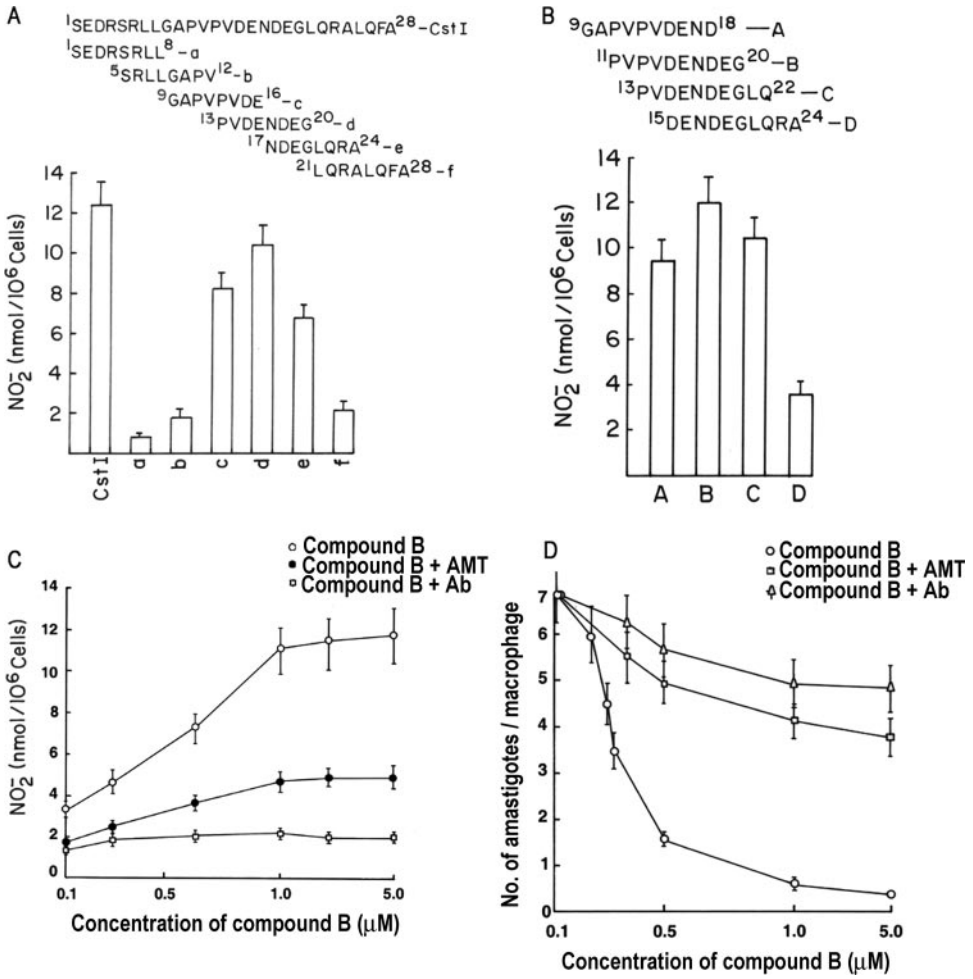

FIG. 4. Minimal peptide sequence with NO-stimulatory potential and its antileishmanial activity. (A) Macrophages were incubated with overlapping synthetic peptides (8-mer) derived from Cst I along with IFN-γ (100 U/ml), and NO_2^- release was quantified. (B) NO_2^- release from macrophages in response to overlapping 10-mer peptides containing the most potent 8-mer peptide in terms of NO production was measured. (C) Dose response curves for various concentrations of compound B in combination with IFN-γ (100 U/ml) representing the release of NO_2^- by macrophages in the presence or absence of either anti-compound B antibody or AMT (10 μM). (D) Infected macrophages were treated with graded concentrations of compound B in combination with IFN-γ (100 U/ml) in the presence or absence of either anti-compound B antibody or AMT (10 μM). Infected controls contained 7.14 ± 0.69 amastigotes/macrophages. Data are means ± SD of results from three experiments.

