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In Vitro Assay To Demonstrate High-Level Erythromycin Resistance of a Clinical Isolate of *Treponema pallidum*

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We have previously demonstrated that cells of *Treponema pallidum* freshly extracted from infected rabbit testes can be intrinsically radiolabeled with [^{35}S]methionine to very high specific activities. In this study we used the inhibition of [^{35}S]methionine incorporation into trichloroacetic acid-precipitable protein in vitro as an assay to test the susceptibilities of three different pathogenic treponemal strains to various antibiotics. In general, the results correlated very well with the known efficacies of these antibiotics in treating human patients with syphilis. One of the strains tested, however, a clinical isolate of *T. pallidum* designated street strain 14, was found to exhibit high-level resistance to erythromycin and a closely related macrolide, roxithromycin (RU 965). Street strain 14 was originally isolated from a human patient with active secondary syphilis who failed to respond to erythromycin therapy. Thus, our results indicate that an erythromycin-resistant strain of *T. pallidum* can be responsible for erythromycin treatment failure. In addition, street strain 14 treponemes were found to be generally less susceptible by this assay to a variety of antibiotics than were treponemes of the *T. pallidum* Nichols strain. These findings suggest that the outer envelope of street strain 14 treponemes may be generally less permeable to antibiotics than is that of Nichols strain treponemes.

Current understanding of *Treponema pallidum*, the etiologic agent of syphilis, is limited due to the inability to culture this spirochete in vitro for sustained periods of time (7, 15). Therapy for syphilis is based largely on empirical clinical trials, in vivo experiments in which the rate of clearance of treponemes from infected rabbit tissues is examined, and in vitro observations of the effect of antibiotics on the motility of treponemes extracted from infected rabbit testes (11–13, 26). The in vitro observation studies are particularly difficult to perform and to interpret, since problems have been encountered in obtaining adequate numbers of treponemes from rabbits experimentally infected with clinical isolates of *T. pallidum* and because motility is not necessarily a reliable indicator of treponemal metabolic activity (1, 18, 20).

Extensive clinical experience has shown that penicillin is the drug of choice for the treatment of syphilis due to its extremely effective treponemicidal activity and its relative lack of toxicity for humans (10, 12, 17, 19). Alternative antibiotics for patients with primary or secondary syphilis who are allergic to penicillin include tetracycline and erythromycin, which are administered orally at a daily dose of 2 g over 15 days (3). Tetracycline is contraindicated for young children and pregnant women (10), however, and erythromycin has been only marginally effective when a total dose of less than 30 g was administered over 2 weeks (2, 4, 21). Erythromycin treatment failures have also been documented when adequate therapy was given for primary syphilis (8), and congenital syphilis has occurred in infants whose mothers responded to this antibiotic (6, 22).

The emergence of penicillin resistance in some microorganisms such as *Streptococcus pneumoniae* and *Streptococcus viridans* emphasizes the fact that antibiotic resistance may develop after decades of exquisite susceptibility (5, 27). Although *T. pallidum* apparently continues to be one of the most penicillin-susceptible microorganisms known, the report (14) of the presence of plasmid DNA in *T. pallidum* Nichols strain suggests that this organism may possess the potential to acquire resistance to antibiotics. Unfortunately, little information is available as to whether the strains of *T. pallidum* that currently cause clinical infections are as susceptible to antibiotics (other than penicillin) as the *T. pallidum* Nichols strain, which was isolated in 1912, before the advent of antibiotic therapy (for a review, see reference 25).

We have previously reported (23) that treponemes freshly extracted from infected rabbit testes can be intrinsically radiolabeled in vitro with [^{35}S]methionine to very high specific activities. In the present study, we used a modification of this procedure to evaluate the effect of various antibiotics on protein synthesis in two strains of *T. pallidum*, the Nichols strain and a more recent clinical isolate of *T. pallidum* designated street strain 14, and *T. pallidum* subsp. *pertenue*, the etiologic agent of yaws. We found that street strain 14 treponemes exhibited high-level resistance to erythromycin in this in vitro assay. This was in marked contrast to the in vitro susceptibility of treponemes of the other two strains and the known antitreponemal efficacy of this antibiotic. To our knowledge, this represents the first demonstration of clinically relevant antibiotic resistance among the pathogenic treponemes.

