ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1996, p. 1301–1303 Vol. 40, No. 5
0066-4804/96/$04.00+0

50S Ribosomal Subunit Synthesis and Translation Are Equivalent Targets for Erythromycin Inhibition in *Staphylococcus aureus*

W. SCOTT CHAMPNEY* AND ROBIN BURDINE

Department of Biochemistry, J. H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614

Received 7 December 1995/Returned for modification 16 January 1996/Accepted 12 February 1996

Macrolide antibiotics like erythromycin can prevent the formation of the 50S ribosomal subunit in growing bacterial cells, in addition to their inhibitory effect on translation. The significance of this novel finding has been further investigated. The 50% inhibitory doses of erythromycin for the inhibition of translation and 50S subunit assembly in *Staphylococcus aureus* cells were measured and were found to be identical. Together they account quantitatively for the observed effects of erythromycin on cell growth rates. There is also a direct relationship between the loss of rRNA from the 50S subunit and its accumulation as oligoribonucleotides in cells. The importance of this second site for erythromycin inhibition of bacterial cell growth is discussed.

Erythromycin and related 14-membered macrolide antibiotics are important antimicrobial agents in the treatment of infectious diseases, particularly those caused by gram-positive microorganisms (8). These drugs prevent bacterial protein biosynthesis by binding to the 50S ribosomal subunit and interfering with the elongation of nascent polypeptide chains (3, 10). The precise mechanism for translational inhibition by erythromycin is not yet completely understood (4, 5). In addition to this well-investigated activity of the macrolides, we have recently shown that they interfere with a second cellular process, the formation of the 50S ribosomal subunit. Both in *Escherichia coli* cells (2) and in *Bacillus subtilis* and *Staphylococcus aureus* cells (1a), a specific effect on 50S subunit assembly was observed. The formation of the 30S particle was not affected, and the breakdown of preexisting subunits was not stimulated. We have now investigated the significance of this second activity of the macrolides by measuring the relative effects of erythromycin on translation and 50S particle formation. Our results demonstrate that these two processes are equivalent targets for the antibiotic, and together, they account for the observed growth inhibition caused by erythromycin.

Measurements of cell growth, subunit assembly, and translation rates. To measure subunit assembly, *S. aureus* RN1786 was grown and labeled with [^{3}H]uridine in the presence and absence of erythromycin as described previously (1a), except that cells were cultured in tryptone broth (TB) (7) instead of tryptic soy broth. At each concentration of erythromycin used, the cells were grown for two doublings to the same final density that they had before being collected. Growth rates were measured by following the increase in cell density in a Klett-Summerson colorimeter. The cell lysis buffer and sucrose gradient buffers were modified to contain only 0.2 mM $MgCl_2$, and the centrifugation time was increased to 2.5 h. The A_{260} and the amount of [^{3}H]uridine radioactivity in each gradient fraction were measured as described previously (1a).

To measure the rates of protein synthesis, cells were grown in TB to the same cell density with or without erythromycin, washed with A salts (7) containing the same concentration of antibiotic, and resuspended in 1 ml of A salts containing 0.2% glucose and erythromycin at the concentration used during cell growth. Cells were incubated at 37°C in the presence of [^{3}H] leucine (1 μCi/ml), and 0.25-ml samples were collected at 2.5-min intervals. The rates of [^{3}H]leucine incorporation into 10% trichloroacetic acid-precipitable material were measured by liquid scintillation counting.

Erythromycin inhibition of growth and protein synthesis. *S. aureus* cells were grown in the presence of different concentrations of erythromycin of between 0.1 and 1.0 μg/ml, and the inhibition of growth rate and protein synthesis rate relative to those for uninhibited controls were measured. Figure 1 shows the decline in cell growth rate as a function of erythromycin concentration. The 50% inhibitory dose (ID_{50}) for 50% inhibition of growth was 0.17 μg of erythromycin per ml. [^{3}H] leucine incorporation into cellular proteins in the presence and absence of erythromycin was also measured. The effect of erythromycin on translation rates is also shown in Fig. 1. The ID_{50} for inhibition of translation was found to be 0.38 μg/ml,

