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Susceptibility of *Giardia lamblia* to Aminoglycoside Protein Synthesis Inhibitors: Correlation with rRNA Structure

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The very limited development of antiparasitic agents targeting protein synthesis stems in part from the belief that parasite and host ribosomes are sufficiently similar to preclude selective toxicity. However, recent studies have revealed that *Giardia lamblia* rRNA has an unusual size and sequence; consequently, this organism and its homogeneous rRNA provide a useful model for the development of protein synthesis inhibitors with antiparasitic activity. In this study, I determined the sequence and secondary structure of the 3′ end of the small-subunit RNA, the target for aminoglycoside inhibitory activity. The primary structure of these 140 nucleotides includes two blocks of sequence highly conserved among other organisms; the remaining sequence, although not conserved, can be folded into a secondary structure common to all rRNAs. The presence of U-1495 within one of the conserved blocks predicts hygromycin susceptibility. Also, a specific base pair (C-1409 · G-1491) implicated in paromomycin susceptibility is present; whereas all procaryotes have this base pair, it is absent in many eucaryotes (including mammals). Conversely, kanamycin and apramycin resistance can be predicted from substitution of A-1408 with G. A growth inhibition assay was used to test the susceptibility of *G. lamblia* to a variety of aminoglycosides. After 48 h, 8 of 11 aminoglycosides tested failed to inhibit growth at a concentration of 200 μg/ml. Paromomycin and hygromycin, however, inhibited growth of three strains tested by 50% at 50 to 60 μg/ml and by close to 90% at 120 μg/ml. These results correlate well with the sequence and secondary-structure analyses. Paromomycin may be clinically useful when the toxicity of standard antigiardial drugs is of concern.

Protein synthesis inhibitors represent a large, diverse, and clinically important group of antibiotics. Many of these agents have broad-spectrum activity, in some cases extending beyond bacteria to eucaryotic cells. With surprisingly few exceptions, protein synthesis has rarely been exploited as a target for treatment of parasitic infections. The significant exceptions include the use of tetracycline or clindamycin, in combination with conventional drugs, as antimalarial agents, use of spiramycin as an alternative therapy for toxoplasmosis, and treatment of amoebiasis with emetine (the toxicity of this drug has reduced its use in recent years) (for review, see reference 24). This limited repertoire of antiparasitic protein synthesis inhibitors to some extent reflects similarities in the ribosomal components of mammalian hosts and certain parasites, which reduce the potential for selective toxicity. However, recent studies involving rRNA sequence analysis have revealed that the protozoa represent a highly diverse group of organisms (1, 14, 17, 26, 28). In light of this finding, it is inappropriate to predict the antiparasitic activity of a drug on the basis of studies of an unrelated protozoan or yeast. Furthermore, selective toxicity appears feasible, given the extensive divergence between the ribosomal components of the host and many parasites.

Giardia lamblia is an anaerobic (mitochondria-lacking) protozoan that colonizes the upper small intestine of a wide variety of mammalian hosts. It is responsible for about 5% of acute and perhaps 20% of chronic cases of diarrheal disease (22). It is endemic in most developing countries but is also responsible for many localized outbreaks in the United States caused by contaminated water supplies. Three drugs are widely used in this country for treating giardiasis: metronidazole, quinacrine, and furazolidone (for reviews, see references 7 and 8). The mechanisms of action of these drugs are not well understood but may ultimately involve DNA damage. Side effects and potential toxicity (including carcinogenicity) associated with these agents have prompted us and others (4, 5) to search for new therapeutic approaches to this organism. When *G. lamblia* rRNA was characterized by gel electrophoresis, cloning of the corresponding gene, and partial sequence analysis, it was apparent that the protein synthesis machinery of this parasite was quite unusual and probably evolutionarily primitive (3, 9). These observations, along with the lack of mitochondria (which would complicate the interpretation of drug susceptibility data, since mitochondria represent a target for many antibiotics), suggest that *G. lamblia* may provide a parasite model for evaluating protein synthesis inhibitors. Once established, *G. lamblia* can be routinely cultured in the laboratory. Consequently, several laboratories have published in vitro drug susceptibility studies (4, 11–13), although relatively few protein synthesis inhibitors have been evaluated.

