ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1976, p. 639-645

Vol. 10, No. 4
Printed in U.S.A.

Multiple Antibiotic Resistance in Clinical Strains of *Neisseria gonorrhoeae* Isolated in South Carolina

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Received for publication 1 March 1976

Minimal inhibitory concentration (MIC) values for penicillin G, tetracycline, amoxicillin, and actinomycin D were determined for 81 strains of *Neisseria gonorrhoeae* isolated from patients attending public health clinics in the Piedmont region of South Carolina. Gonococcal isolates were also screened for high-level resistance to streptomycin. Significant positive correlations ($r \geq 0.45$, $P \leq 0.01$) were found between all possible pairs of the antibiotics penicillin G, tetracycline, and amoxicillin. The MIC values of actinomycin D showed no significant positive correlations with the MIC values of the other antibiotics. Gonococcal strains that were resistant to streptomycin tended to be resistant to penicillin G, tetracycline, and amoxicillin. Of the 81 isolates, 18.5% were multiply resistant to penicillin G, tetracycline, amoxicillin, and streptomycin. Spontaneous mutants with reduced antibiotic susceptibility, selected for decreased susceptibility to penicillin G, displayed small decreases in susceptibility to tetracycline, amoxicillin, and actinomycin D. Spontaneous mutants selected for decreased susceptibility to actinomycin D displayed small losses in susceptibility to penicillin G. The results show that multiple antibiotic resistance occurs in clinically isolated gonococcal strains in South Carolina. The results further suggest the presence of a common genetic mechanism determining antibiotic resistance in *N. gonorrhoeae*.

The inherent ability of *Neisseria gonorrhoeae* to develop resistance to antibiotics is a major contributing factor to the continuing spread and marked increases in reported gonococcal infections in the past 20 years (20). Sensitivities of random isolates of *N. gonorrhoeae* to such clinical antibiotics as penicillin, tetracycline, and streptomycin have significantly decreased in many areas (9, 20, 24). In addition, several investigators have reported positive correlations between the susceptibilities of individual isolates of *N. gonorrhoeae* to a wide variety of antibiotics, including penicillin, tetracycline, erythromycin, streptomycin, spiramycin, and chloramphenicol (12, 14, 15). Multiply antibiotic-resistant gonococcal strains pose a serious problem in the treatment and control of gonorrhea. At this time, the underlying basis for multiple resistance has not been conclusively established. The current need for regional surveillance of gonococcal antibiotic susceptibility levels and regional analysis of levels of multiple antibiotic resistance has been pointed out by several investigators (3, 9, 12, 20, 24).

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For the years 1972, 1973, and 1974, South Carolina has ranked first, third, and fifth, respectively, among states in the number of reported gonococcal infections per 100,000 population, and can be considered a problem area in terms of gonorrhea control (1, 2). In this investigation, we sought to measure the antibiotic susceptibility levels of random isolates of *N. gonorrhoeae* from a particular region of South Carolina and determine the extent to which these strains were multiply antibiotic resistant. The antibiotics used in this investigation were penicillin G, tetracycline, amoxicillin, streptomycin, and actinomycin D. These antibiotics were chosen for their chemical dissimilarity and their varying modes of action. In addition, multiply antibiotic-resistant mutants were isolated by specific selection for penicillin G and actinomycin D resistance. Our results provide evidence for a common mechanism for multiple antibiotic resistance in *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacterial strains. Fifty-four randomly isolated strains of *N. gonorrhoeae* were collected in July and August 1974 at the Greenville County Public Health Department, Greenville, S.C. Twenty-seven addi-

tional strains were collected during the same period at the Anderson County Public Health Department, Anderson, S.C. The study population consisted of 81 symptomatic male patients. No further screening or treatment follow-up was done. Cultures were confirmed as *N. gonorrhoeae* by colonial morphology, Gram stain, positive oxidase reaction using *N,N*-dimethyl-*p*-phenylenediamine monohydrochloride (J. T. Baker Chemical Co.), degradation of glucose but not maltose, and failure to grow on nutrient agar. Three reference strains possessing known antibiotic susceptibilities were kindly provided by P. F. Sparling, University of North Carolina, Chapel Hill.

