Comparison of Antibacterial and Antiimmune Effects of Certain Rifamycins

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Comparison of the in vivo and in vitro immunosuppressive activities of the five rifamycins with their in vitro antibacterial and anti-ribonucleic acid polymerase activities indicated that correlation was poor. Examination of their activities on mitogen-induced blastogenesis in human peripheral blood leukocytes and inhibition of delayed-type hypersensitivity to partially purified protein derivative in immunized mice demonstrated that correlation was usually good. Antibacterial activity in cultures and the activities of the rifamycins inhibiting deoxyribonucleic acid-dependent ribonucleic acid polymerase appeared to correlate well. However, when these two types of activity, antiimmune and antibacterial, were compared, correlation was poor on occasion and indicated that the antiimmune activities and antibacterial activities of the rifamycins are probably not related.

It has been reported previously (6) that some members of the ansamycin class of antibiotics, including certain rifamycins and streptovaricins, share the well-recognized immunosuppressive properties of rifampin (3, 4, 8, 11). It was noted in that report that when the in vitro antibacterial activity of these substances was compared with their in vivo immunosuppressive activity, correlation was poor, suggesting that the two activities are unrelated.

The following is a report of studies of the immunosuppressant activity of five rifamycins, chosen because they had large differences in antibacterial activity. Comparison of the antibacterial and immunosuppressant activities of these compounds demonstrated that their in vitro antimicrobial activity was often unrelated to their in vivo and in vitro immunosuppressive effects.

MATERIALS AND METHODS

Rifamycins used. The following rifamycins were kindly supplied by Gruppo Lepetit, Milano, Italy (the minimal inhibitory concentrations required for *Mycobacterium tuberculosis*, as determined in Lepetit's laboratory, are within parentheses): rifampin (0.5 µg/ml), rifamycin SV (0.05 µg/ml), rifamide (0.2 µg/ml), 8 methyl-rifamycin (5 µg/ml), and rifamycin formo-S (5 µg/ml). (See Results.)

Rifamycins were prepared for use by dissolving them in a minimal volume of a 1:10 mixture of dimethyl sulfoxide (Me₂SO) (Mallinckrodt, St. Louis, Mo.) and fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.). This solution was further diluted with a volume of physiological saline sufficient to provide the desired concentration of drug. Solutions used for control studies contained a concentration of Me₂SO and fetal calf serum in saline equal to the drug-containing solutions.

Inhibition of in vitro blastogenesis. The effects of the rifamycins studied on mitogen-induced blastogenesis were assayed by using human peripheral blood leukocytes. Blood was drawn from healthy donors into heparinized tubes, and the leukocytes were isolated and cultured by the method of Thompson et al. (14) and by the microculture technique with 5×10^4 cells in 0.2 ml of Eagle medium supplemented with pooled human AB plasma, penicillin, streptomycin, and L-glutamine. When 5 µg of concanavalin A (Con A) (Calbiochem, San Diego, Calif.) per ml and 1 µg of phytohemagglutinin (PHA) (Wellcome Reagents Ltd., Beckenham, England) per ml were used, the mitogens were added to the cell cultures at the same time as the rifamycins. After 72 h of incubation, the cells were pulsed with [³H]thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and harvested with a Brandel model M12V cell harvestor (Brandel Inc., Rockville, Md.). The cell-incorporated radioactivity was counted in a Beckman liquid scintillation counter, model LS-100C (Beckman Instruments, Inc., Cambridge, Mass.) with toluene with Omnifluor (New England Nuclear Corp.).

To look for a nonspecific toxic effect of the various rifamycins, the effect of these substances on survival of cells in culture was determined. The cells were incubated under the same conditions as in the mitogen studies, with the same concentrations of the rifamycins. After 72 h of incubation with rifamycin, the cells were exposed to 0.2% trypan blue, and viability was visually determined by noting exclusion of the dye. More than 90% of the cells survived culture with 100 µg of either rifampin, rifamycin SV, 8 methyl-rifamycin, or rifamide per ml. Cultures with 10 µg of rifamycin formo-S per ml yielded at least 85% surviving cells.