There is a growing body of knowledge regarding specific relationships of rRNA sequence or secondary structure to drug activity. This information is derived from sequence analysis of drug-resistant mutations (20, 27), from the mapping of methylation sites conferring drug resistance or susceptibility (2, 16), and from chemical footprinting of drug-binding sites on purified ribosomes (23). I have applied this information to sequence analysis of the 3′ end of *G. lamblia* 16S-like small-subunit (SS) RNA. This region plays a crucial role in protein synthesis (binding of mRNA and tRNA) and has been characterized as the site of action of several aminoglycoside antibiotics. The results of this sequence analysis were then correlated with experimental determination of drug susceptibility.

MATERIALS AND METHODS

Sequence analysis. Cloning and mapping of the rRNA gene from *G. lamblia* WB were previously described (9). The sequences of the *Bam*HI and *Rsa*I restriction fragments spanning the 3′ end of the SS RNA were determined by dideoxynucleotide sequencing of M13 subclones. SS RNA sequences (compiled by Dams et al. [6]) were manually aligned with reference to conserved secondary structures. Percent similarities were calculated for unambiguously aligned nucleotides only.

Drug susceptibility assay. *G. lamblia* WB, PO, and LT (25) were obtained from P. Smith and D. Keister, National Institutes of Health, Bethesda, Md. Trophozoites were cultured at 37°C in filled glass tubes containing Diamond TYI-S-33 supplemented with 10% fetal bovine serum, bile, and cysteine hydrochloride (19). Streptomycin (100 μg/ml), piperacillin (250 μg/ml), and amphotericin B (0.25 μg/ml) were added routinely to prevent microbial contamination; these drugs have no effect on *Giardia* growth (11). Unattached cells were discarded along with the spent medium. Adherent cells (in log phase) were detached by addition of cold medium and incubation on ice for 15 min. Cells were counted in a hemacytometer and diluted with medium (minus streptomycin) to 0.5×10^4 to 1×10^4 cells per ml. Diluted cells were used to fill 2-ml glass vials containing 0 to 40 μl of H_2O or drug (10 mg/ml); duplicate or triplicate vials were prepared for each drug dilution. Vials were incubated in an upright position at 37°C. At 48 h, the vials were chilled for 0.5 to 1.5 h in ice water and mixed well, and a small portion was removed for counting. Results are expressed as a percentage of control untreated cells. Doubling times for control cells were 7 to 8 h. Antibiotics were obtained as follows: gentamicin, hygromycin, kasugamycin, neomycin, kanamycin, ribostamycin, and tobramycin, Sigma Chemical Co., St. Louis, Mo.; paromomycin and butirosin, Warner-Lambert, Ann Arbor, Mich.; apramycin, Eli Lilly & Co., Indianapolis, Ind.; and sisomicin, Schering Corp., Bloomfield, N.J.

RESULTS AND DISCUSSION

Sequence analysis. The nucleotide sequence corresponding to the 3′ end of the SS RNA of *G. lamblia* WB (Afghanistan) is presented in Fig. 1A. This sequence is identical to the preliminary (one-strand) sequence reported by Boothroyd et al. (3) for the PO (Portland) strain. It was previously noted that the SS RNA 3′ end includes two long stretches of sequence universally conserved among cytoplasmic rRNAs (centered at positions 1400 and 1499 [*Escherichia coli* numbering]) as well as one stretch of highly variable sequence (centered at position 1450) (15). These sequences were also evident in alignments of the *G. lamblia* sequence with several other SS RNA sequences representing human, yeast, another protozoan, archaebacterial, and eubacterial RNAs. As previously noted by RNA gel electrophoresis (9), the *G. lamblia* sequence was considerably shorter than the corresponding sequences of other eucaryotes, somewhat shorter even than the eubacterial sequence, but comparable to those of several archaebacteria. Within this stretch of sequence, *G. lamblia* displayed the least degree of sequence conservation with the eubacteria (60%); with respect to several other protozoa, yeasts, archaebacteria, and mammals, the extent of conservation fell within the range 68 to 73%. The sequence most highly related to that of *G. lamblia* was the sequence of *Chlamydomonas reinhardtii* (78%). Comparisons of longer sequences and comparisons with additional protozoan representatives are required to more clearly define the taxonomy and evolution of *Giardia* spp.