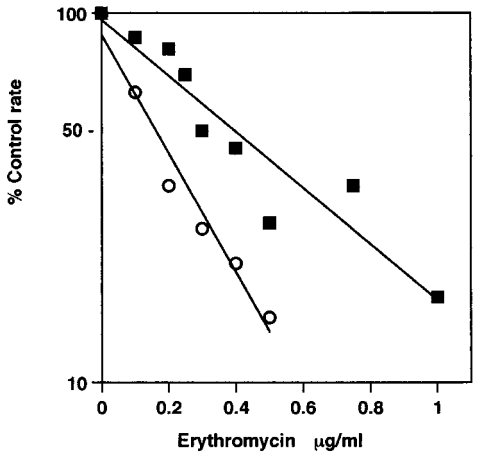

FIG. 1. Decline in the rate of cell growth and protein synthesis with increasing erythromycin concentration. The percent inhibition of the control growth rate (○) and the control protein synthesis rate (■) are indicated. The doubling time for control cells was 1.6 ± 0.15 h (n = 4), with an average error of ±10%. The rate of [^{3}H]leucine incorporation for control cells was 3,200 ± 164 cpm/10 min (n = 3), with an average error of ±5%.

* Corresponding author. Phone: (615) 929-6211. Fax: (615) 975-8235.

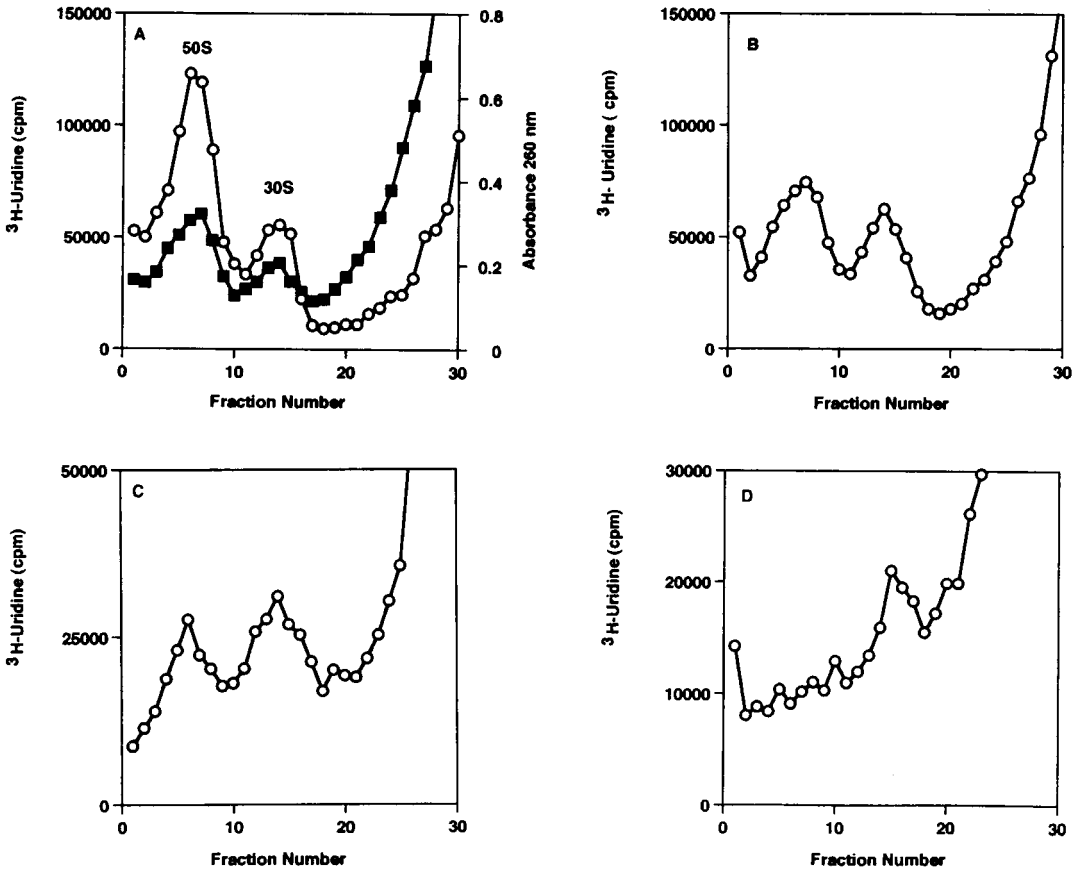

FIG. 2. Sucrose gradient profiles of cell lysates from control and erythromycin-treated cells. The A_{260} (■) and counts per minute of [^{3}H]uridine (○) were measured for each gradient fraction. (A) Gradient profile for untreated cells. (B) Gradient profile for cells grown in 0.2 μg of erythromycin per ml. (C) Gradient profile for cells grown in 0.3 μg of erythromycin per ml. (D) Gradient profile for cells grown in 1.0 μg of erythromycin per ml. For clarity, only the counts per minute of [^{3}H]uridine are shown for the gradients in panels B to D.

which is twice the concentration needed to obtain an equivalent inhibition of growth.