Culture media and growth. Primary isolation and purification of *N. gonorrhoeae* isolates were done on enriched chocolate agar (ECA), which consisted of GC medium base (Difco) containing 2% hemoglobin (Difco) and 1% Difco supplement C. Routine subculture and culture maintenance were done on GC medium base containing 1% concentrations of supplements 1 and 2 of White and Kellogg (23). Sugar degradations were performed by using cystine tryptic agar (Difco) to which 1% filter-sterilized (Morton filter) sugars were added. Broth dilutions were made in either tryptic soy broth (Difco) or GC base broth (proteose-peptone no. 3, 1.5 g; corn starch, 0.1 g; K_2HPO_4, 0.4 g; KH_2PO_4, 0.1 g; NaCl, 0.5 g; distilled water, 100 ml). All cultures were incubated at 36°C in a moist atmosphere of 5% CO_2 unless otherwise specified. Strains were stored at −70°C in tryptic soy broth (Difco) plus 20% glycerol.

Testing for susceptibility to antibiotics. The wells of a replicator-inoculating device, similar in design to a Steers replicator (21), were filled with standardized suspensions of gonococcal cells (approximately 10^6 colony-forming units [CFU]/ml). Approximately 10^3 CFU of each suspension was transferred with the replicator to a series of ECA plates containing doubling dilutions of antibiotic and at least three ECA control plates containing no antibiotic. The minimal inhibitory concentration (MIC) value was defined as the lowest concentration that prevented visible growth of more than one oxidase-positive colony after 24 h of incubation. All MIC determinations were performed in duplicate, and results were reproducible within twofold limits on replicate determinations. Gonococcal isolates were screened for high-level resistance to streptomycin by observing growth or no growth on an antibiotic plate containing 300 μg of streptomycin per ml. Three control strains possessing known antibiotic susceptibilities were included in each test.

Selection of antibiotic-resistant mutants. Multistep spontaneous antibiotic-resistant mutants of *N. gonorrhoeae* were obtained by subculturing on increasing concentrations of either penicillin G or actinomycin D. Cells were suspended in broth to a turbidity greater than 100 Klett units (Klett-Summerson colorimeter, no. 54), and 0.25-ml portions were spread onto a series of ECA plates containing doubling concentrations of the selected antibiotic. Selected colonies from the plate containing the highest concentration of antibiotic showing growth were subcultured to plates containing that concentration of antibiotic. The selection process was then repeated, using the selected mutant cultures, until a series of antibiotic-resistant mutants was obtained. Finally, MIC values for selected and nonselected antibiotics were determined for each antibiotic-resistant mutant.

Determination of cell survival on increasing concentrations of antibiotics. Gonococcal cells were suspended in broth and adjusted to a turbidity corresponding to 80 to 85 Klett units (approximately 3×10^8 CFU/ml). Suspensions were agitated using a Vortex mixer to minimize cell clumping. Suspensions were 10^{-5} diluted, and 0.1-ml portions were spread onto the surface of a series of ECA plates containing increasing concentrations of antibiotic and also onto control plates containing no antibiotic. All plating was done in duplicate, and survival determinations were repeated two or more times for each gonococcal strain.

Data analysis. The coefficient of correlation (r) was computed for each pair of antibiotics with the aid of a Monroe 1930 programed calculator, and t tests were used to determine which of the r values differed significantly from zero (19).

Antibiotics. Sources of antibiotics were: Lilly, penicillin G and streptomycin; Beecham-Massengill, amoxicillin; Lederle, tetracycline; and Merck Sharp & Dohme, actinomycin D.

RESULTS

Antibiotic susceptibility. The measured antibiotic susceptibilities to four antibiotics for 81 strains of *N. gonorrhoeae*, isolated in Greenville and Anderson, S.C., are shown in Fig. 1. Penicillin G MIC values ranged from very susceptible (0.004 μg/ml) to very resistant (2.0 μg/ml) and were unevenly distributed. Of the 81 isolates 32.1% were resistant to penicillin G (MIC > 0.3 μg/ml). Amoxicillin MIC values ranged from 0.015 to 1.0 μg/ml and showed an uneven distribution similar to that of penicillin G. Tetracycline MIC values showed a normal distribution and peaked in a range corresponding to tetracycline resistance (1.0 μg/ml). Of the isolates, 23.3% were highly resistant to tetracycline (MIC > 1.0 μg/ml). The range of actinomycin D MIC values was very narrow, with the majority of the gonococcal strains being inhibited by 2.0 μg/ml. Thirty-three (41%) of the 81 gonococcal isolates displayed high-level resistance to streptomycin (MIC > 300 μg/ml). Fifteen (18.5%) of the 81 isolates were multiply resistant to penicillin G (MIC > 0.3 μg/ml), tetracycline (MIC ≥ 1.0 μg/ml), amoxicillin (MIC ≥ 0.5 μg/ml), and streptomycin (MIC > 300 μg/ml.)