MATERIALS AND METHODS

Bacterial strains. The sources of *T. pallidum* subsp. *pallidum* Nichols strain and street strain 14 and *T. pallidum* subsp. *pertenue* Gauthier strain and the cultivation of these treponemes in infected rabbit testes have been described previously (24). *T. pallidum* Nichols strain was isolated in 1912 and has been maintained by passage in rabbits since that time (26). *T. pallidum* street strain 14 was isolated by J. Clark of the Centers for Disease Control in 1977 from a patient with secondary syphilis (see Discussion section).

Antibiotics. Chloramphenicol, erythromycin, lincomycin

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hydrochloride, midecamycin, oleandomycin phosphate, rifampin, spiramycin, tetracycline, and tylosin tartrate were obtained from Sigma Chemical Co., St. Louis, Mo. Streptomycin sulfate was obtained from Pfizer, Inc., New York, N.Y.; penicillin G was obtained from E. R. Squibb & Sons, Princeton, N.J.; and clindamycin phosphate was obtained from Upjohn Manufacturing Co., Barceloneta, P.R. Roxithromycin (RU 965) was a gift from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J. With the exception of tetracycline and penicillin G, antibiotic stock solutions were prepared at a concentration of 5 mg/ml shortly before use and filter sterilized. Chloramphenicol, erythromycin, midecamycin, roxithromycin, spiramycin, and tylosin were prepared by dissolving 25 mg of each antibiotic in 1 ml of absolute ethanol, followed by the addition of phosphate-buffered saline to produce the final stock concentration. Clindamycin, lincomycin, and oleandomycin were prepared as stock solutions in phosphate-buffered saline. Rifampin was prepared in methanol. The tetracycline stock solution was prepared by dissolving 20 mg in 1 ml of methanol and diluting with phosphate-buffered saline to a final concentration of 2 mg/ml. Penicillin was prepared in distilled water at a final stock concentration of 10 U/ml.

Extraction and radiolabeling of treponemes; in vitro assays for antibiotic susceptibility. Treponemes were aseptically extracted from the testes of experimentally infected rabbits in medium containing cycloheximide to inhibit protein synthesis by any contaminating host cells (23). We have previously established (23) that this drug has no effect on treponemal protein synthesis. The treponemes were standardized to a density of 3×10^8 to 6×10^8 cells per ml. Volumes of 1 ml of treponemes were transferred into sterile, screw-cap glass vials containing either antibiotic solution or an equal control volume of the corresponding solvent. [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) was added to each vial at the indicated times and concentrations. Following incubation at 34°C for the indicated time period, incorporation of label into treponemal proteins was determined by trichloroacetic acid (TCA) precipitation of a 0.1-ml portion from each vial as described previously (23). In addition, a sample of treponemes from each vial was examined by dark-field microscopy, and the remaining organisms were processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). With regard to the assays for antibiotic susceptibility, insufficient treponemes were obtained from infected rabbits to permit these assays to be done simultaneously in duplicate. However, all experiments described in this study were done at least twice and essentially identical results were obtained. The percent inhibition values provided in Tables 1 and 2 (see Results section) were derived from individual experiments.

SDS-PAGE and fluorography. The SDS-PAGE system used in this study has been described previously (24). Solubilized, radiolabeled treponemal extracts were mixed with sample buffer and electrophoresed on 15% acrylamide slab gels. The gels were stained, destained, and processed for fluorography as described previously (24).

RESULTS

Radiolabeling of *T. pallidum* Nichols strain in the absence or presence of chloramphenicol. To establish conditions for in vitro antibiotic susceptibility assays, we used our previously described radiolabeling procedure (23) to compare the incorporation of [^{35}S]methionine into TCA-precipitable protein by freshly extracted cells of *T. pallidum* Nichols strain in the absence or presence of 20 μg of chloramphenicol per ml. In agreement with results of earlier studies (23), [^{35}S]methionine was incorporated into TCA-precipitable material in a linear fashion throughout the 10-h labeling period when antibiotic was not present (Fig. 1). Nearly 1.4×10^7 TCA-precipitable cpm of [^{35}S]methionine was incorporated into approximately 3.6×10^8 treponemes during this 10-h period. In marked contrast, incorporation of label was significantly reduced when chloramphenicol was added to the treponemes at the beginning of the incubation period. Furthermore, there was negligible incorporation of label into treponemal protein after the first 2 h of incubation. These results indicate that a 2-h incubation period is sufficient for 20 μg of chloramphenicol per ml to totally inhibit treponemal protein synthesis in this in vitro system.

