Published in final edited form as:
Biochem Pharmacol. 2001 February 15; 61(4): 467–476.

Increased efficacy of antileishmanial antisense phosphorothioate oligonucleotides in *Leishmania amazonensis* overexpressing ribonuclease H

Manjari Mishra[a], **Jabbar R. Bennett**[b], and **Gautam Chaudhuri**[a,*]

[a] Department of Microbiology, Meharry Medical College, 1005 D. B. Todd Jr. Blvd., Nashville, TN 37208, USA

[b] Department of Anatomy & Physiology, Meharry Medical College, 1005 D. B. Todd Jr. Blvd., Nashville, TN 37208, USA

Abstract

Ribonuclease H (RNase H), an enzyme that cleaves an RNA sequence base-paired with a complementary DNA sequence, is proposed to be the mediator of antisense phosphorothioate oligonucleotide (S-oligo) lethality in a cell. To understand the role of RNase H in the killing of the parasitic protozoan *Leishmania* by antisense S-oligos, we expressed an episomal copy of the *Trypanosoma brucei RNase H1* gene inside *L. amazonensis* promastigotes and amastigotes that constitutively express firefly luciferase. Our hypothesis was that S-oligo-directed degradation of target mRNA is facilitated in a cell that has higher RNase H activity. Increased inhibition of luciferase mRNA expression by anti-luciferase S-oligo and by anti-miniexon S-oligo in these stably transfected promastigotes overexpressing RNase H1 was correlated to the higher activity of RNase H in these cells. The efficiency of killing of the RNase H overexpressing amastigotes inside *L. amazonensis*-infected macrophages by anti-miniexon S-oligo was higher than in the control cells. Thus, RNase H appears to play an important role in the antisense S-oligo-mediated killing of *Leishmania.* Chemical modification of S-oligos that stimulate RNase H and/or co-treatment of cells with an activator of RNase H may be useful for developing an antisense approach against leishmaniasis. The transgenic *Leishmania* cells overexpressing RNase H should be a good model system for the antisense-mediated gene expression ablation studies in these parasites.

Keywords

Leishmania; Amastigote; Promastigote; Differentiation; Ribonuclease H; Antisense phosphorothioate oligonucleotide

1. Introduction

The etiological agent for the human parasitic disease leishmaniasis is the protozoon of the genus *Leishmania.* This obligate intracellular parasite of mammalian macrophages has a digenetic life cycle [1]. These cells are exposed to a variety of environmental factors during their transmission from sandfly vectors to the human body, their establishment of infection in human macrophages, and their propagation as amastigotes inside macrophage phagolysosomes [1]. In its life cycle, *Leishmania* alternates between an insect vector and a

*Corresponding author. Tel.: +1-615-327-6499; fax: +1-615-327-5559. gchaudhuri@mail.mmc.edu (G. Chaudhuri).

vertebrate host. The parasite lives as a motile, flagellated promastigote in the gut of various sandfly vectors, characterized by its elongated shape (10–12 μm) and a single, long anterior flagellum. When delivered into the mammalian body by the bite of an infected sandfly, the promastigotes are engulfed by macrophages by a receptor-mediated process [1]. The promastigotes end up in the phagolysosomes of macrophages where they transform into non-motile amastigotes [1].

Among the many distinguishing molecular and biochemical features [2,3] that differentiate this parasite from its mammalian host is a 39 nt conserved sequence of RNA post-transcriptionally added at the 5′-ends of all leishmanial mRNAs and related cells [4]. This unique 5′-end sequence (miniexon) is methylated to form the ‘cap4’ structure, which is essential for the entry of ribosomes to translate the mRNA [4,5]. We and others [6–9] have targeted this miniexon sequence with antisense phosphorothioate oligonucleotides to ablate the translation of mRNAs of the parasite resulting in the demise of the parasite and survival of the host cells. Adoption of a macrophage scavenger receptor-mediated delivery system for the antisense oligonucleotide to *Leishmania*-infected macrophage phagolysosomes yielded better efficacy of amastigote killing [9]. Although we have clues from other eukaryotic systems, we do not yet have any information about the mechanism of antisense action in *Leishmania.* Since RNase H is implicated [10] in the modality of antisense phosphorothioate action in ablation of gene expression in other cells, we decided to study the effect of overexpression of this enzyme on the efficacy of antisense oligos in these cells.

RNase H is a widely distributed enzyme found in organisms ranging from retroviruses to mammals [11–16]. This enzyme hydrolyzes RNA in RNA/DNA heteroduplexes [16]. RNase H isotypes vary significantly in molecular size and associated functional activities. On the other hand, the nuclease properties of all RNase Hs are similar. All RNase Hs require a divalent cation for activity [16]. Thus far, three classes of RNase Hs have been discovered in bacterial cells. They are RNase H1, H2, and H3 [17]. RNase H1 and H3 share similar divalent cation preferences, and RNase H2 and H3 have significant similarity at the amino acid sequence level [16,17]. RNase H2 is the most ubiquitous of the three [16]. No single bacterium has been found to have all three RNase H isotypes [16]. *Escherichia coli* RNase H1, which is involved in DNA replication [18–23], is the best characterized among the prokaryotic enzymes. The key amino acids involved in divalent cation binding, substrate binding, and catalysis have been identified and are highly conserved in this family of enzymes [22,24–26]. In retroviral replication, RNase H acts as a prosthetic domain of the reverse transcriptase and helps convert the retroviral RNA into double-stranded DNA by generating RNA primers during second strand cDNA synthesis [24,27]. Retroviral RNase H domains share homology with *E. coli* RNase H1 [24]. Thus far, mammalian cells have been shown to have two isotypes of RNase H [12–16,25,26,28,29]. These two isotypes differ from each other by Mg^{2+} and Mn^{2+} requirements and sensitivity towards sulfhydryl reagents [16].

Although RNase H is likely to be present in *Leishmania,* it has not been shown in these cells until this study. RNase H has been cloned from organisms related to *Leishmania* [30–34]. Along with the universal RNase H catalytic domain in these protozoal enzymes, there is also a double-stranded RNA binding domain that is typical to eukaryotic RNase H [33,34]. The latter domain is absent in *E. coli* RNase H and, hence, is not needed for the functions of this enzyme in this cell. The physiological role of this domain of RNase H in eukaryotes is not clearly understood [33,34]. A single gene seems to be responsible for at least two forms of the *Crithidia fasciculata* RNase H1 [32,33]. Using the renaturation gel assay for RNase H activity, species of 38 and 45 kDa are obtained, both of which disappear in strains whose *RNH1* gene is deleted [32,33].

In this paper, we provide evidence that RNase H may be involved in antisense phosphorothioate oligonucleotide-mediated gene expression ablation in pathogenic *Leishmania* promastigotes and amastigotes.