Because it was also considered possible that the effect of the rifamycins on mitogen-induced blastogenesis could be the result of binding of the mitogen by the rifamycins, two experiments were conducted to assess this possibility. Rifamycin SV (one of the more active compounds studied) at 100 µg/ml was passed

alone or together with 50 μg of Con A per ml through Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden), packed in a column 1 by 20 cm with phosphate-buffered saline (pH 7.0). The column was then eluted with saline, and the progress of the colored band was noted. The measured volume of exclusion of rifamycin SV was the same in both experiments, and only a single band was observed in the column effluent regardless of the presence or absence of Con A.

In addition, 8 μg of rifamycin SV per ml and 120 μg of Con A per ml were dissolved in phosphate-buffered saline, pH 7.0, and dialyzed at 2°C across a semipermeable membrane (Union Carbide, Chicago, Ill.) impermeable to Con A. The movement of rifamycin SV across the membrane, as reflected by spectrophotometric measurement of the concentrations of antibiotic at the end of 24 and 72 h, dialyzed alone or mixed with Con A, was the same. Thus, binding of Con A to the rifamycins seems an unlikely explanation of their diminished activity as mitogens in these experiments.

In vivo studies. Assay of the in vivo effect of the rifamycins on delayed-type hypersensitivity (DTH) was performed in mice by methods described in detail elsewhere (6). In brief, the animals were immunized with the R_1R_v strain of *M. tuberculosis*, and the resulting immunity was tested by injecting 250 units of partially purified protein derivative (Connaught Laboratories, Willowdale, Ontario, Canada) into the hind footpad. The induced swelling was measured 24 h later with calipers. Rifamycins were administered intraperitoneally once a day (see Table 2 for doses), and their effects on DTH, as measured by inhibition of footpad swelling, was measured after 7 and 14 days of therapy. Therapy was then stopped, and after 7 drug-free days, DTH was measured again.

Susceptibility of bacteria to rifamycin. The methods used to determine the in vitro susceptibility of *M. tuberculosis* to the five rifamycins were identical to those previously published (6). Growth of the organisms was measured 7 days after adding the antibiotics. The in vitro susceptibility of *Listeria monocytogenes* (kindly provided by F. M. Collins, Trudeau Laboratories, Saranac Lake, N.Y.) to the rifamycins was determined in Typticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). The drugs were added at the time of inoculation, and the organisms were incubated for 24 h at 37°C. Bacterial growth was assessed visually.

Effect of rifamycins on DNA-dependent RNA polymerase. Deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase activity was measured by the method of Nakamura and Yura (9). *Escherichia coli*, strain K-12 (kindly supplied by Sam Donta, University of Iowa, Iowa City), was grown in medium E (16), supplemented with 0.5% glucose and 0.01% Difco Casamino Acids, at 37°C until it was in the logarithmic phase of growth. The culture was then centrifuged, and the harvested bacteria were washed and suspended in fresh medium with 1% Me₂SO. At time 0, the rifamycins to be studied (in a final concentration of 100, 50, or 10 μg/ml), together with nalidixic acid (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.), were added, and the final mixture was shaken at 37°C. After 2, 4, or 6 min, the bacterial suspensions were pulsed for 1 min with 5 μg of [³H]uridine (40 Ci/mmol; New England Nuclear Corp.). The reaction was

stopped, and the nucleoprotein was precipitated with 10% trichloroacetic acid. The nucleoprotein was harvested on a Millipore HA 0.4-μm filter (Millipore Filter Corp., Bedford, Mass.), washed with 5% trichloroacetic acid, and dried. The incorporated radioactivity was counted in the Beckman liquid scintillation counter, model LS-100C. Each determination of activity was done in triplicate.

Controls were provided by identical bacterial suspensions which were incubated without rifamycins. The results were recorded as a percent of the control (experimental results × 100 ÷ control values).

RESULTS

In vitro blastogenesis. The effects of the five rifamycins on mitogen-induced blastogenesis in cultures of human peripheral blood leukocytes are shown in Table 1. All of the rifamycins studied inhibited both PHA- and Con A-induced blastogenesis in this system, but there was a substantial difference in the relative activity of these compounds. Of the five compounds studied, rifamycin formo-S had the greatest suppressive effect with either mitogen. Both rifamide and rifampin had much less activity than the other rifamycins used in this system. Exposure of the cells to 100 μg of rifamide per ml produced less inhibition of mitogen-induced blastogenesis than exposure to 10 μg of rifamycin formo-S per ml or to 50 μg of rifamycin SV per ml. The results with rifampin were similar to those noted by Nilsson (10) in experiments with identical cells and PHA.