In vitro susceptibility of treponemal protein synthesis to various antibiotics. We investigated the in vitro susceptibility of three different pathogenic treponemal strains to several different antibiotics that either directly or indirectly inhibit protein synthesis. A modification of the radiolabeling procedure described above was employed. Treponemes were preincubated with test concentrations of each antibiotic for 4 h prior to the addition of radiolabel. After this initial 4-h period, [^{35}S]methionine was added and incubation was continued for an additional 4-h period. The incorporation of label into protein was then determined and compared with that in a control sample that was incubated without antibiotic. In these experiments, we used two different concentrations of each antibiotic. The lower concentration roughly corresponded to a clinically achievable, high (peak) level in serum in patients taking the drug according to currently recommended regimens. The higher concentration represented 10 times this therapeutic level.

Chloramphenicol and tetracycline effectively inhibited

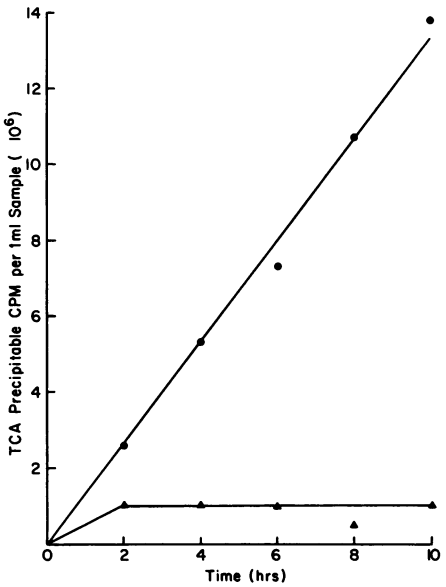

FIG. 1. Incorporation of [^{35}S]methionine into protein by Nichols strain treponemes in the absence or presence of chloramphenicol. Treponemes freshly extracted from rabbit testes were suspended in medium to a cell density of 3.6×10^8 cells per ml. Treponemes were continuously incubated in the presence of [^{35}S]methionine (final concentration, 130 μCi/ml), either without (●) or with (▲) chloramphenicol (final concentration, 20 μg/ml). Samples (0.1 ml) were removed at 2, 4, 6, 8, and 10 h; precipitated with TCA; and counted, as described in the text.

protein synthesis in cells of all three treponemal strains (Table 1). Streptomycin had essentially no effect on *T. pallidum* street strain 14, and its modest effect on *T. pallidum* Nichols strain and *T. pallidum* subsp. *pertenue* was particularly evident at only the higher concentration. Rifampin had little effect on protein synthesis in all three strains. Erythromycin effectively inhibited protein synthesis in cells of *T. pallidum* Nichols strain and *T. pallidum* subsp. *pertenue* at both concentrations. Somewhat surprisingly, erythromycin had virtually no effect on protein synthesis in street strain 14 treponemes, even at the higher (100-μg/ml) concentration tested. Finally, due to the exquisite susceptibility of pathogenic treponemes to penicillin G in vivo, it was of interest to include two different concentrations of this antibiotic in this analysis. Some inhibition of [^{35}S]methionine incorporation was observed for all three strains, although, as was the case for most of the other antibiotics tested, street strain 14 treponemes were inhibited the least. For all three strains, the observed inhibition of protein synthesis presumably was a reflection of the treponemistatic or -cidal activity of penicillin. Also, no obvious differences in the motility of the treponemes in any of the antibiotic-treated samples compared with that in the appropriate control samples were observed.

We also employed a second method of evaluating the effects of these antibiotics on treponemal protein synthesis.