2. Materials and methods

2.1. *Leishmania* amastigotes and promastigotes

The promastigotes of *L. amazonensis* were grown at 25° in Medium M199 with 10% HIFBS [9]. The amastigotes of *L. amazonensis* were obtained from tail-base lesions of infected Balb/C mice [35]. The amastigotes were also isolated from infected, cultured mouse macrophages (J774G8 cells) [9]. Axenic amastigotes of *L. amazonensis* were grown in axenic medium at pH 4.5 and at 33° [36,37]. Amastigotes were allowed to transform into promastigotes at 25° in Medium M199 containing 10% HIFBS. *Leishmania* and J774G8 cells were gifts from Prof. K-P. Chang of the Chicago Medical School.

2.2. Oligonucleotides

The following phosphorothioate ODNs (S-oligos) were used in this study: anti-miniexon antisense oligo ASM, 5′-CTGATACTTATATAGCG-3′ [9], the corresponding sense oligo SSM, 5′-CGCTATATAAGTATCAG-3′ [9], anti-luciferase antisense oligo LUAS, 5′-ATGCCCATA-CTGTTGAG-3′, and the corresponding sense oligo LUS, 5′-CTCAACAGTATGGGCAT-3′. The following phosphodiester ODNs were used for PCR amplifications: TbRHF, 5′-CCTTCTCTGCTCGCTTTTTAAC-3′;TbRHR, 5′-TGT-CTGTGCCACGTTAGCC-3′; Mex2, 5′-GGATCCAGT-TTCTGTACTTTATTG-3′ and LUAS, 5′-ATGCCCATA-CTGTTGAG-3′. The nucleotide sequence of firefly luciferase was obtained from the nucleotide sequence of pGL3-Basic plasmid (Promega), GenBank Accession Number U47295. The sense and antisense primers were designed using MacVector software (Oxford Molecular Inc.).

2.3. Amplification, cloning, and sequence verification of the *Trypanosoma brucei RNase H1* gene

The *RNase H1* gene of *T. brucei* was amplified from the genomic DNA templates of the procyclic form of the parasite. Primers for the amplification (TbRHF and TbRHR) were designed using the MacVector program (Oxford Molecular Inc.) from the published *T. brucei RNase H1* gene sequence [31]. These primers encompass a 1018 bp region of the *T. brucei RNase H1* gene, of which 99 bp are upstream of the translational start site, 905 bp are coding sequence, and 14 bp are downstream of the translational stop site. The amplification reaction was carried out following standard PCR protocol [38] using *Pfu* DNA polymerase (Stratagene). The PCR reaction product was gel-purified [38] and incubated with AmpliTaq DNA polymerase and dATP for 30 min at 65° to add 3′-A overhangs. This PCR product was then cloned into the pCRII-TOPO vector following the TOPO-TA-cloning protocol (Invitrogen). The nucleotide sequence of the insert of the recombinant phage-mid pCR-TbRH1 was verified by automated sequencing in a 310 Genetic Analyzer (PE Biosystems).

2.4. Development of *T. brucei RNase H1* constructs for stable expression of this enzyme in *L. amazonensis* promastigotes

The insert of the phagemid pCR-TbRH1 (see above) was cut out with *Kpn*I and *Xho*I and was subcloned at the *Kpn*I/*Xho*I sites of pGL3-Basic plasmid to get the pGL3-TbRH plasmid. This *T. brucei RNase H1* gene insert was then cut out of the pGL3-TbRH plasmid with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pX63-Neo plasmid ([4,39], a gift from Prof. S. Beverley) to obtain pXNeo-TbRH1F and pXNeo-TbRH1R clones (Fig. 1, B and C). These plasmids are *E. coli/Leishmania* shuttle vectors and thus contain all the

elements needed for replication in both *E. coli* and *Leishmania* [4,39]. In *Leishmania*, genes are thought to be transcribed from the plasmid DNA without requiring any specific promoter elements [4], but the appropriate export of the message from the nucleus to the cytosol and its translation into protein are thought to be dependent upon proper *trans*-splicing and polyadenylation of the message. The presence of a *trans*-splicing signal at the 5′-flank of the pre-mRNA, which is a poly-pyrimidine stretch followed by an 'AG' sequence, is critical for *trans*-splicing [4,39]. There are two such *trans*-splicing sites in pX63-Neo: one in front of the '*Neo*' gene (confers G418-resistance to *Leishmania*) and the other is in front of the unique *Bam*HI site [39]. Thus, in *Leishmania* cells transfected with the plasmid constructs, the *Neo* gene and the *TbRH1* gene cloned at the *Bam*HI site are expected to be expressed. The orientation of the insert was determined by nucleotide sequencing with a T3 primer [38].

2.5. Development of a firefly luciferase construct for the stable expression of this enzyme in *L. amazonensis* promastigotes

The firefly luciferase gene was cleaved from pGL3-Basic plasmid and was cloned, along with a hygromycin-resistance marker gene, into pBluescript KS(+) (Stratagene) to construct the pXHyg-Luci phagemid (Fig. 3A). Briefly, a 2 kb DNA fragment containing a 1 kb upstream sequence (US) of the *L. major* dihydrofolate reductase gene, cloned in front of the hygromycin-resistance marker gene, was cut out of the pX63-Hyg plasmid ([4,39], a gift from Prof. S. Beverley) and cloned into the *Sal*I/*Bam*HI sites of pBlue-script KS(+) to create the phagemid pBS-HygDhfr. In another experiment, a 580 bp *Kpn*I/*Sac*I fragment containing the US of the *L. donovani* leishmanolysin (*gp63*) gene was cloned at the *Kpn*I/*Sac*I sites, in front of the firefly luciferase gene in proper orientation, in pGL3-Basic to create the pGL3bK1 plasmid. Then, the pBHygDhfr plasmid was cut by digestion with *Kpn*I/*Sal*I, and the resulting *Kpn*I/*Sal*I fragment, containing *gp63* US in front of firefly luciferase gene, was cloned [38] to create the pXHyg-Luci plasmid (Fig. 3A).

2.6. Transfection of *Leishmania* cells

Late-log phase cells were washed and resuspended in high ionic strength electroporation buffer (20 mM HEPES, pH 7.2, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose) to a final concentration of 3.3×10^8 cells/mL [40,41]. Cells (0.3 mL) were mixed in a 0.2-cm electroporation cuvette (Bio-Rad) with plasmid DNA (25 μg/10^8 cells) and were exposed to an exponential discharge of 2250 V/cm from a 500 μF capacitor (Gene Pulsar, Bio-Rad) [40]. Electroporated cells were incubated on ice for 10 min, transferred into culture medium (10 mL), and incubated at 25° for 20–24 hr before growth in selective medium. Cells were selected in increasing concentrations of antibiotic, starting from 1 to 200 μg/mL.