The secondary structure of rRNA is more highly conserved than its sequence (15). The *G. lamblia* SS RNA 3′ end was found to be readily folded into the two-hairpin structure common to all SS RNAs (Fig. 1B). Within the region studied here, the unusually small size of *Giardia* rRNA can be accounted for by a short 1409-to-1491 hairpin. The hairpin is, however, rich in G · C base pairs and therefore very stable. Nearly all of the nucleotide changes within this highly variable region represent A or U changes to C or G.

The 3′ terminus of *G. lamblia* SS RNA has not been precisely determined. The bars in Fig. 1 represent the ranges of lengths found in other cytoplasmic SS RNAs, as compiled by Dams et al. (6). Surprisingly, the 3′-terminal AUUA/G found, with little variation, in all other eucaryotes was absent. Rather, there was a close match (CCUCGC) with the bacterial sequence that base pairs with the translational initiation site of bacterial mRNA (Shine-Dalgarno sequence; CCUCC, underlined in Fig. 1A). Determination of the sequences of representative mRNA initiation sites, along with precise sequence determination of the SS RNA 3′ end, are required to examine the role of complementarity in initiation site selection in *G. lamblia*.

From analysis of the sequence and secondary-structure model shown in Fig. 1, certain predictions (based on the assumptions listed below) can be made regarding antibiotic susceptibility. In all cases, these predictions involve members of the aminoglycoside group. (1) The first antibiotic for which an rRNA target was implicated was kasugamycin (16); rRNA from resistant *E. coli* mutants lacked the two methyl groups normally added to A-1518 and A-1519. *G. lamblia* rRNA, like rRNAs of all organisms, includes the AA dinucleotide, although we have no indication of methylation status. (2) Hygromycin-resistant mutants of *Tetrahymena* in which U-1495 is altered have been isolated (27). The adjacent G-1494 is protected by this drug in chemical footprinting assays (23). These nucleotides lie within the highly conserved 1492-to-1506 sequence, also present in *G. lamblia*; therefore, susceptibility can be predicted. (3) Paromomycin-resistant rRNA mutations have been isolated in tetrahymena (27) and yeast mitochondria (20). These mutations are at two sites separated with respect to sequence but at the same location with respect to secondary structure (Fig. 1B). Apparently, the 1409-to-1491 base pair, and not a specific sequence, confers susceptibility to paromomycin. Both cytoplasmic and mitochondrial rRNAs from most higher eucaryotes (including mammals) and some protozoa lack this base pair (6, 15) and are naturally resistant. Paromomycin susceptibility can be predicted for *G. lamblia* on the basis of the presence of this base pair. (4) Upon methylation of A-1408, bacteria lose susceptibility to kanamycin and apramycin (2); *G. lamblia*, like other eucaryotes, has G in this position. On the assumption that this base change has the same effect as methylation, *G. lamblia* should be resistant to these two agents. Similarly, methylation of G-1405 confers resistance to gentamicin, an aminoglycoside whose activity is considered limited to bacteria. All organisms retain this G, however, which implies that there are additional, uncharacterized requirements for gentamicin activity.

Drug susceptibilities. Fulfillment of the predictions made above is based on several assumptions: (i) the *G. lamblia* membrane does not pose a selective barrier to aminoglycoside penetration; (ii) the drug is not efficiently inactivated within the cell; and (iii) requirements for drug action, other