cysteine proteases such as cathepsins B, H, L, and S. These proteases, found in all species of *Leishmania*, play a dominant role in parasite virulence, the modulation of the host's immune response, and parasite differentiation. Hence, inhibitors that would efficiently target the cysteine proteases of the parasite, while maintaining some selectivity distinguishing them from the homologous host enzyme, would be ideal drug leads. Cystatin C has also been shown to exhibit antiviral functions, as suggested from experiments done with polio, herpes simplex, and corona virus-infected cell lines (3, 5). We previously elucidated the dual role of cystatin of suppressing the functional differentiation of Th2 type $CD4^+$ T cells, leading to the augmentation of the Th1 response, and up-regulating NO, resulting in the elimination *Leishmania* parasites in both in vitro and in vivo murine models of visceral leishmaniasis (6). In the present work, we have confirmed the efficacy of cystatin along with a suboptimal dose of IFN-γ against experimental visceral leishmaniasis in BALB/c mice, with effects involving an up-regulation of NO and a favorable T-cell response. These effects could be reversed by AMT (an iNOS-specific inhibitor), reflecting thereby that increased microbicidal activity was attained via a nitrogen-dependent mechanism involving the induction of iNOS. It has been a well-established fact that NO produced by cytokine-activated murine macrophages exhibits microbicidal action during intracellular infections (23). The ability of cystatin to generate NO from stimulated macrophages was unaffected even when the cysteine protease-inhibitory site was blocked with reduced papain. Human stefin B and T-kininogen, members of the cysteine protease inhibitor family, also demonstrated the same biological ability, while aprotinin, an unrelated protease inhibitor, exhibited no such action, indicating that the NO-inducing effect is unrelated to the protease-inhibitory effect. An appreciable amount of work has been devoted to the biochemical characterization of the cysteine protease-inhibitory function of cystatin (18, 13). Based on such findings, peptide segments derived from consensus