2.7. RT–PCR

Poly(A)$^+$ RNAs were isolated from *L. amazonensis* promastigotes using the PolyATtract® mRNA Isolation System (Promega). The RNA was treated with RNase-free DNase (Promega) to remove contaminating DNA. Complementary DNAs were synthesized from these RNAs (1 μg) using Superscript II reverse transcriptase (GIBCO-BRL) and oligo(dT) primers in a total volume of 20 μL, following a recommended protocol (GIBCO-BRL). An aliquot (1 μL) of these cDNAs was used for PCR amplification using AmpliTaq DNA polymerase (Qiagen) [38]. For the amplification of *T. brucei* RNase H1 cDNA, primers TbRHF and TbRHR were used. For 5′-RACE (rapid amplification of cDNA ends) analyses of *T. brucei* RNase H1 mRNA and firefly luciferase mRNA isolated from transgenic *Leishmania* cells, the miniexon-specific sense primer Mex2 and gene specific antisense primer (TbRHR or LUAS, see above) were used. The PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and photographed [38]. In some

experiments, the RT–PCR products were gel-purified, cloned into the pCRII-TOPO vector (Invitrogen), and sequenced using T7 and SP6 primers [38].

2.8. Luciferase assay

The luciferase assay was performed using the Luciferase Reporter Assay System (Promega). G418/Hygromycin-resistant cells (promastigotes or amastigotes) were incubated with the phosphorothioate oligonucleotide (LUS or LUAS) for 20 hr in complete culture medium under appropriate growth conditions. Cells were washed with ice-cold PBS and lysed with Passive Lysis Buffer (Promega, 100 μL/10^7 cells) at room temperature for 15 min on a rocker platform. The lysates were centrifuged at 16,000 *g* for 10 min. Aliquots (20 μL) of the supernatant were added to the substrate LARII (Promega, 100 μL) and mixed, and luminescence was assayed for 10 sec in a Turner Designs model 20/20 Luminometer [42].

2.9. Assay for RNase H activity in *Leishmania* cell extracts

[^{32}P]Poly(rA):poly(dT) was used as the substrate of RNase H [43]. *E. coli* RNA polymerase (6 μL, 10 units/μL, Amersham Pharmacia Biotech) was added to a mixture containing 5 A_{260} units poly(dT), 5% glycerol, 50 mM Tris · HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 4 mM dithiothreitol, 30 μM ATP, and 100 μCi [α-^{32}P]ATP (3000 Ci/mmol, Amersham) in a total volume of 3 mL. The reaction mixture was extracted twice with an equal volume of water-saturated phenol (GIBCO-BRL). The pooled phenol phases were extracted with an equal volume of 50 mM Tris · HCl, pH 8.0. The aqueous phases were pooled and extracted with an equal volume of chloroform/isoamyl alcohol (24/1). Absolute ethanol was added to the aqueous phase to a final concentration of 35%, and the resulting solution was loaded onto a 2-mL column of cellulose (CF11, Sigma). The column was pre-equilibrated with a 35:65 mixture of ethanol:'buffer A' (50 mM Tris · HCl, pH 7.5/0.1 M NaCl, 50 mM EDTA). After sample loading, the column was washed extensively with this ethanol:buffer A mixture (~50 mL) until all unbound radioactivity was removed (<1000 cpm/10 μL wash). Then the bound nucleic acid polymer was eluted from the column with 5 mL of buffer A, and 500-μL fractions were collected in microfuge tubes. Fractions with high radioactivity (generally tubes 3–7) were pooled, and radioactivity was measured by scintillation counting [38]. A typical preparation usually gave us ~125,000 cpm/10 μL. The hybrid solution was divided into aliquots and stored at −20° for later use. *Leishmania* promastigotes or amastigotes were washed in PBS and lysed in the Passive lysis buffer (Promega, 10^8 cells/mL) for 15 min on ice. The lysate was cleared by centrifugation at 16,000 *g* for 10 min at 4°, and the supernatant was assayed for RNase H activity. Protein concentration was measured using the Bradford reagent (Bio-Rad). For the RNase H solution assay, the cell lysate (1 μg protein), in 10 mM Tris · HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, and 0.1 mM dithiothreitol, was incubated with the substrate (5 μL) at 37° for 30 min in a total volume of 25 μL. The reaction was terminated by the addition of EDTA (50 mM, final concentration) and glycogen (1 μg/μL, final concentration). The unreacted substrates and proteins were precipitated with trichloroacetic acid (5%, final concentration, 10 min at 4°) and were centrifuged at 16,000 *g* for 10 min at 4° [43]. The radioactivity in the supernatant was determined by liquid scintillation counting [38], and the data were used as a measure of RNase H activity.

2.10. Preparation of MBSA-coated liposomes

BSA was maleylated with maleic anhydride [9]. Di-palmitoyl phosphatidylethanolamine (Sigma Chemical Co.) (100 mg, dissolved in 0.15 M NaCl by ultrasonication) was coupled to MBSA (30 mg) by a 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (100 mg)-mediated reaction [44,45], in a total volume of 3 mL. DMPC was used to prepare liposomes for encapsulation of ASM or SSM [9]. Briefly, DMPC (10 mg) was made into vesicles with cholesterol (9 μmol) and dicetyl phosphate (1.2 μmol) in 1 mL HBS (HEPES buffer, 25 mM;

NaCl, 0.15 M; sometimes contained oligonucleotide, 50 μmol/mL). Typically, 0.1 to 0.2 μmol oligonucleotides became trapped in 1 mg of liposomal phospholipids using this procedure. The amount of phospholipids in each liposomal preparation was assayed by estimating its phosphate content [9]. For coating the liposomes with phospholipid-tailed MBSA (which binds via the phospholipid part), 200 μg of the protein was incubated with 1 mg of liposomes in HBS for 20 hr at 4°. Bound MBSA was separated from unbound molecules by centrifugation at 100,000 *g* for 1 hr at 4°, as described previously [9].

2.11. Assay of leishmanicidal activity of ASM

Monolayers of J774G8 cells in 24-well plastic tissue culture plates were incubated at 37° with *L. amazonensis* promastigotes (parasite-to-macrophage ratio of 10:1) in RPMI 1640 medium containing 20% HIFBS for 16 hr [9]. The infected macrophages were then incubated in the growth medium at 35° for 48 hr. The growth medium was then replaced with fresh medium, and the test compound was added. After incubation of the parasite-laden macrophages at 35° for 20 hr, the number of amastigotes per 100–200 macrophages were counted microscopically, as described [9]. Axenic amastigotes were harvested by centrifugation (3000 *g*, 10 min, 4°), resuspended (10^6 cells/mL) in growth medium, and incubated with or without the oligonucleotide in a 24-well plastic tissue culture plate (500 μL/well) for 20 hr with gentle rocking (100 rpm). Aliquots (10 μL) were withdrawn from the wells, and the cells were counted in a hemocytometer [9].