Erythromycin inhibition of 50S subunit formation. The biosynthesis of the 50S ribosomal subunit in cells growing in erythromycin was examined as described previously (1a). Cells were labeled with [^{3}H]uridine, and the distribution of radioactivity in the 30S and 50S subunits was measured. Figure 2 shows representative sucrose gradient profiles of ribosomal subunits from control and erythromycin-inhibited cells. The control gradient profile shown in Fig. 2A reveals a 2-to-1 ratio of [^{3}H]uridine radioactivity in the 50S and 30S subunit peaks and a specific activity ratio (counts per minute of ^{3}H/A_{260}) of 1.0, as observed previously (1a). Growth in increasing concentrations of erythromycin resulted in a sharp decline in the amount of [^{3}H]uridine in RNA sedimenting in the 50S subunit region of the sucrose gradient, as shown in Fig. 2B to D. The gradient profile for cells grown at 0.4 μg/ml was shown previously (1a). At 1.0 μg of erythromycin per ml, no 50S subunit peak was detected, although an apparent 30S subunit peak was found (Fig. 2D). The percentage of the total radioactivity was calculated for the 50S, 30S, and top regions of the gradient (fractions 21 to 30). The reduction in the counts per minute in the 50S and 30S subunits as a function of erythromycin concentration is shown in Fig. 3. The decline in the 50S subunit occurred with a slope equivalent to the observed decline in the translation rate, with an ID_{50} of 0.36 μg/ml. The reduction in 30S particle formation was much diminished, with a slope one-third

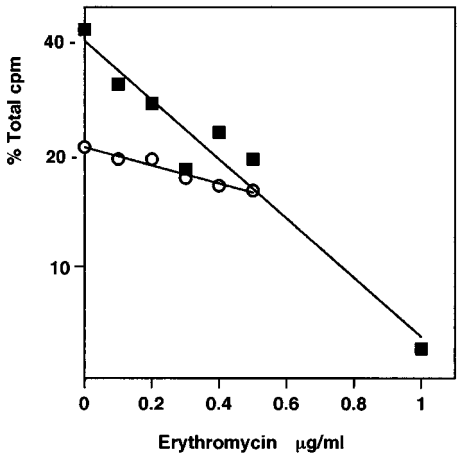

FIG. 3. Decline the amount of [^{3}H]uridine radioactivity in 50S and 30S subunits with increasing erythromycin concentration. The fraction of total radioactivity in the 50S (fractions 4 to 8) (■) and 30S (fractions 12 to 16) (○) regions of each sucrose gradient was calculated for cells grown at each concentration of erythromycin. For control cells, the 50S region contained 44% ± 1% of the total counts per minute, and the 30S region contained 21% ± 1% of the total. The average error in duplicate experiments was ±2%.

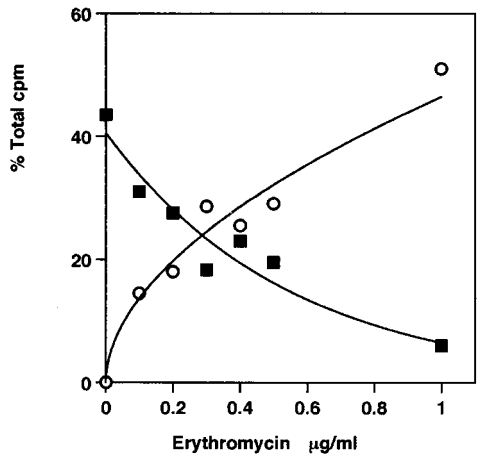

FIG. 4. Apparent precursor-product relationship between the decline of the amount of [^{3}H]uridine radioactivity in the 50S subunit and the increase in the amount of [^{3}H]uridine radioactivity in the top gradient fractions. The decline in the fraction of total counts per minute in the 50S region (■) and the increase in the counts per minute in the top gradient fractions (fractions 21 to 30) (○) are displayed as a function of increasing erythromycin concentration. The average error in duplicate experiments was ±3%.