TABLE 1. In vitro susceptibilities of three pathogenic strains to various antibiotics

Antibiotic and concn (μg/ml)	% Inhibition of protein synthesis of[a]:		
	T. pallidum		*T. pallidum* subsp. *pertenue* Gauthier
	Nichols	Street strain 14	
Chloramphenicol			
20	92.5	68.9	86.4
200	93.3	87.9	94.6
Tetracycline			
4	86.9	78.3	88.8
40	94.5	96.9	96.3
Erythromycin			
10	86.8	0.0	83.7
100	89.5	7.1	86.5
Streptomycin			
50	32.6	6.4	6.7
500	60.9	8.3	42.4
Rifampin			
10	24.8	0.0	0.0
100	41.5	3.0	0.2
Penicillin G			
0.03[b]	51.7	0.9	29.8
0.3	64.6	35.3	NT[c]

[a] Freshly extracted treponemes were preincubated in the presence of individual antibiotics for 4 h and then radiolabeled with [^{35}S]methionine (final concentration, 65 μCi/ml) for an additional 4 h. See text for additional experimental details. The percent inhibition of protein synthesis was calculated by dividing the TCA-precipitable counts per minute obtained in the presence of antibiotic by the TCA-precipitable counts per minute obtained in the appropriate control sample for each strain, multiplying by 100, and then subtracting from 100%. Each control sample yielded TCA-precipitable counts per minute in excess of 10^6/ml.

[b] Values are in units per milliliter.

[c] NT, Not tested.

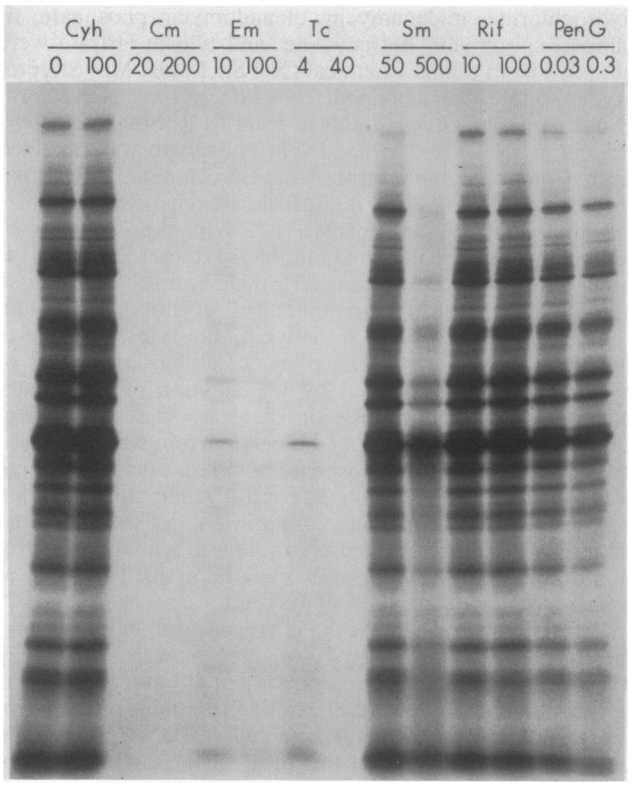

FIG. 2. Inhibition of protein synthesis in the *T. pallidum* Nichols strain during in vitro incubation in the presence of various antibiotics. Treponemes treated with the various antibiotics listed in Table 1 were analyzed by SDS-PAGE and fluorography. The antibiotics tested are listed at the top of the figure and are abbreviated as follows: Cyh, cycloheximide; Cm, chloramphenicol; Em, erythromycin; Tc, tetracycline; Sm, streptomycin; Rif, rifampin; and PenG, penicillin G. The final concentration of each antibiotic used in the assays is indicated in micrograms per milliliter (except for penicillin G, which is in units per milliliter). Cycloheximide was present in all samples except the first one to inhibit any residual protein synthesis by contaminating rabbit testicular material, although this control likely was not necessary (23). Note that identical amounts of protein were loaded into each lane; all lanes were exposed for fluorography for an identical time period. See text for additional experimental details.