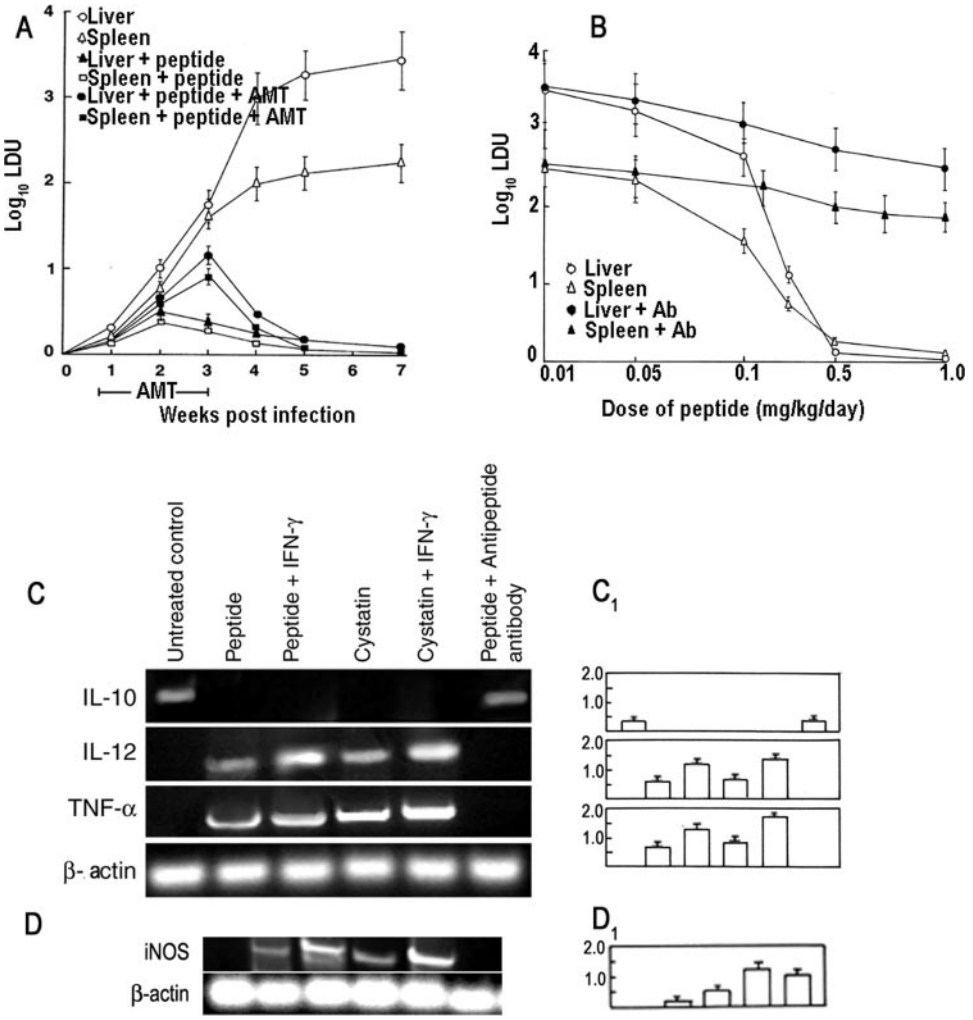

FIG. 5. Effect of compound B treatment on visceral infection in BALB/c mice. (A) Various doses of compound B ranging from 0.05 to 1 mg/kg/day were given i.v. along with IFN-γ (10^4 U/mouse) for 4 consecutive days beginning 10 days after infection. The parasite burdens in livers and spleens were then determined at 45 days after infection. Anti-peptide antibody was given along with compound B and IFN-γ in a separate set of experiments. (B) The course of visceral reinfection was studied by i.v. administration of 10^7 promastigotes into naïve, age-matched BALB/c mice and cured (1-mg/kg/day-compound B-treated) mice. In one group of cured mice, AMT (5 mg/kg/day) was i.v. administered 1 week after reinfection for 2 weeks. Determining liver and spleen parasite burdens, expressed as the $\log_{10}$ numbers of LDU, allowed for the monitoring of the progression of infection in all the cases. Results are from three experiments and indicate the means ± SD for 5 to 7 mice at each time point. Ab, antibody. (C and D) Cytokine profiles of *L. donovani*-infected mice as analyzed by RT-PCR. The levels of expression of IL-10, IL-12, TNF-α, iNOS, and β-actin mRNA by spleen cells of infected mice treated i.v. with the peptide and/or IFN-γ in the presence or absence of anti-peptide antibody were determined and compared to those by spleen cells of mice receiving cystatin and IFN-γ treatment. RT products were visualized by ethidium bromide staining. RNA samples were obtained from five mice in each group. Results shown are representative of those for five separate samples. β-Actin expression levels were used as controls for RNA content and integrity. Band intensities were analyzed by densitometry (C_1 and D_1).

sequences of the inhibitory site of cystatin have been used as models to design and develop specific substrates and selective inhibitors of cysteine proteinases and have been shown to exert activity against pathogens like *Porphyromonas gingivalis* (4, 28).

The characterization of the chicken cystatin-encoding gene has revealed the gene to be ~ 2.4 kb in length and to contain three exons, two introns, and two polyadenylation signals. We made use of recombinantly expressed fragments of chicken cystatin to identify and characterize the region for NO generation in activated macrophages. The aim was to design an immunostimulatory peptide moiety that would not only minimize drug doses per se but also be used as a natural immunomodulator in macrophage-related disorders requiring a NO-promoting response. A total of three separate fragments (~30 aa each) spanning the entire length of functional cystatin were expressed as fusion proteins, purified, and used to test their efficacy in generating NO in activated macrophages. The N-terminal fragment (Cst I), which did not possess the well-documented cysteine protease-inhibitory site (18), was found to be the most potent of the three fragments in terms of NO generation. Further analysis of the NO-up-regulatory response by using a minimal 10-mer peptide synthetically derived from this region revealed NO generation capability both in vitro and in vivo. When assessed for leishmanicidal potential, the 10-mer peptide corresponded to a pattern of elevated iNOS transcript levels in parallel with a disease-regressing Th1 cytokine (IL-12

and TNF-α) expression pattern, thereby suppressing the amastigote proliferation not only in infected peritoneal macrophages but also in a 45-day murine model of visceral leishmaniasis as effectively as the whole molecule. This 10-mer peptide mimicked the effect of the whole cystatin molecule both in terms of NO up-regulation and antileishmanial action, as this effect was completely reversed in the presence of the peptide-specific antibody, which held true for the whole cystatin molecule as well. Taken together, the present study identifies the functional domain of cystatin that holds promise for the design of an immunomodulatory biopeptide-based therapy for visceral leishmaniasis and similar macrophage-associated diseases which involve a down-regulation of NO. It may be mentioned in this regard that the expression of iNOS and the consequent production of NO have been shown to correlate with leishmaniasis resistance in a murine model as well as in human patients (10). Moreover, several other recent reports suggest an antileishmanial function of iNOS in human leishmania infection in vivo (8, 24).

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