of the value for the 50S subunit and an ID_{50} of 1.2 μg/ml. Because of the extensive breakdown of 50S subunits which extended into the 30S region of the gradient, no accurate measure of the counts per minute of the 30S subunit could be made at greater than 0.5 μg of erythromycin per ml (Fig. 2D). A summation of the measured ID_{50}s by the relationship $1/ID_{50\ (growth)} = 1/ID_{50\ (translation)} + 1/ID_{50\ (assembly)}$ accounts for the total effect of erythromycin on cell growth. It is apparent that erythromycin has an equivalent inhibitory effect on translation and 50S subunit assembly in this organism. The small reduction in 30S particle formation may be the consequence of erythromycin inhibition of the translation of mRNAs for ribosomal proteins, which would be an indirect effect of the antibiotic, affecting the synthesis of both ribosomal subunits (6, 9).

rRNA breakdown. Inspection of the distribution of radioactivity in the sucrose gradient profiles revealed an increase in the amount of acid-precipitable radioactivity in the top fractions from drug-treated cells in excess of the amount in the control. The increase seen was proportional to the decline in the 50S subunit region. An apparent precursor-product relationship was found when the fraction of the total radioactivity in the two regions was compared (Fig. 4). This result is presumptive evidence for the model that we have presented previously, suggesting that erythromycin interferes with an early step in 50S formation. Presumably, cellular RNases break down the free 23S and 5S rRNAs into oligoribonucleotides which accumulate in the top gradient fractions. Analysis of these fractions by polyacrylamide gel electrophoresis revealed several discrete bands of RNA unique to the drug-treated cells (1).

These results verify the importance of 50S subunit assembly inhibition as a second target for macrolide antibiotic inhibition in cells. Inhibition of translation and 50S subunit assembly account for the total effect of the drug on the ribosome. These results may help to explain the observed requirement for a greater concentration of erythromycin for inhibition of cell-free protein synthesis relative to that for cell growth inhibition (3). These data suggest that other macrolides which affect 50S subunit formation will have a significant inhibitory effect on cells on the basis of our previous findings of equivalent assembly inhibition at similar drug concentrations (1a, 2). This seems to be true for azithromycin, since we have observed a twofold difference in ID_{50} for growth inhibition compared with the ID_{50} for inhibition of translation by this macrolide (1).

The presence of this second target for erythromycin explains why the growth of erythromycin-resistant strains of *E. coli* (2) and *B. subtilis* (1a) can still be inhibited by elevated concentrations of the antibiotic. Presumably, the features of the assembly inhibition site and the translational inhibition site are not identical. Future studies on new macrolides should focus on this additional feature of drug function in the design and testing of new compounds against antibiotic-resistant cells.

REFERENCES

1. **Champney, W. S.** Unpublished data.
1a. **Champney, W. S., and R. Burdine.** 1995. Macrolide antibiotics inhibit 50S ribosomal subunit assembly in *Bacillus subtilis* and *Staphylococcus aureus*. Antimicrobial Agents Chemother. **39:**2141–2144.
2. **Chittum, H. S., and W. S. Champney.** 1995. Erythromycin inhibits the assembly of the large ribosomal subunit in growing *Escherichia coli* cells. Curr. Microbiol. **30:**273–279.
3. **Corcoran, J. W.** 1984. Mode of action and resistance mechanisms of macrolides, p. 231–259. *In* S. Omura (ed.), Macrolide antibiotics. Academic Press, Inc., Orlando, Fla.
4. **Cundliffe, E.** 1987. On the nature of antibiotic binding sites in ribosomes. Biochimie **69:**863–869.
5. **Cundliffe, E.** 1990. Recognition sites for antibiotics within rRNA, p. 479–490. *In* W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner (ed.), The ribosome: structure, function and evolution. American Society for Microbiology, Washington, D.C.
6. **Dodd, J., J. M. Kolb, and M. Nomura.** 1991. Lack of complete cooperativity of ribosomal assembly in vitro and its possible relevance to in vivo assembly and the regulation of ribosomal gene expression. Biochimie **73:**757–767.
7. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Nakayama, I.** 1984. Macrolides in clinical practice, p. 261–300. *In* S. Omura (ed.), Macrolide antibiotics. Academic Press, Inc., Orlando, Fla.
9. **Osawa, S., E. Otaka, T. Itoh, and T. Fukui.** 1969. Biosynthesis of 50s ribosomal subunit in *Escherichia coli*. J. Mol. Biol. **40:**321–351.
10. **Vazquez, D.** 1979. Inhibitors of protein biosynthesis. Springer-Verlag, Berlin.