2.12. Statistical procedures

Each value is presented as the mean ± SEM. The statistical significance of a difference between two series of data was tested by determining the *P* value [46]. If the *P* value was less than 0.05, the difference was considered significant [46].

3. Results

3.1. Overexpression of *T. brucei* RNase H in *L. amazonensis*

The *RNase H1* gene has been cloned from *T. brucei* by genetic complementation in RNase H negative *E. coli* [31]. Primers were designed from the published [31] (GenBank Accession Number U74470) nucleotide sequence of the *T. brucei RNase H1* (*TbRH1*) gene. The gene was amplified by PCR using *T. brucei* genomic DNA as the template (Fig. 1A). A DNA polymerase with a proofreading function was used in the amplification to avoid any inadvertent mutation. The PCR product was sequenced to confirm that there was no change in nucleotide sequence. Two plasmid constructs were generated with the *TbRH1* gene cloned into the *Bam*HI/*Bgl*II sites of the *Leishmania*/*E. coli* shuttle vector pX63-Neo (Fig. 1, B and C). The *L. amazonensis* promastigotes were electroporated individually with those plasmids. G418-selected clones were checked for the presence of plasmid DNA and the expression of RNase H1 mRNA. As shown in Fig. 2A, the primers used only amplified DNA of the expected size from *L. amazonensis* cells transfected with the *TbRH1* gene. RT–PCR also showed expression of the *TbRH1* gene in cells transfected with pXNeo-TbRH1F plasmid and not in those transfected with pXNeo-TbRH1R plasmid, as expected (Fig. 2A).

Like other trypanosomatids, genes are transcribed in *Leishmania* as homo- or hetero-polycistronic RNAs, which then are *trans*-spliced and polyadenylated to produce mono-cistronic translatable messages [2–4]. Although a few candidates for RNA polymerase II promoters have been reported in *Leishmania* and *T. brucei* [47–49], their identification remains controversial [3]. *Trans*-splicing seems to be regulated by a pyrimidine-rich region upstream of the coding sequence, which guides the miniexon to attach to a nucleotide next to an ‘AG’ sequence near this regulatory region [5]. The smaller ribosomal subunit seems to be entering the cap4 structure at the 5′-end of the mature mRNA and, by the scanning

mechanism [50], finds the AUG codon, usually following the Kozak rule (−3 is a purine and +4 is a guanine nucleotide) [50]. The plasmid pX63-Neo has two strong *trans*-splicing signals: one before the '*Neo* ' gene and the other before the *Bam*HI site [39]. Thus, the mRNA transcribed from the *TbRH1* gene in *L. amazonensis* should have been *trans*-spliced in the expected site. We checked the *trans*-splicing site of the TbRH1 mRNA by 5′-RACE analysis. The first strands of the cDNAs were synthesized using oligo(dT) primers. This ensured that only mature transcripts would participate in the cDNA synthesis reaction. The nucleotide sequence of the miniexon end of the PCR product showed that *trans*-splicing occurred at the expected site as directed by the *trans*-splicing signal in the plasmid (Fig. 2B).

Thus, our PCR data showed that the *TbRH1* gene cloned behind the *trans*-splicing signal in pX63-Neo was expressed as a polyadenylated and *trans*-spliced mRNA. Although the antisense strand of the DNA is also known to be transcribed in *Leishmania* [51], we did not detect any mature antisense transcript of the *TbRH1* gene in *L. amazonensis* promastigotes transfected with the pXNeo-TbRH1R plasmid (data not shown). When we transformed the $TbRH1^{+}$ promastigotes into amastigotes in axenic culture medium and analyzed them for expression of the *TbRH1* gene, a similar expression pattern of the polyadenylated and *trans*-spliced TbRH1 transcripts was observed (data not shown).

Finally, we evaluated whether RNase H activity in the *Leishmania* cell extract increases with the episomal expression of the *TbRH1* gene in these cells. Using a poly(dT): [^{32}P]poly(rA) substrate, we detected significant RNase H activity in the cell-free extract of *L. amazonensis* promastigotes and axenic amastigotes (Fig. 2C). This RNase H is optimally active at 37° and requires Mg^{2+}. Episomal expression of the *TbRH1* gene in these cells increased the RNase H activity 2- to 3-fold over that present in the cell (Fig. 2C). The axenic amastigotes of *L. amazonensis* had 3-to 4-fold higher RNase H activity than the promastigotes (Fig. 2C). However, episomal expression of TbRH1 in these amastigotes also boosted the RNase H activity about 2-fold (Fig. 2C). Further characterization of *Leishmania* RNase H will offer a better explanation for these observations.

3.2. Development of stable transfectants of *L. amazonensis* promastigotes expressing firefly luciferase

The G418-resistant transgenic *L. amazonensis* promastigotes expressing the *TbRH1* gene were electroporated with a firefly luciferase construct, pXHyg-Luci, which contains the hygromycin-resistant gene (Fig. 3A). Like pX63-Neo, pXHyg-Luci also has two strong *trans*-splicing signals: one in front of the Hyg^{r} gene (imported from pX63-Hyg [39]) and the other just before the luciferase gene (from upstream of the *L. donovani* stationary phase-specific *gp63* gene) (Fig. 3A). The *gp63* upstream sequence has been shown by others to direct the expression of the luciferase gene in transfected *Leishmania* [5]. The hygromycin/G418-resistant (200 μg/mL each) *L. amazonensis* promastigotes were analyzed for luciferase transcripts by RT–PCR. Oligo(dT) primers were used in the reverse transcription of mRNA to cDNA. The 5′-end of the luciferase cDNA was then amplified with a miniexon-specific sense primer (Mex2) and a luciferase-specific antisense primer (LUAS). As shown in Fig. 3B, the luciferase gene was *trans*-spliced at the expected splicing site. Our data thus suggest that in these stable transfectants of *L. amazonensis* promastigotes, firefly luciferase is episomally expressed as a *trans*-spliced and polyadenylated transcript. We transformed these transgenic promastigotes into amastigotes. As expected, amastigotes also expressed the mature luciferase transcript (data not shown). Cell-free lysates from transgenic *Leishmania* promastigotes and amastigotes contained significant luciferase activity (Fig. 3C). These cells continued to express TbRH1 mRNA and to display enhanced RNase H activity in both the promastigotes and the axenic amastigotes, even after several passages in G418-free growth medium (over a period of 3 weeks).