After the 4-h incubation period of treponemes with antibiotic and label, a portion of each sample was solubilized and subsequently analyzed by SDS-PAGE and fluorography. We used this technique previously (23) to demonstrate that the majority of treponemal proteins are synthesized in vitro during the incubation period in radiolabeling medium. The results obtained for *T. pallidum* Nichols (Fig. 2) complemented those presented above. For example, only a few faint bands could be detected for treponemes that were radiolabeled in the presence of chloramphenicol, erythromycin, or tetracycline. Likewise, some inhibition by streptomycin was only detectable at the higher concentration. Similar results were obtained for the other two treponemal strains (data not shown). To further illustrate the erythromycin resistance exhibited by *T. pallidum* street strain 14, extracts prepared from each treponemal strain incubated without antibiotic or with low or high concentration of erythromycin were analyzed on the same gel (Fig. 3). These results demonstrate that, in stark contrast to the other two strains, protein synthesis in street strain 14 treponemes is

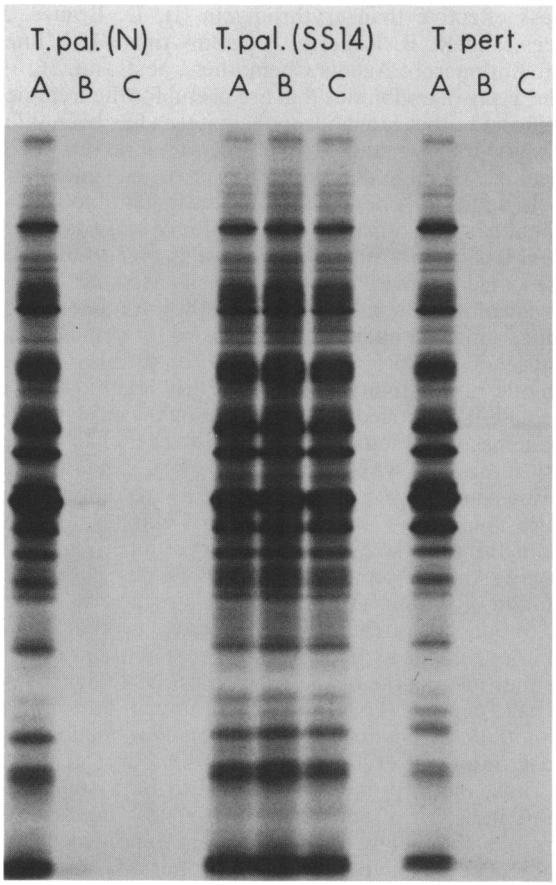

FIG. 3. In vitro inhibition of protein synthesis in pathogenic treponemes incubated in vitro in the presence of erythromycin. Treponemes of all three pathogenic strains were tested with erythromycin, as shown in Table 1, and were analyzed by SDS-PAGE and fluorography. Lanes: A, control, no erythromycin; B, 10 μg of erythromycin per ml; C, 100 μg of erythromycin per ml. Note that erythromycin effectively inhibited the incorporation of label into protein for both *T. pallidum* Nichols strain and *T. pallidum* subsp. *pertenue* treponemes at both concentrations tested. See legend to Fig. 2 and text for additional experimental details. Abbreviations: T.pal. (N), *T. pallidum* Nichols; T.pal. SS14, *T. pallidum* street strain 14; T. pert., *T. pallidum* subsp. *pertenue*.

strongly resistant to inhibition by both erythromycin concentrations tested.

Susceptibility of treponemal strains to macrolide and lincosamide antibiotics. The high-level erythromycin resistance of *T. pallidum* street strain 14 prompted us to investigate the in vitro susceptibility of this strain to higher erythromycin concentrations. We found that protein synthesis in street strain 14 cells was only slightly inhibited by an erythromycin concentration as high as 400 μg/ml (Table 2). In the same experiment, we also tested the susceptibilities of both street strain 14 and Nichols strain treponemes to a number of additional macrolide and lincosamide antibiotics (Table 2). For each of these antibiotics, the concentrations tested were 10 and 100 μg/ml. We found that street strain 14 treponemes, but not Nichols strain organisms, were also highly resistant to roxithromycin, a new macrolide antibiotic with a structure and antimicrobial spectrum similar to those of erythromycin (11). Protein synthesis in treponemes of both strains was strongly inhibited by spiramycin, tylosin, and midecamycin. Oleandomycin, lincomycin, and clindamycin inhibited protein synthesis somewhat in both strains, although in each case the effect on Nichols strain organisms was somewhat stronger.