Correlations between MIC values. Significant positive correlations ($r \geq 0.45$, $P \leq 0.01$) were found between the susceptibilities of the 81 isolates of *N. gonorrhoeae* to all pairs of the antibiotics penicillin G, tetracycline, and

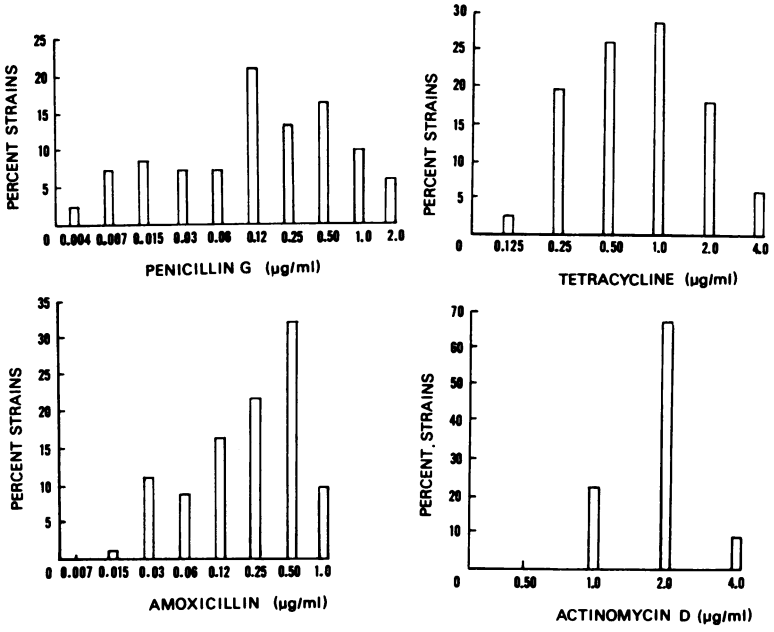

FIG. 1. *Distribution of penicillin G, amoxicillin, tetracycline, and actinomycin D MIC values for 81 random isolates of Neisseria gonorrhoeae.*

amoxicillin (Table 1). Representative scattergram plots of penicillin G versus tetracycline and penicillin G versus amoxicillin reveal roughly proportional and continuous relationships over the ranges of MIC values observed (Fig. 2 and 3). No significant positive correlations ($r < 0.28$, $P > 0.01$) were found between the susceptibilities to actinomycin D and penicillin G, amoxicillin, or tetracycline. The scattergram plot of penicillin G versus actinomycin D is representative and shows no proportional relationship (Fig. 4).

The percentage of streptomycin-resistant strains at each susceptibility level of penicillin G, amoxicillin, tetracycline, and actinomycin D is shown in Table 2. The results show that gonococcal strains that were less susceptible to either penicillin G, amoxicillin, or tetracycline tended to be resistant also to streptomycin. Roughly equal proportions of streptomycin-resistant strains were present at the lowest and highest levels of actinomycin D resistance. These results suggest that positive correlations exist between gonococcal susceptibilities to streptomycin and those to penicillin G, amoxicillin, and tetracycline. There is no apparent correlation, however, between streptomycin and actinomycin D susceptibilities in *N. gonorrhoeae*.

Spontaneous antibiotic-resistant mutants. The phenotypes of selected penicillin G and actinomycin D mutants are shown in Table 3.