3.3. Increased inhibition of luciferase mRNA expression by anti-luciferase antisense phosphorothioate ODN in promastigotes and axenic amastigotes overexpressing RNase H

We tested whether an anti-luciferase antisense phosphorothioate ODN ablates luciferase activity more efficiently in the transgenic TbRH1$^+$ *L. amazonensis* axenic amastigotes (which have pXNeo-TbRH1F plasmid) than in the TbRH1$^-$ amastigotes (which have pXNeo-TbRH1R plasmid). *L. amazonensis* amastigotes growing in axenic culture medium containing 200 μg/mL each of G418 and hygromycin were incubated with the anti-luciferase antisense S-oligo (LUAS) for 20 hr. Control incubations were performed with sense S-oligo (LUS) in parallel. The effect of LUS on cell growth was insignificant. In the presence of LUAS, the cells expressing *T. brucei* RNase H demonstrated less luciferase activity than those without the *T. brucei* enzyme (Fig. 4). The antisense S-oligo was more effective in amastigotes than in promastigotes, perhaps because of higher RNase H activities in the former (Fig. 2C).

3.4. Increased efficacy of antileishmanial antisense phosphorothioate oligonucleotides in the stable transfectants

We tested whether overexpression of RNase H in *Leishmania* amastigotes affects their sensitivity against anti-miniexon antisense S-oligo (ASM). Initially, we tested the effect of ASM on the growth of TbRH1$^-$ and TbRH1$^+$ *L. amazonensis* amastigotes cultured in axenic medium. Control incubations were done with the S-oligo with sense nucleotide sequence (SSM) in parallel. SSM at 10 or 20 μM had no significant effect on the growth of the amastigotes. At both concentrations tested, ASM killed the axenic amastigotes more efficiently when the cells expressed the *T. brucei* RNase H (Fig. 5). We then tested whether ASM was more efficient in killing TbRH1$^+$ amastigotes inside infected macrophages. We previously developed a targeting strategy to deliver S-oligo to the phagolysosomes of *Leishmania*-infected J774G8 macrophages [9]. The S-oligo was encapsulated inside liposomes, and the liposomes were then coated with phosphatidylethanolamine-anchored MBSA. MBSA is an effective ligand for macrophage scavenger receptor type II, which is lysosomotropic [44,45]. When offered to *Leishmania*-infected macrophages, ASM encapsulated-MBSA-coated liposomes readily bind to the macrophage scavenger receptor. The liposomes are endocytosed, the resulting endosome fuses with parasitophorous vesicles, and the drug is released following digestion of the liposomes by hydrolases. When targeted this way, the parasite cells are exposed to high concentrations of ASM [9]. In this study, ASM killed the TbRH1$^+$ amastigotes more efficiently than the TbRH1$^-$ amastigotes (Fig. 6). In contrast, SSM had no significant effect on the growth of amastigotes.

4. Discussion

Our aim was to understand, optimize, and evaluate the feasibility of the antisense approach against the expression of leishmanial genes. We previously demonstrated the practicality of the antisense approach against leishmaniasis [9]. We used anti-miniexon ODN as our test compound. We packaged the ODN inside MBSA-coated liposomes (MBSA-liposomes) and offered the liposomes to *Leishmania*-infected macrophages or mice. This modality of drug delivery proved to be very effective as the drug is quickly and selectively dispatched to macrophages without exposing other biomolecules of the host, thus reducing the possibility of secondary reactions [10].

Although antisense S-oligo worked effectively against *Leishmania*, we do not know the mode of action of the ODN in this parasite. Nucleotide sequence specificity of the effective S-oligo indicates that the mode of action involves hybridization of the antisense S-oligo to the complementary sense sequence of its target mRNA. The heteroduplex formed between the sense RNA and the antisense DNA may result in inhibition of ribosome binding,

capping, polyadenylation, *trans*-splicing, and/or transport of the mRNA from the nucleus to the cytosol, depending upon the structural distortion caused by the heteroduplex [10]. RNase H is implicated in aiding this process [10]. RNase H recognizes and binds to the heteroduplex and cleaves its RNA component. Originally, it had been thought that antisense oligonucleotides produced their effect by blocking or sterically hindering their target mRNAs. It was soon recognized [10], however, that RNase H may play a prominent role in the mode of action of S-oligos [10].

In this study, using antisense phosphorothioate oligos designed against two different targets, we demonstrated that the efficacy of these antisense S-oligos is enhanced in *Leishmania* promastigotes and amastigotes overexpressing RNase H. We expressed the *T. brucei RNase H1* gene in *Leishmania*. This gene has all the conserved features for RNase H1 including an N-terminal RNA binding subdomain, conserved RNase H1 catalytic residues, and other invariant amino acid residues conserved at the C-terminus of the enzyme molecule [34]. The recombinant protein expressed from this gene has been shown recently to have RNase H activity by a solution assay using [α-^{32}P]RNA: DNA duplex as a substrate [34]. We also have shown in this study that *Leishmania* cells transfected with the *T. brucei RNase H1* gene had higher RNase H activity than the control cells transfected with the gene cloned in the reverse orientation (with respect to the *trans*-splicing signal). Thus, *T. brucei* RNase H1 appeared to be expressed as an active enzyme inside the recombinant *Leishmania* cells.

We used S-oligos because these molecules are known to mediate their action in an RNase H-dependent manner as opposed to other types of chemical modifications, e.g. morpholino antisense oligomers, which mediate their actions via RNase H-independent mechanisms [52]. The specificity of the antisense S-oligos used in this study was documented using appropriate control S-oligos that did not have any significant target ablation effects. We previously documented the specificity of ASM against *Leishmania* cell growth using additional control oligonucleotides [9].

The study described herein indicates a possible pivotal role for RNase H of *Leishmania* promastigotes and amastigotes in the antisense phosphorothioate ODN-mediated killing of these cells. Thus, conceptually, co-treatment of *Leishmania* with an activator of RNase H and an antisense oligonucleotide should accentuate the efficacy of the latter and provide an efficient chemotherapy against leishmaniasis.

Acknowledgments

We thank Prof. Robert C. Crouch of the National Institutes of Health and Dr. Andrew Campbell of Providence for the suggestions on RNase H assays, Dr. Minu Chaudhuri for *T. brucei* genomic DNA, and Mrs. Angelika K. Parl for technical assistance. This work was supported by the National Institutes of Health, USA, Grants 5-R01AI42327–03 and 2S06GM08037–24 to G.C.