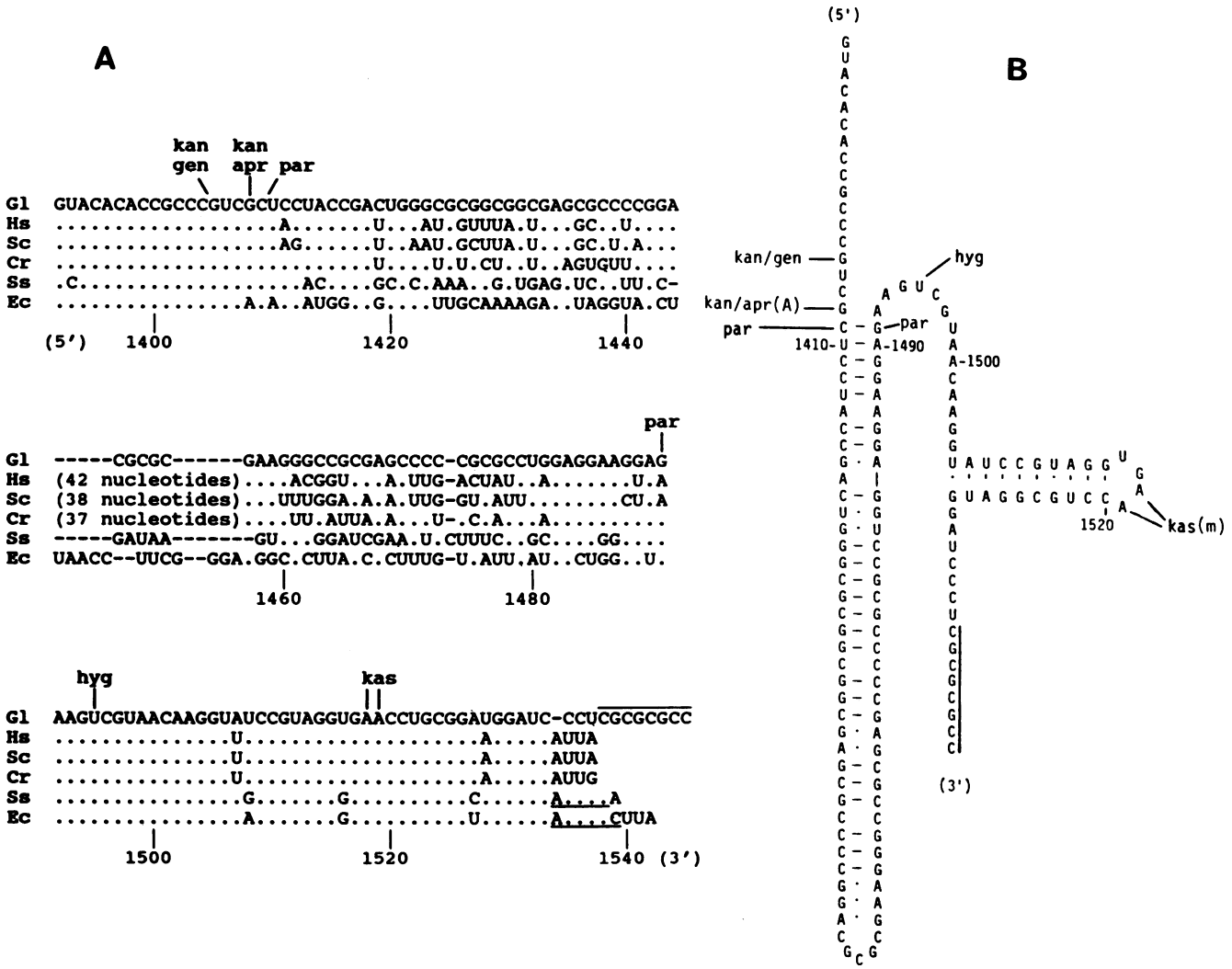

FIG. 1. Sequence (A) and secondary structure (B) of the 3′ end of *G. lamblia* (Gl) SS RNA. The sequence (represented as RNA) is aligned with other SS RNA sequences (Hs, human; Sc, *Saccharomyces cerevisiae*; Cr, *Chlamydomonas reinhardtii*; Ss, *Sulfolobus solfataricus*; Ec, *Escherichia coli*). Symbols: · · ·, identity to the *Giardia* sequence; – – –, lack of a corresponding nucleotide. Portions of the human, *S. cerevisiae*, and *C. reinhardtii* hairpin sequences are not shown. The *Giardia* RNA 3′ terminus has not been determined; the bar represents the ranges of lengths observed in cytoplasmic SS RNA (see reference 6 for compilation). Shine-Dalgarno sequences in *S. solfataricus* and *E. coli* are underlined. Numbering is that used for *E. coli* (15). Nucleotides whose alteration confers aminoglycoside resistance are indicated (kan, kanamycin; gen, gentamicin; apr, apramycin; par, paromomycin; hyg, hygromycin; kas, kasugamycin) (see text for references).

than those cited above (e.g., ribosomal proteins and other rRNA sequences or structures) are not lacking in *G. lamblia* cells.