TABLE 1. *Correlation coefficients (r values) for MIC values of the various pairs of antibiotics for 81 strains of Neisseria gonorrhoeae*

Antibiotic	Antibiotic[a]			
	pen	tet	amx	act
pen		0.79	0.62	0.14[b]
tet	0.79		0.45	0.23[b]
amx	0.62	0.45		0.06[b]
act	0.14[b]	0.23[b]	0.06[b]	

[a] Abbreviations: pen, penicillin G; tet, tetracycline; amx, amoxicillin; act, actinomycin D.
[b] *r* values not significant ($P > 0.01$).

The results showed that decreasing the susceptibility to penicillin G 16-fold in vitro was sufficient to cause a concomitant fourfold decrease in amoxicillin susceptibility and a twofold decrease in tetracycline susceptibility. Decreased penicillin susceptibility did not change the MIC values for actinomycin D or result in a loss of streptomycin susceptibility. Fourfold decreases in resistance to actinomycin D did not alter the MIC values of penicillin G, tetracycline, or amoxicillin and did not cause a loss of susceptibility to streptomycin.

Survival on increasing concentrations of antibiotics. Survival of wild-type parent gonococcal isolate GV77 is compared with that of four GV77 less penicillin-susceptible mutants on increasing concentrations of actinomycin D in Fig. 5. At 0.75 μg of actinomycin D per ml,

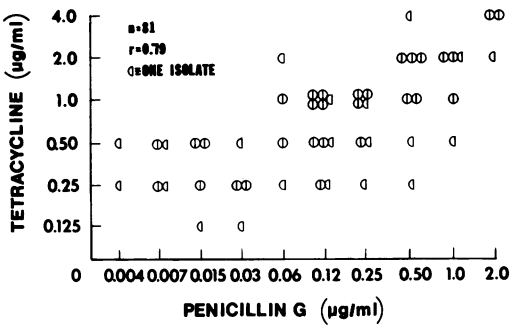

FIG. 2. *Scattergram of penicillin G versus tetracycline MIC values for 81 isolates of Neisseria gonorrhoeae.*

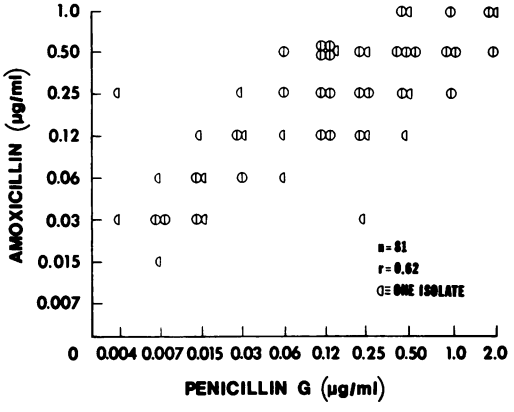

FIG. 3. *Scattergram of penicillin G versus amoxicillin MIC values for 81 isolates of Neisseria gonorrhoeae.*

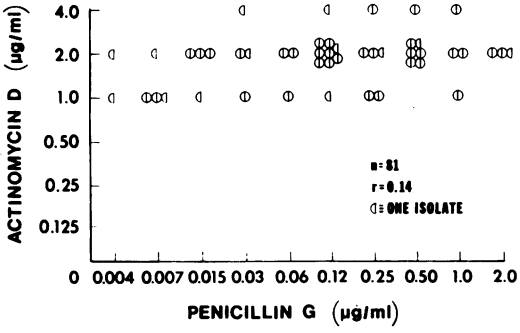

FIG. 4. *Scattergram of penicillin G versus actinomycin D MIC values for 81 isolates of Neisseria gonorrhoeae.*

less than 10% of the average number of CFU counted on control plates containing no antibiotics was present for wild-type strain GV77. At this same level, however, 30 to 35% of the less penicillin-susceptible mutants GV77Pen.03 (penicillin MIC + 0.03 μg/ml), GV77Pen.06, and GV77Pen.12 and 64% of GV77Pen.25 was present. At 1.0 μg of actinomycin D per ml, all strains were inhibited except GV77Pen.25, which showed 65% survival.

The survival, on increasing concentrations of penicillin G, of the GV77 wild-type strain and two less actinomycin D-susceptible mutants is shown in Fig. 6. At 0.0087 μg of penicillin G per ml, GV77Act4.0 (actinomycin D MIC + 4.0 μg/ml) showed 49% survival and GV77Act8.0 showed 83% survival. No colonies of the GV77 wild-type strain were observed at this level.