Abbreviations

RNase H	ribonuclease H
S-oligo	phosphorothioate-modified oligodeoxyribonucleotide
LUAS	anti-luciferase antisense S-oligo
LUS	S-oligo complementary to LUAS
ASM	S-oligo antisense to *Leishmania* miniexon
SSM	S-oligo complementary to ASM
HIFBS	heat-inactivated fetal bovine serum

TbRH1	*Trypanosoma brucei RNase H1* gene
RACE	rapid amplification of cDNA ends
MBSA	maleylated bovine serum albumin
ODN	oligodeoxyribonucleotide
PCR	polymerase chain reaction
DMPC	dimyristoyl phosphatidylcholine

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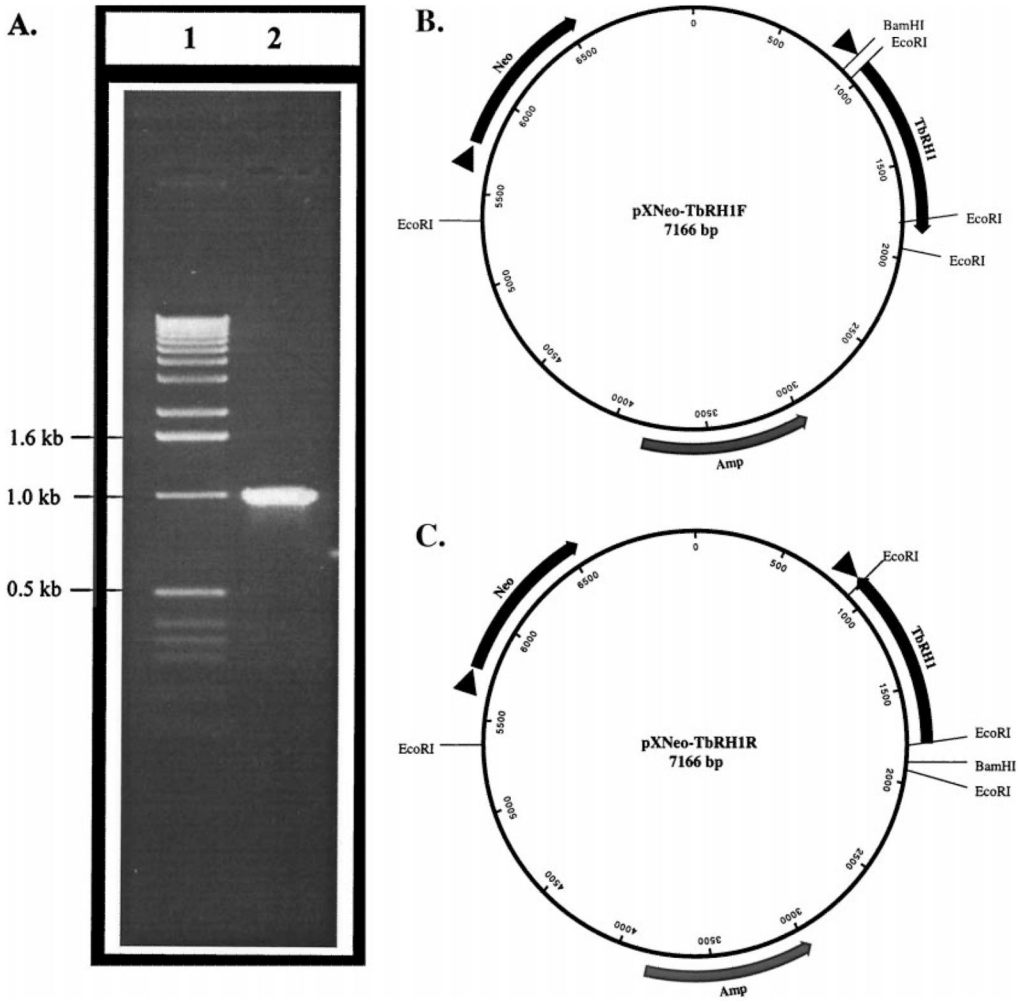

Fig. 1.

Amplification and cloning of *T. brucei RNase H1* in pX63-Neo. (A) Ethidium bromide-stained agarose gel showing the PCR amplification product of *T. brucei RNase H1* (lane 2); lane 1, 1-kb ladder molecular size marker (GIBCO-BRL). (B) Map of the pXNeo-TbRH1F plasmid. (C) Map of the pXNeo-TbRH1R plasmid. *Amp*, *Neo* and *TbRH1* indicate the *beta*-lactamase, neomycin phosphotransferase, and *T. brucei* ribonuclease H1 genes, respectively. Arrowheads before the genes indicate *Leishmania trans*-splicing signals. These *trans*-splicing signals are needed for the expression of the genes cloned behind them. In these plasmids, nucleotide sequences 0–821, 4752–5710, and 6619–7166 are originated from the *Leishmania DHFR* locus [39]. The *TbRH1* gene sequence is 822–1925. The rest of the sequences are from bacterial plasmids [39]. The orientations of the genes with respect to their promoter (*Amp* gene) or *trans*-splicing signal (*Neo* or *TbRH1* genes) are indicated as solid arrows.

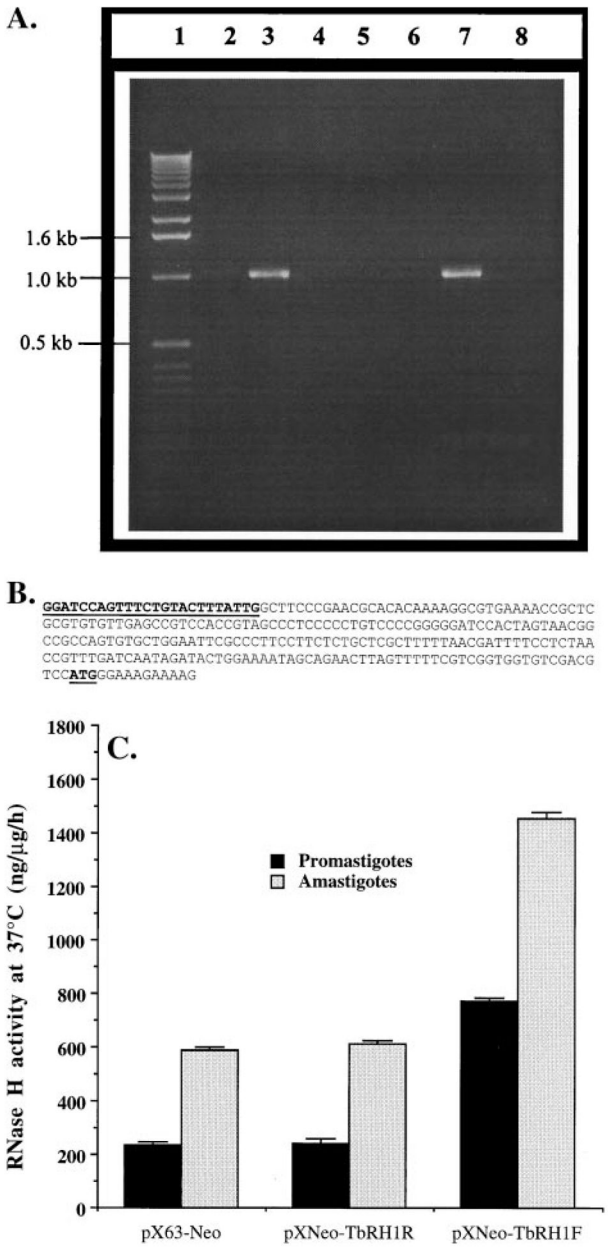

Fig. 2.