DISCUSSION

We used an in vitro radiolabeling procedure to evaluate the effect of a number of antibiotics on protein synthesis in cells of three different pathogenic treponemal strains freshly extracted from infected rabbit testes. Protein synthesis in both strains of *T. pallidum* and one strain of *T. pallidum* subsp. *pertenue* was strongly inhibited by both chloramphenicol and tetracycline at concentrations that were clinically significant. These results are consistent with the findings of previous clinical trials and published reports (10, 18, 26) of the efficacy of these two antibiotics in the treatment of human syphilis and experimental rabbit syphilis, but are at variance with the results of Baseman and Hayes (1), who reported that chloramphenicol does not inhibit the incorporation of ^{3}H-labeled amino acids by Nichols strain treponemes under their radiolabeling conditions. Due to its adverse side effects, chloramphenicol is no longer a recommended alternative treatment for syphilis, and the

TABLE 2. In vitro susceptibility of *T. pallidum* to macrolide and lincosamide antibiotics

Antibiotic and concn (μg/ml)	% Inhibition of protein synthesis of the following *T. pallidum* strains[a]:	
	Nichols	Street strain 14
Macrolides		
Erythromycin		
10	79.7	4.2
100	87.1	0.0
200	NT[b]	0.0
400	NT	39.0
Roxithromycin		
10	85.2	0.0
100	77.6	0.0
Midecamycin		
10	90.6	74.1
100	92.1	82.8
Oleandomycin		
10	39.5	23.4
100	69.7	28.6
Spiramycin		
10	88.5	90.8
100	86.3	96.2
Tylosin		
10	79.6	56.7
100	88.0	76.7
Lincosamides		
Clindamycin		
10	26.0	16.0
100	57.0	26.6
Lincomycin		
10	68.1	22.4
100	84.4	54.3

[a] See footnote *a* of Table 1.
[b] NT, Not tested.

recommended uses of tetracyclines are also somewhat limited (10). Our results also correlated with those of previous studies (9, 10, 18) in which it was indicated that rifampin and streptomycin are clinically ineffective against treponemal infections.

Erythromycin is the only recommended alternative antibiotic for the treatment of syphilis in penicillin-allergic patients, for whom the use of tetracycline is contraindicated (3). We found that protein synthesis in cells of *T. pallidum* Nichols strain and *T. pallidum* subsp. *pertenue* was strongly inhibited by therapeutic levels of erythromycin. Baseman and Hayes (1) have also reported that erythromycin (5 μg/ml) inhibited the in vitro incorporation of ^{3}H-labeled amino acids into protein of Nichols strain treponemes. In contrast to these results, we found that protein synthesis in street strain 14 treponemes was refractory to even very high levels of this antibiotic. Analysis of solubilized extracts of these treponemes incubated with [^{35}S]methionine in the absence or presence of up to 100 μg of erythromycin per ml revealed no difference in the intensity or number of radiolabeled proteins, strongly indicating that this *T. pallidum* clinical isolate is highly resistant to this antibiotic.

The finding of an erythromycin-resistant strain of *T. pallidum* is not necessarily surprising if past reports of erythromycin treatment failures in human patients with syphilis are considered (2, 4, 8, 21), and it prompted us to investigate the origin of street strain 14. We subsequently learned in a personal communication from D. S. Kellogg (Centers for Disease Control, Atlanta, Ga.) that *T. pallidum* street strain 14 was isolated from a penicillin-allergic patient with an active case of secondary syphilis that had been unsuccessfully treated with erythromycin. This patient first received a total of 10 g of erythromycin over a 5-day period in the hospital and then a total of 20 g over a 10-day period as an outpatient. Because of a persistent vasculitic rash and a positive result in the Venereal Disease Research Laboratory test, the patient was readmitted to the hospital and treated with 500 mg of erythromycin administered four times daily for 30 days. While still under hospital observation after this additional therapy, the patient developed scrotal lesions that were positive by dark-field microscopy. It was at this time that a sample was taken that resulted in the isolation of street strain 14 by passage in rabbit testes. Since street strain 14 was clearly resistant to erythromycin in our in vitro assay, the evidence strongly indicates that erythromycin-resistant strains of *T. pallidum* are responsible for at least some instances of erythromycin treatment failure.