DISCUSSION

The ranges and distributions of gonococcal antibiotic susceptibilities found in this investi-

TABLE 2. *Streptomycin-resistant strains at each susceptibility level of penicillin G, amoxicillin, tetracycline, and actinomycin D*

MIC (μg/ml)	No. of strains	% Streptomycin resistant[a]
Penicillin		
0.004	2	0
0.007	6	0
0.015	7	0
0.03	6	16
0.06	6	50
0.12	17	53
0.25	11	57
0.50	13	54
1.0	8	63
2.0	5	100
Total	81	41
Tetracycline		
0.125	2	0
0.25	16	19
0.50	21	14
1.0	23	65
2.0	14	57
4.0	5	80
Total	81	41
Amoxicillin		
0.015	1	0
0.03	9	0
0.06	7	14
0.12	13	15
0.25	17	17
0.50	26	73
1.0	8	88
Total	81	41
Actinomycin D		
1.0	18	28
2.0	55	47
4.0	8	25
Total	81	41

[a] Streptomycin resistant = MIC > 300 μg/ml.

TABLE 3. *Phenotypes of selected antibiotic-resistant spontaneous mutants of Neisseria gonorrhoeae*

Strain	MIC (μg/ml)[a]				
	pen	tet	amx	act	str
GV77 wild type	0.015	0.25	2.0	≤300	
GV77Pen.03	0.03	0.25	2.0	≤300	
GV77Pen.06	0.06	0.25	2.0	≤300	
GV77Pen.12	0.12	0.25	2.0	≤300	
GV77Pen.25	0.25	0.50	2.0	≤300	
GV77Pen4.0	0.015	0.25	4.0	≤300	
GV77Pen8.0	0.015	0.25	8.0	≤300	

[a] Abbreviations: pen, penicillin G; tet, tetracycline; amx, amoxicillin; act, actinomycin D; str, streptomycin

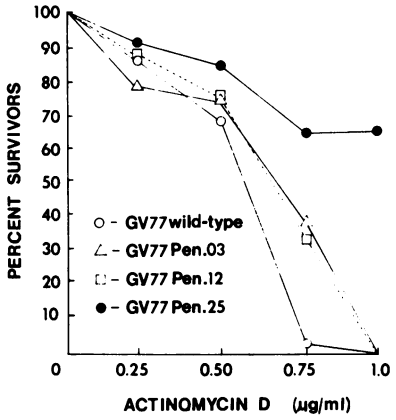

FIG. 5. *Survival of less penicillin G-susceptible mutants on increasing concentrations of actinomycin D. Approximately 300 CFU equals 100% survival.*

gation in the Piedmont region of South Carolina support previous observations by other investigators that regional variations in antibiotic susceptibilities do exist. The percentage of penicillin-resistant strains (MIC > 0.3 μg/ml) found in patients in the present study (32%) was significantly lower than the penicillin-resistant percentage of those tested in Philadelphia, Pa., in 1973 (54%) (11). However, the percentage of penicillin-resistant isolates was higher than the latest reported national averages (17.4%) (6). In addition, a higher percentage of penicillin-resistant strains was found in the Greenville-Anderson area than in any individual location examined by the cooperative national survey (6). The percentage of gonococcal isolates resistant to tetracycline (MIC > 1.0 μg/ml) in the present study (23%) was slightly higher than the latest reported national average (14.0%) (6). The percentage of tetracycline-resistant strains in the Greenville-Anderson area was also higher than those from the 1973 Philadelphia survey (7%) (11) and the 1972 Vermont state survey (0%) (4). On the basis of this study, the high reported gonorrhea case rate per 100,000 population for South Carolina, when compared with other states, does positively correlate with a higher percentage of penicillin- and tetracycline-resistant strains. However, a direct cause and effect relationship between antibiotic resistance and prevalence of gonococcal infection cannot be assumed on the basis of our data.

Amoxicillin in vitro activity was greater than penicillin G activity against the most penicillin-resistant gonococcal strains (Fig. 3). This effect has been observed with other broad-spectrum penicillins, especially ampicillin (12, 13).

Actinomycin D is not used to treat clinical infections due to its excessive toxicity. It was used in this study because of its chemical structure and mode of action, as well as its unique ability to provide a tool for studying the effects of an antibiotic to which the gonococci have never been previously exposed in vivo. There have been no previous reports of the susceptibility of *N. gonorrhoeae* to actinomycin D.