Effect of rifamycins on DTH in Mice. Treatment of mice with the five rifamycins resulted in a significant suppression of DTH as measured by their footpad reactions to partially purified protein derivative in several instances. This suppression was similar to that previously noted with other ansamycins (6).

However, one of the compounds used was inactive in the doses employed. As shown in Table 2, rifamide, in a daily dose of 200 mg/kg, had no detectable suppressive activity after 14 days. Rifampin, rifamycin SV, and 8 methylrifamycin appeared to be approximately equal in activity in this immunological model and produced a significant reduction in footpad swelling after 14 days of therapy. Rifamycin formo-S was active when the mice received a dose of 100 mg/kg per day. This was a dose of antibiotic which had been shown to be ineffective with rifampin used under identical conditions in previous experiments (6). Thus, it can be said that although the in vivo differences in activity were not great, rifamycin formo-S appeared to be the most active substance studied in vivo, as it was in vitro, and that rifamide was the least active substance studied in both systems.

Where immunosuppressive activity was

TABLE 1. *Effect of rifamycins on mitogen-induced blastogenesis[a]*

Mitogen	Rifampin		Rifamycin SV		8 Methyl-rifamycin		Rifamide		Rifamycin formo-S	
	100 µg/ml	50 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml	10 µg/ml	100 µg/ml	50 µg/ml	10 µg/ml	5 µg/ml
PHA	51.9 ± 2.0 (37)	106.6 ± 2.1 (77)	0.5 ± 0.2 (1.0)	122.7 ± 3.3 (89)	10.1 ± 8.5 (7)	114.0 ± 5.9 (82)	89.2 ± 2.7 (64)	119.1 ± 2.0 (86)	1.4 ± 0.6 (1.0)	112.9 ± 4.7 (81)
Con A	12.7 ± 1.7 (93)	23.5 ± 4.1 (174)	1.3 ± 2.0 (10)	20.7 ± 3.2 (154)	1.1 ± 1.0 (8)	18.3 ± 4.4 (136)	9.0 ± 3.0 (67)	23.3 ± 5.5 (181)	0.4 ± 0.3 (3)	2.2 ± 6.0 (16)

[a] cpm, Counts per minute; SD, standard deviation. Numbers within parentheses are percent of stimulated control.

TABLE 2. *Effect of rifamycin on hypersensitivity to partially purified protein derivative in mice*

Drug treatment (dose)[a]	Mean footpad diameter ± standard deviation (mm) at:		
	Day of treatment		Posttreatment[b]
	7	14	
Control	2.7 ± 0.2	2.7 ± 0.2	2.7 ± 0.1
Rifampin (200)	2.7 ± 2.0	2.4 ± 0.1[c]	2.7 ± 0.1
Rifamycin SV (200)	2.5 ± 0.2	2.4 ± 0.2[d]	2.6 ± 0.1
8 methyl-rifamycin (200)	2.7 ± 0.2	2.4 ± 0.2[d]	2.8 ± 0.1
Rifamide (200)	2.6 ± 0.2	2.6 ± 0.1	2.6 ± 0.1
Rifamycin formo-S (100)	2.6 ± 0.3	2.3 ± 0.1[c]	2.6 ± 0.2

[a] Each treatment group consisted of 10 animals, except the rifampin group which consisted of 9. Doses are shown as milligrams per kilogram of body weight per day.

[b] Drug treatment discontinued for 7 days.

[c] $P < 0.01$, based on Donnet's test.

[d] $P < 0.05$, based on Donnet's test.

found, it was noted to be reversible. Pretreatment levels of DTH in the suppressed animals, as measured by their response to partially purified protein derivative, returned 7 days after therapy was stopped (Table 2).

Antibacterial activity. Rifampin, rifamycin SV, and rifamide had essentially the same antimycobacterial activity and inhibited growth at a concentration of 0.02 µg/ml. This minimal inhibitory concentration of rifampin and rifamide required for mycobacteria was the same as that found by others (7, 13).

