In Vitro Development of Rifampin Resistance in Clinical Isolates of *Haemophilus influenzae* Type b

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Although all of 14 clinical isolates of *Haemophilus influenzae* type b strains demonstrated rifampin susceptibility in vitro (minimal inhibitory concentration ≤0.4 μg/ml) when an inoculum of 10⁶ colony-forming units (CFU) was used, 10 of the 14 strains manifested resistance to this agent when an inoculum of 10⁸ CFU was tested. The mutation rate for rifampin resistance ranged from 1 resistant colony per 3.5 × 10⁶ CFU to 1 per 4 × 10⁷ CFU. The emergence of rifampin-resistant mutants was prevented when trimethoprim was combined with rifampin. This finding suggests that when used alone for prophylaxis of *H. influenzae* type b nasopharyngeal carriers, rifampin is likely to lead to the emergence of resistant strains.

The association between nasopharyngeal carriage of *H. influenzae* type b (HIB) and cases of secondary systemic infections due to this bacterium in closed pediatric populations prompted antibiotic trials in attempts to eradicate this agent from the nasopharynx. Recent data suggested rifampin to be the most promising chemo-prophylactic agent for treatment of close contacts of individuals with HIB infections (3, 7, 9). However, other bacteria, such as *Staphylococcus aureus*, have been reported to develop both in vitro (1, 6) and in vivo (5) resistance to rifampin when this drug is used alone. The present in vitro study was designed to examine whether clinical isolates of HIB will develop resistance to rifampin.

MATERIALS AND METHODS

Fourteen strains of HIB isolated from blood (5), cerebrospinal fluid (6), and nasopharynx (3) of pediatric patients were studied. Ten strains were ampicillin susceptible, and four were ampicillin resistant. Minimal inhibitory concentrations (MIC) of rifampin (kindly supplied by Ciba Pharmaceutical Co.) for these strains were determined by using supplemented Mueller-Hinton broth (sMH) containing 10 μg of hemin per ml and 1% IsoVitaleX. Ten milligrams of rifampin was dissolved in methanol and diluted with sMH broth to obtain final concentrations ranging from 0.1 to 12.5 μg/ml. From an overnight growth of HIB on a chocolate agar plate, three colonies were inoculated into 5 ml of sMH and shaken for 4 h at 35°C. Dilutions were made for a final inoculum of 10⁶ colony-forming units (CFU) per ml. One milliliter of inoculum was added to each antibiotic tube, with a final volume of 2 ml. The tubes were incubated overnight at 35°C with 5% CO₂. The MIC was considered to be the lowest concentration which prevented visible growth. The minimal bactericidal concentration (MBC) was read as the lowest concentration of drug in which there was no growth of the organism after plating 10 μl from each tube into chocolate agar and incubating for 18 h at 35°C with 5% CO₂.

To study the development of resistance to rifampin, two experiments were performed. In one experiment, the effect of subinhibitory concentrations of rifampin on growth of HIB was evaluated by overnight incubation in the presence of one-quarter to one-half MIC of rifampin for a given strain. After overnight incubation, the MIC was determined as described previously. HIB colonies were serially exposed to subinhibitory concentrations of rifampin in this fashion 8 to 10 times, with determination of the MIC each time.

In the second experiment, 1 ml of sMH broth with 10⁶ CFU of HIB per ml was added to chocolate agar plates containing rifampin (1.56 μg/ml). After 36 h of incubation at 35°C with 5% CO₂, if growth was noted, three to five colonies were picked, and each colony was subcultured separately on chocolate plate in the absence of rifampin. After 18 h of incubation, the MICs of colonies were again determined as before, using rifampin concentrations ranging from 1.56 to 100 μg/ml. To test if a second antibiotic like trimethoprim would prevent emergence of rifampin-resistant mutants, the second experiment was repeated, except that 1 ml of sMH broth with 10⁶ CFU of HIB per ml was added to chocolate agar plates containing 3.12 μg of trimethoprim per ml, with or without 1.56 μg of rifampin per ml.

RESULTS

All 14 HIB strains were susceptible to rifampin when an inoculum of 10⁶ CFU/ml was used. Seven strains were highly susceptible to rifampin with MICs of ≤0.1 μg/ml. Four strains were inhibited by 0.2 μg/ml, and the remaining three strains were inhibited by 0.4 μg/ml. The MBCs
of all strains tested exceeded the respective MIC values by two- to fourfold. Trimethoprim MICs for the 14 strains ranged from $\leq 0.05$ to 0.2 $\mu g/ml$, with MBCs of $>50$ $\mu g/ml$.