By plotting MIC values on scattergrams and calculating *r* values, we have shown significant positive correlations between antibiotic susceptibilities to penicillin G, tetracycline, and amoxicillin in natural isolates of *N. gonorrhoeae*. Our calculated coefficient of correlation for penicillin G versus tetracycline ($r = 0.79$) agrees with similar studies done by Maier et al. (12), who calculated an *r* value of 0.75 for their penicillin-versus-tetracycline MIC values. It is somewhat surprising that penicillin G MIC values correlated more closely with those of tetracycline ($r = 0.79$) than with those of amoxicillin ($r = 0.62$), since penicillin G and amoxicillin are structurally related. We cannot explain this

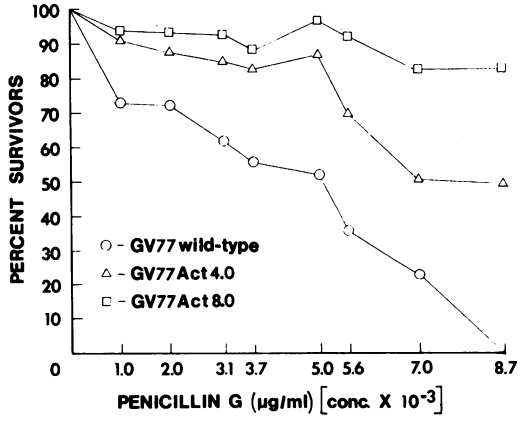

FIG. 6. *Survival of less actinomycin D-susceptible mutants on increasing concentrations of penicillin G. Approximately 300 CFU equals 100% survival.*

on the basis of our experiment other than to point out that gonococcal strains that showed the highest levels of observed resistance to any one of these three antibiotics were resistant to the others as well.

Our demonstration that spontaneous mutants, selected for decreased susceptibility to penicillin G, were less sensitive to tetracycline and amoxicillin agrees with similar studies by Maness and Sparling (14) and Maier et al. (12) and supports the hypothesis that resistance to dissimilar antibiotics may be under the control of a common genetic mechanism.

Our hypothesis that small decreases in actinomycin D susceptibility were occurring in conjunction with decreases in penicillin G susceptibility was demonstrated by observing differences in the average number of CFU observed as standard inocula of wild-type and mutant *N. gonorrhoeae* cells were spread on a series of plates containing increasing concentrations of a specific antibiotic (Fig. 5 and 6). The results suggest that simultaneous decreases in susceptibility to penicillin G and actinomycin D in *N. gonorrhoeae* can occur as a result of one or several spontaneous mutations. The increased survival capabilities probably reflect decreases in susceptibility to each antibiotic in the mutant cultures, resulting in less selective pressure from the restrictive antibiotic plates. These decreases in susceptibility were most likely small because no changes in susceptibility were detectable by standard MIC procedures.

The most likely mechanism for common resistance to these two antibiotics would be a mutation, or possibly several distinct mutations, that affects the permeability properties of the cell envelope. Several investigators have proposed that differences in cell envelope permeability were responsible for spontaneous losses in antibiotic susceptibility to penicillin G and several other antibiotics in *N. gonorrhoeae* (5, 12, 14). Also, bacterial resistance to actinomycin D has been found to result from differences in cell envelope permeability (7, 8, 10, 18). A cell envelope permeability hypothesis for antibiotic resistance is supported by the fact that no enzymes have been found that actively degrade actinomycin D in bacterial cells (22) and by the observation that the gonococci do not produce penicillinase (16). Alterations in specific target sites or transport systems as possible explanations for common resistance patterns are also unlikely due to the chemical and structural dissimilarity of the penicillin G and actinomycin D molecules. The lack of correlation between penicillin G and actinomycin D MIC values in clinical isolates is most likely due to the standard MIC procedure not being sufficiently sensitive to detect small changes in actinomycin D susceptibility. The results of the present study suggest that actinomycin D may be useful in the further study of multiple antibiotic resistance mechanisms in *N. gonorrhoeae*.

ACKNOWLEDGEMENT

Robert K. Shockley ably assisted with some of the work.

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