Expression of *T. brucei* RNase H1 in *L. amazonensis.* (A) Evidence for the presence of *T. brucei* RNase H1 DNA and mRNA in recombinant *L. amazonensis.* Lane 1, 1-kb ladder DNA marker (GIBCO); lanes 2 and 3, PCR products from plasmid DNA templates isolated from TbRH1^{-} (lane 2) and TbRH1^{+} (lane 3) cells; lanes 4–8, RT–PCR data. Lanes 4–6 represent RT–PCR products from RNA(−), RT(−), and AmpliTaq(−) reaction controls; lanes 7 and 8, RT–PCR products from RNA isolated from TbRH1^{+} and TbRH1^{-} cells, respectively. PCR amplifications were done with TbRHF and TbRHR primers. The identity of the amplified products was confirmed by nucleotide sequencing. The faint DNA band in lane 2 was a PCR artifact. (B) Nucleotide sequence (5′ to 3′) of the miniexon end (261 bp) of the 5′-RACE product with Mex2 and TbRHR. The Mex2 sequence and the RNase H1 mRNA translation start site (AUG) are boldfaced and underlined. (C) Overexpression of

RNase H activity in transgenic *Leishmania.* The plasmids that were used to transfect *L. amazonensis* cells are shown on the x-axis. RNase H activity in the cell extracts is expressed as nanograms of substrate degraded per microgram of cell extract protein per hour. Results are means ± SEM (N = 3). The difference in RNase H activity between amastigote and promastigote cell extracts was statistically significant ($P < 0.01$).

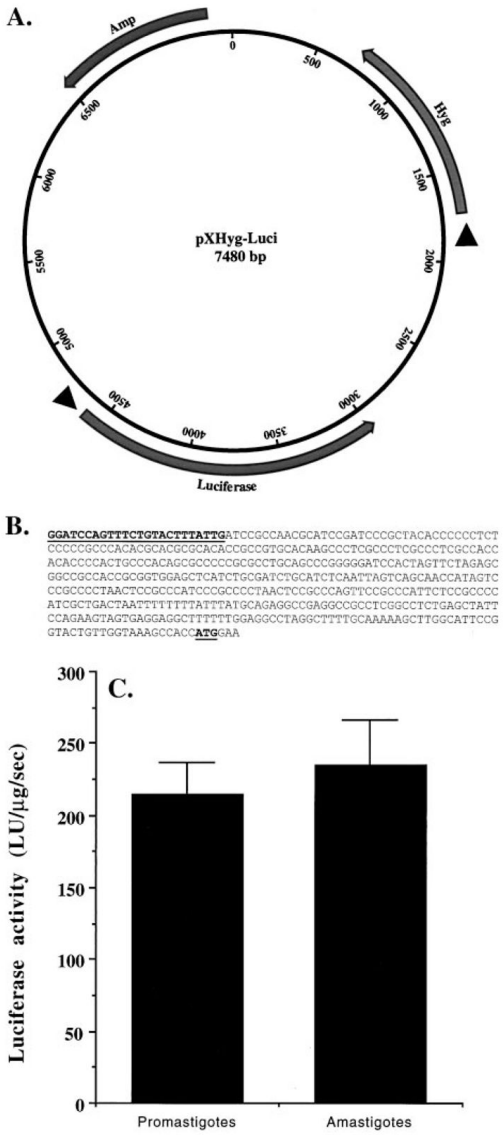

Fig. 3.

Stable expression of firefly luciferase in *L. amazonensis* promastigotes overexpressing *T. brucei RNase H1*. (A) Map of the plasmid pXHyg-Luci; *Amp*, *Hyg*, and *Luciferase* indicate the *beta*-lactamase, hygromycin phosphotransferase, and firefly luciferase genes. Arrowheads before the genes indicate *Leishmania trans*-splicing signals. These *trans*-splicing signals are needed for the expression of the genes cloned behind them. The orientations of the genes with respect to their promoter (*Amp* gene) or *trans*-splicing signal (*Hyg* or *Luciferase* genes) are indicated as solid arrows. (B) Nucleotide sequence (5′ to 3′) (453 bp) of the miniexon end of the 5′-RACE product from the mRNA of the recombinant cells using Mex2 and LUAS as primers. The Mex2 sequence and the luciferase mRNA translation start site (AUG) are boldfaced and underlined. (C) Expression of luciferase activity in the recombinant promastigotes and amastigotes. LU = light units. Results are means ± SEM (N = 6). The difference in luciferase activity between the amastigote and promastigote cell extracts was not statistically significant.

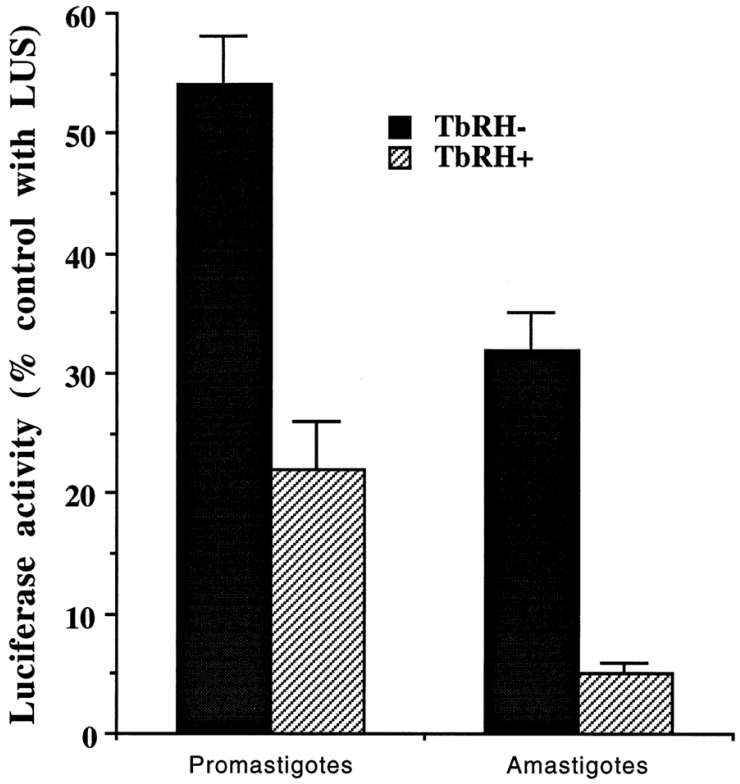

Fig. 4.

Inhibition of the expression of luciferase activity in RNase H overexpressing *L. amazonensis* promastigotes and amastigotes by anti-luciferase. Results are means ± SEM (N = 6). Luciferase activities in the controls were 5326 ± 159 and 3648 ± 221 light units/10^6 cells for promastigotes and amastigotes, respectively. The differences in luciferase activities between $TbRH1^+$ and $TbRH1^-$ cell extracts were statistically significant ($P < 0.05$).