Several methods have been used to test *Giardia* drug susceptibility in vitro: loss of mobility and morphology (18), colony formation in agarose (12), [^{3}H]thymidine uptake (4), and growth in liquid culture followed by cell counting in situ (11). I have used 48-h liquid cultures, followed by detachment and cell counting with a hemacytometer, since this is a simple yet highly quantitative method. Most of the results reported here are for the widely used *G. lamblia* WB (originating in Afghanistan). WB has been in culture for several years (25); the results should be extrapolated with caution to other isolates.

The aminoglycosides are a large and diverse group of antibiotics, a number of which are known to target the SS RNA 3′ end (10, 23). One member of this group, streptomycin, is routinely used during isolation and culture of *G. lamblia*, and three others have previously been tested for antigiardial activity by one of the assays mentioned above. Amikacin (11) and gentamicin (12) have little or no activity in the 300-μg/ml range. In this laboratory, we have used amikacin in routine cultures at 125 μg/ml without effect (21). With use of the liquid culture assay described in Materials and Methods, there was a similar lack of inhibition by gentamicin at 200 μg/ml (Fig. 2). In contrast to these three aminoglycosides, paromomycin is clearly active; 50% inhibitory doses (ID_{50}s) or MICs of 10 to 100 μg/ml have been reported for most isolates (4, 12, 13). The range of susceptibilities reported may be due to differences in the assays used or to strain-to-strain variation (the latter factor was particularly apparent in the study of Gordts et al. [13]). With the assay used in this study, I determined the effects of paromomycin on *G. lamblia* WB. At 200 μg/ml, growth was inhibited to 7% of control levels (Fig. 2). From dose-response curves, an ID_{50} of 60 μg/ml was determined (data

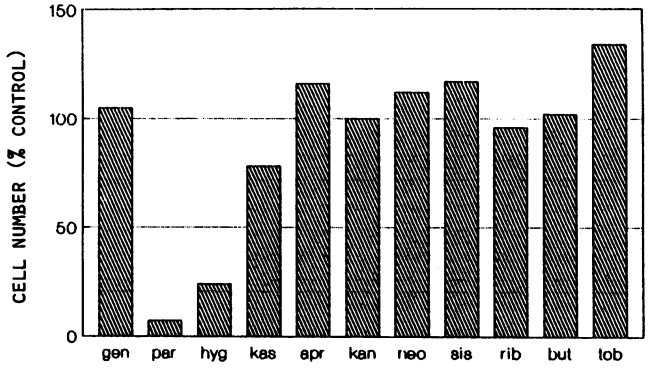

FIG. 2. Susceptibility of *G. lamblia* WB to various aminoglycosides at 200 μg/ml. Cell number after 48 h of culture in the presence of drug is expressed as percentage of control value. Results are averages of two determinations and are representative of three similar experiments. Antibiotics tested: gen, gentamicin; par, paromomycin; hyg, hygromycin; kas, kasugamycin; apr, apramycin; kan, kanamycin; neo, neomycin; sis, sisomicin; rib, ribostamycin; but, butirosin; tob, tobramycin.

not shown). Two unrelated *G. lamblia* strains, PO (originating in Portland, Oreg.) and LT (Puerto Rico), were also tested for paromomycin susceptibility. Similar ID_{50}s of 50 to 60 μg/ml were obtained.