In this study, we also investigated the effect of other macrolide and lincosamide antibiotics on in vitro treponemal protein synthesis. We found that street strain 14 treponemes were also highly resistant to roxithromycin, a new macrolide antibiotic that is structurally similar to erythromycin and which was recently shown to provide effective therapy for *T. pallidum* Nichols-infected rabbits (11). Erythromycin and roxithromycin were the only two antibiotics tested for which we could clearly demonstrate significant differences in susceptibility between two different pathogenic treponemal strains. Protein synthesis in street strain 14 treponemes was somewhat more resistant to oleandomycin, lincomycin, and clindamycin than was protein synthesis in Nichols strain treponemes. Both strains were strongly inhibited by spiramycin, tylosin, and midecamycin. In 1965 and 1966, spiramycin was used successfully for the treatment of early syphilis in clinical trials (10). We are unaware of any clinical trials for the treatment of human syphilis with the other antibiotics. Clindamycin was tested in rabbits but appears to be less effective than erythromycin (B. D. Brause, J. S. Borges, and R. B. Roberts, Program Abstr. 15th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 36, 1975).

The assay of antibiotics that are useful for the treatment of syphilis and other human treponematoses has been difficult, and relatively few studies have been undertaken, although clinical use of some antibiotics has provided some information. In addition, a new method of testing antibiotics by use of Nichols strain organisms that multiply in a tissue culture system has much potential (16). Strains of *T. pallidum* that cause clinical infections are not easily isolated, and even after adaptation to rabbit passage, they frequently do not produce sufficient numbers of organisms for detailed studies. Because of this problem, we were only able to test the antibiotic susceptibility of one relatively recent (1977) clinical isolate. Our procedure for examining the susceptibility of treponemes to various antibiotics, however, has proven its utility in several ways. First, it is more expeditious, less laborious, and more economical than in vivo rabbit trials that require many days and a number of animals to generate meaningful results. Second, our procedure quantitatively measures the susceptibility of treponemes based on the inhibition of protein synthesis. In this regard, we observed that treponemes treated with effective protein synthesis inhibitors remain as actively motile as untreated controls over the time course of the in vitro assay. Other investigators had reported (1, 20) that erythromycin does not affect the motility of Nichols strain treponemes incubated with concentrations of erythromycin that inhibit protein synthesis. Thus, motility does not appear to be a sensitive and reliable indicator of antibiotic effectiveness in vitro. Third, the results obtained with Nichols strain treponemes in our in vitro procedure have certainly corroborated prior clinical observations on the antitreponemal efficacy of chloramphenicol, tetracycline, erythromycin, rifampin, streptomycin, and spiramycin.

Finally, with regard to *T. pallidum* street strain 14, we have demonstrated for the first time clinically relevant antibiotic resistance in a pathogenic treponemal isolate. Results of our studies did not provide any information concerning the mechanism of high-level erythromycin resistance exhibited by *T. pallidum* street strain 14 or the nature of the resistance determinant; this is being investigated further. Our results do indicate, however, that resistance is specific for erythromycin and closely related derivatives. In view of the potential limitations in the use of erythromycin, other therapeutic alternatives should be investigated further. We also found in this study that, in our in vitro assay, street strain 14 treponemes were somewhat less susceptible to most of the antibiotics tested when compared with the susceptibilities of the other two strains. This finding suggests that the outer envelope of treponemes of this fairly recent clinical isolate may be generally less permeable to antibiotics. It is tempting to speculate that this represents an adaptive response of *T. pallidum* to the antibiotic pressures it has encountered since the widespread use of such agents became prevalent in the middle of this century. This also bears further investigation.

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