To investigate the possibility that subinhibitory concentrations of rifampin may induce development of resistance to this antibiotic, HIB strains were grown in sMH broth containing one-half and one-quarter concentrations of rifampin as compared with the respective MIC. Although all 14 strains were passaged 8 to 10 times in broth containing subinhibitory concentrations of rifampin, only two strains showed an increase in their MICs, from 0.1 to 0.4 $\mu g/ml$ and from 0.4 to 1.56 $\mu g/ml$, respectively. This increase in MIC might represent laboratory variation. In contrast, when large inocula ($10^8$ CFU) were plated into chocolate agar containing 1.56 $\mu g$ of rifampin per ml, 10 of 14 HIB strains showed growth after 36 h of incubation. The number of colonies of HIB ranged from 12 to 240 per plate. When random samples of these colonies were used as the inoculum ($10^6$ CFU) for repeat broth susceptibility determinations, all proved to be highly resistant to rifampin. Two strains had MICs of 12.5 $\mu g/ml$, compared with $\leq 0.1$ $\mu g/ml$ originally, and eight had MICs greater than 100 $\mu g/ml$, compared with $\leq 0.4$ $\mu g/ml$ previously. From the number of CFU plated and the number of colonies which subsequently grew, the frequency of mutation to rifampin resistance ranged from $1.35 \times 10^6$ to $1.4 \times 10^7$. The remaining four strains did not show any growth on chocolate agar plates with rifampin, although repeated attempts were made to induce resistance in these strains. When trimethoprim (3.12 $\mu g/ml$) was incorporated onto the chocolate agar plates, confluent growth of all 10 HIB strains was noted when an inoculum of $10^6$ CFU was used. In contrast, no growth was seen with any of the 10 HIB strains when the same inocula were plated onto chocolate agar containing rifampin (1.56 $\mu g/ml$) with trimethoprim (3.12 $\mu g/ml$). Thus, this combination prevented the emergence of rifampin-resistant mutants.

**DISCUSSION**

*H. influenzae* type b has been reported to be susceptible to rifampin. Bannatyne found that 100% of 26 strains tested were inhibited at rifampin concentrations of $\leq 0.5$ $\mu g/ml$ (2). Similar results were obtained by McDougal, who found the modal MIC for 30 HIB strains to be 0.5 $\mu g/ml$ (L. K. McDougal and C. Thornsberry, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C48, p. 282), and by Zweighaft, who demonstrated the median MICs and MBCs for rifampin in 11 HIB strains to be 0.3 $\mu g/ml$ (T. R. Zweighaft and G. H. McCracken, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, A4, p. 1). In the present study, all 14 strains studied were inhibited by $\leq 0.4$ $\mu g$ of rifampin per ml. In all of these experiments, an inoculum of $10^3$ to $10^5$ CFU per ml was used. With this inoculum size, the development of resistance to rifampin could not be demonstrated even when the bacteria were grown repeatedly in the presence of subinhibitory concentrations of this antibiotic. In contrast, we found that when an inoculum of $10^8$ CFU was plated on chocolate agar containing 1.56 $\mu g$ of rifampin per ml, 10 of 14 strains of HIB could be shown to have developed a small number of colonies which were highly resistant to rifampin. The rifampin concentration of 1.56 $\mu g/ml$ in chocolate agar was selected because it represents the concentration which was achieved in the saliva of children treated orally with 10 mg of rifampin per kg, a dose which is recommended for prophylaxis of HIB nasopharyngeal carriers (7).

It appears that the low number of HIB organisms present in the nasopharynx of carriers is the main reason that in vivo development of resistance to rifampin has been so rarely observed (8). We previously reported semiquantitative data concerning the number of HIB present in the nasopharynx of children. Most carriers had low numbers, usually less than $10^3$ CFU (10). In addition, Halsey reported in the infant rat model a density of nasopharyngeal colonization of $\geq 2.5 \times 10^4$ CFU of nasal wash per ml. When those rats were treated with rifampin, no emergence of resistant strains was noted (4). Although the number of colonized bacteria is probably $10^3$ to $10^4$ below the frequency of mutation to rifampin resistance found in the present studies (1 in $3.5 \times 10^6$ to 1 in $4 \times 10^6$), the recent report of emergence of rifampin-resistant HIB after prophylaxis suggests that such strains may emerge (8). Since we were able to prevent the emergence of rifampin-resistant mutants in vitro by adding trimethoprim to rifampin, administration of this agent together with rifampin may prove to be an effective means of preventing the development of resistance to rifampin.

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