The relative susceptibility of *G. lamblia* WB to paromomycin fulfills the prediction made above on the basis of the presence of the C-1409/G-1491 base pair. It was clearly of interest to examine the activity of the four additional aminoglycosides, listed above, for which an rRNA target has been defined. *G. lamblia* WB was clearly susceptible to hygromycin (Fig. 2) (ID_{50}, 50 μg/ml; data not shown). Indeed, the spectrum of hygromycin activity may extend to all organisms, since its target nucleotides are universally conserved. Strain WB was partially susceptible to kasugamycin at 200 μg/ml (Fig. 2). Although the AA dinucleotide target is present in *G. lamblia* (and all sequenced SS RNAs), its methylation status is unknown; most eucaryotes are resistant to this agent (10). *G. lamblia* WB was not susceptible to apramycin and kanamycin at 200 μg/ml (Fig. 2), fulfilling the predictions made above.

Finally, it was of interest to examine additional aminoglycosides for possible activity, and hence potential clinical utility, against *G. lamblia*. Neomycin, sisomicin, ribostamycin, butirosin, and tobramycin all failed to display activity against strain WB at 200 μg/ml (Fig. 2).

In conclusion, I have tested the feasibility of predicting drug susceptibility of a parasitic protozoan on the basis of rRNA sequence and structure analysis. The susceptibility of *G. lamblia* to hygromycin is not of clinical utility because of the lack of selective toxicity displayed by this aminoglycoside. On the other hand, paromomycin susceptibility has been known for some time, although the drug is rarely used to treat giardiasis (7, 8). One reason for this may be the relatively high ID_{50} that paromomycin displays in vitro, roughly 100-fold higher than that observed for metronidazole. However, paromomycin, like all aminoglycosides, is poorly absorbed from the gut, whereas the standard antigiardial agents are readily absorbed. Therefore, the concentration of paromomycin should remain high where it is needed. In support of this relationship, in vivo studies conducted by Boreham et al. (5) with neonatal mice revealed paromomycin to be about four- to fivefold more active than metronidazole and quinacrine. This minimal absorption, reducing the risk of systemic toxicity, has also led to the recommended use of paromomycin in pregnancy (7). The molecular basis for the selective toxicity of paromomycin against *G. lamblia* that is provided here engenders further confidence in the use of this agent. Furthermore, these results suggest that rRNA drug target analysis is a viable approach to predicting antiparasitic drug activity.

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LITERATURE CITED

1. **Baroin, A., R. Perasso, L.-H. Qu, G. Brugerolle, J.-P. Bachellerie, and A. Adoutte.** 1988. Partial phylogeny of the unicellular eukaryotes based on rapid sequencing of a portion of 28S ribosomal RNA. Proc. Natl. Acad. Sci. USA **85**:3474–3478.
2. **Beauclerk, A. A. D., and E. Cundliffe.** 1987. Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. J. Mol. Biol. **193**:661–671.
3. **Boothroyd, J. C., A. Wang, D. A. Campbell, and C. C. Wang.** 1987. An unusually compact ribosomal DNA repeat in the protozoan *Giardia lamblia*. Nucleic Acids Res. **15**:4065–4084.
4. **Boreham, P. F. L., R. E. Phillips, and R. W. Shepherd.** 1985. A comparison of the *in vitro* activity of some 5-nitroimidazoles and other compounds against *Giardia intestinalis*. J. Antimicrob. Chemother. **16**:589–595.
5. **Boreham, P. F. L., R. E. Phillips, and R. W. Shepherd.** 1986. The activity of drugs against *Giardia intestinalis* in neonatal mice. J. Antimicrob. Chemother. **18**:393–398.
6. **Dams, E., L. Hendriks, Y. Van de Peer, J.-M. Neefs, G. Smits, I. Vandenbempt, and R. De Wachter.** 1988. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. **16**(Suppl.):r87–r173.
7. **Davidson, R. A.** 1984. Issues in clinical parasitology: the treatment of giardiasis. Am. J. Gastroenterol. **79**:256–261.
8. **Dupont, H. L., and P. S. Sullivan.** 1986. Giardiasis: the clinical spectrum, diagnosis, and therapy. Pediatr. Infect. Dis. **5**:S131–S138.
9. **Edlind, T. D., and P. R. Chakraborty.** 1987. Unusual ribosomal RNA of the intestinal parasite *Giardia lamblia*. Nucleic Acids Res. **15**:7889–7901.
10. **Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring.** 1981. The molecular basis of antibiotic action, 2nd ed. John Wiley & Sons, Inc., New York.
11. **Gault, M. J., D. S. Reiner, and F. D. Gillin.** 1985. Tolerance of axenically cultured *Entamoeba histolytica* and *Giardia lamblia* to a variety of antimicrobial agents. Trans. R. Soc. Trop. Med. Hyg. **79**:60–62.
12. **Gillin, F. D., and L. S. Diamond.** 1981. Inhibition of clonal growth of *Giardia lamblia* and *Entamoeba histolytica* by metronidazole, quinacrine, and other antimicrobial agents. J. Antimicrob. Chemother. **8**:305–316.
13. **Gordts, B., J. De Jonckheere, W. Kasprzak, A. C. Majewska, and J.-P. Butzler.** 1987. In vitro activity of antiprotozoal drugs against *Giardia intestinalis* of human origin. Antimicrob. Agents Chemother. **31**:670–673.
14. **Gunderson, J. H., T. F. McCutchan, and M. L. Sogin.** 1986. Sequence of the small subunit ribosomal RNA gene expressed in the bloodstream stages of *Plasmodium berghei*: evolutionary implications. J. Protozool. **33**:525–529.
15. **Gutell, R. R., B. Weiser, C. R. Woese, and H. F. Noller.** 1985. Comparative anatomy of 16S-like ribosomal RNA. Prog. Nucleic Acid Res. Mol. Biol. **32**:155–216.
16. **Helser, T. L., J. E. Davies, and J. E. Dahlberg.** 1971. Change in methylation of 16S ribosomal RNA associated with mutation to kasugamycin resistance in *Escherichia coli*. Nature (London) New Biol. **233**:12–14.
17. **Johnson, A. M., P. J. Murray, S. Illana, and P. J. Baverstock.** 1987. Rapid nucleotide sequence analysis of the small subunit ribosomal RNA of *Toxoplasma gondii*: evolutionary implica-