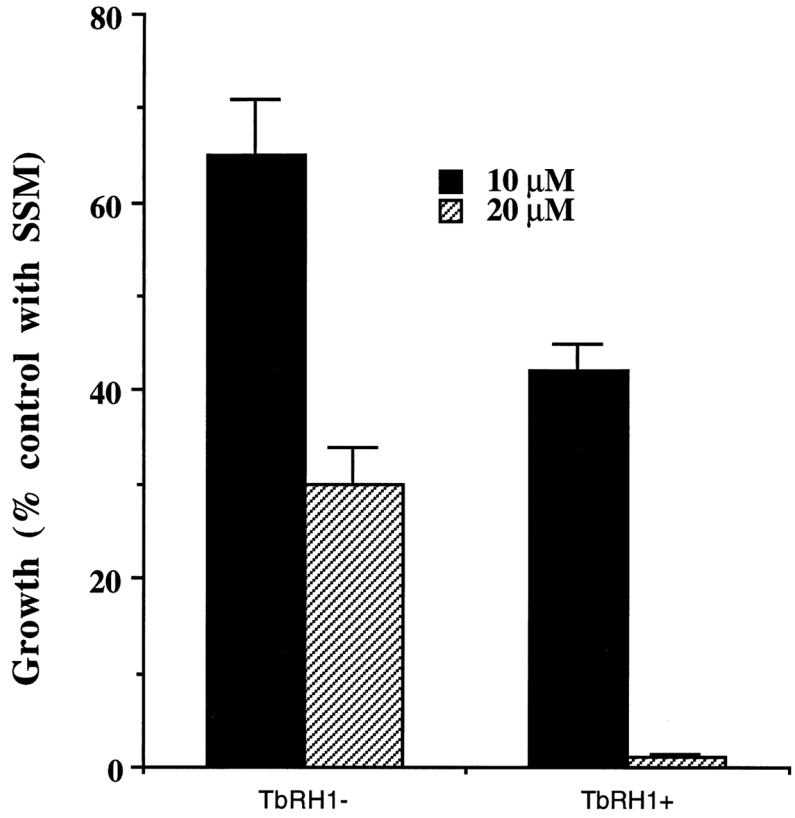

Fig. 5.

Inhibition of growth of RNase H-overexpressing axenic amastigotes of *L. amazonensis* by anti-miniexon antisense S-oligo. Results are means ± SEM (N = 4). TbRH1^{+} and TbRH1^{-} amastigotes incubated in parallel with 20 μM SSM had 2.3×10^{7} and 2.2×10^{7} cells/mL of culture medium, respectively. The numbers of amastigotes per milliliter of culture, when incubated without S-oligo or with 10 μM SSM, were very similar, and the differences were statistically insignificant. The differences of antisense activities of ASM between TbRH1^{+} and TbRH1^{-} cells were statistically significant ($P < 0.05$).

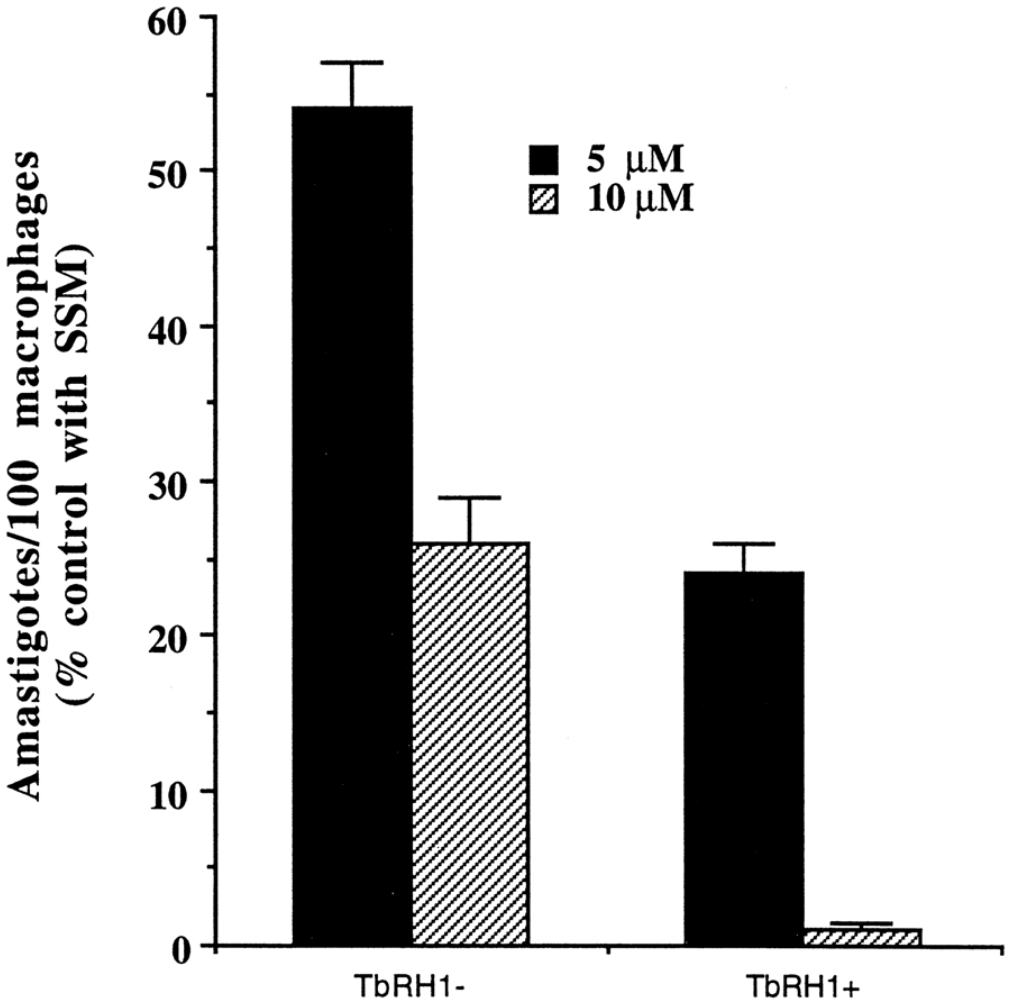

Fig. 6.

Killing of RNase H-overexpressing *L. amazonensis* amastigotes by anti-miniexon antisense S-oligo inside macrophage phagolysosomes. Results are means ± SEM (N = 4). Macrophages incubated in parallel with 10 μM SSM had 546 ± 32 and 539 ± 22 amastigotes/100 cells, respectively, for TbRH1^{+} and TbRH1^{-} cells. The numbers of amastigotes/100 macrophages incubated without S-oligo or with 5 μM SSM were very similar, and the differences were statistically insignificant. The differences of antisense activities of ASM between TbRH1^{+} and TbRH1^{-} cells were statistically significant ($P < 0.01$).