tions for the Apicomplexa. Mol. Biochem. Parasitol. **25**:239–246.
18. **Jokipii, L., and A. M. M. Jokipii.** 1980. In vitro susceptibility of *Giardia lamblia* trophozoites to metronidazole and tinidazole. J. Infect. Dis. **141**:317–325.
19. **Keister, D. B.** 1983. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans. R. Soc. Trop. Med. Hyg. **77**:487–488.
20. **Li, M., A. Tzagoloff, K. Underbrink-Lyon, and N. C. Martin.** 1982. Identification of the paromomycin-resistance mutation in the 15S rRNA gene of yeast mitochondria. J. Biol. Chem. **257**:5921–5928.
21. **Lindley, T. A., P. R. Chakraborty, and T. D. Edlind.** 1988. Heat shock and stress response in *Giardia lamblia*. Mol. Biochem. Parasitol. **28**:135–144.
22. **Mata, L.** 1986. *Cryptosporidium* and other protozoa in diarrheal disease in less developed countries. Pediatr. Infect. Dis. **5**: S117–S130.
23. **Moazed, D., and H. F. Noller.** 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature (London) **327**:389–394.
24. **Pratt, W. B., and R. K. Fekety.** 1986. The antimicrobial drugs. Oxford University Press, New York.
25. **Smith, P. D., F. D. Gillin, N. A. Kaushal, and T. E. Nash.** 1982. Antigenic analysis of *Giardia lamblia* from Afghanistan, Puerto Rico, Ecuador, and Oregon. Infect. Immun. **36**:714–719.
26. **Sogin, M. L., H. J. Elwood, and J. H. Gunderson.** 1986. Evolutionary diversity of eukaryotic small-subunit rRNA genes. Proc. Natl. Acad. Sci. USA **83**:1383–1387.
27. **Spangler, E. A., and E. H. Blackburn.** 1985. The nucleotide sequence of the 17S ribosomal RNA gene of *Tetrahymena thermophila* and the identification of point mutations resulting in resistance to the antibiotics paromomycin and hygromycin. J. Biol. Chem. **260**:6334–6340.
28. **Vossbrinck, C. R., J. V. Maddox, S. Friedman, B. A. Debrunner-Vossbrinck, and C. R. Woese.** 1987. Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. Nature (London) **326**:411–414.