Neither 8 methyl-rifamycin nor rifamycin formo-S was as active as rifampin, rifamide, or rifamycin SV with *M. tuberculosis* (strain R_1R_v), and concentrations 1,000 times greater than those of rifampin were required to inhibit growth under the conditions used. Growth, as determined in our laboratory, was not inhibited by concentrations of 50 µg of 8 methyl-rifamycin per ml; 25 µg of rifamycin formo-S per ml was required to achieve this result.

The susceptibility of *L. monocytogenes* to the five rifamycins was also determined, and the pattern of activity of each of the five rifamycins was similar to the one obtained with *M. tuberculosis*. It was found that 0.01 µg of rifamycin SV per ml inhibited the in vitro growth of *L. monocytogenes*. Rifampin and rifamide inhibited this microorganism at a concentration of 0.1 µg/ml. Rifamycin formo-S and 8 methyl-rifamycin were the least active rifamycins tested, and concentrations of 10 and 100 µg/ml, respectively, were required to produce the same effect.

Effect on RNA polymerase. In an attempt to see if there was a correlation between the antiimmune or antibacterial activities of the five

rifamycins and the activity of these substances as inhibitors of bacterial DNA-dependent RNA polymerase, their effect on this enzyme was observed by using suspensions of *E. coli*. In general, the effect of these substances on DNA-dependent RNA polymerase appeared to parallel their antibacterial activity rather than their antiimmune effect. At a concentration of 50 μg/ml, rifampin, rifamide, and rifamycin SV inhibited polymerase activity after 2 min of incubation to about 50% of that of the control cell suspensions (48, 50, and 60% of control, respectively). Rifamycin formo-S and 8 methyl-rifamycin were not active at this concentration, but appeared to stimulate the enzymatic activity (157 and 106% of control, respectively). Similar studies with the five rifamycins, at concentrations of 100 and 20 μg/ml and adding the rifamycins at 2, 4, and 6 min after initiation of the reaction, yielded similar results. For example, when 100 μg of rifampin or rifamycin SV per ml were added, DNA polymerase activity at 2 min was 23 and 27% of the control, respectively, but the rate with rifamycin formo-S at the same concentration was 89% of control.

Although these experiments with RNA polymerase are not elaborate, they indicate that there were substantial differences in the abilities of the five rifamycins to inhibit DNA-dependent RNA polymerase in vitro. There also appears to be a reasonable correlation between their activity in this regard and their antibacterial activity observed with *M. tuberculosis* and *Listeria*, but not with their activities with the two immune systems studied.

DISCUSSION

Comparison of the activities of the five rifamycins used in these experiments indicates that there was poor correlation between their immunosuppressive effects and their antibacterial activity and inhibition of DNA polymerase. Two of the compounds, 8 methyl-rifamycin and rifamycin formo-S, were relatively weak antibacterial substances, but inhibited DTH in mice and mitogen-induced blastogenesis in cell cultures as well as or better than any rifamycin tested. Rifamycin SV was relatively active as an antibacterial substance and also inhibited both DTH and mitogen-induced blastogenesis under the conditions used. As compared with rifamycin SV, rifamide was as active as an antibiotic, but much less active as an immunosuppressant.

Comparison of the inhibiting activity of the rifamycins observed with *E. coli* DNA polymerase with their antibacterial activity observed with *M. tuberculosis* and *Listeria* indicated that

correlation of the two activities is good. The mechanism of action of rifampin in bacteria, including *M. tuberculosis*, has been shown to be the result of the binding of rifampin to DNA polymerase with inhibition of RNA synthesis (12, 15, 17). In this regard, it would seem reasonable to speculate that the antienzyme and antibacterial activities of the rifamycins are linked and that their antibacterial activity is related to their activity with this or a similar enzyme in both *M. tuberculosis* and *Listeria*.

It has also been observed that a permeability barrier may be another factor in rifampin resistance in mycobacteria (5). This reported permeability barrier was diminished when Tween 80 was added to the medium used to determine rifampin susceptibility (5). The medium used in the study reported here contained Tween 80, suggesting that a permeability barrier was not an important factor in the results of these experiments.

Correlation between suppression of mouse DTH and mitogen-induced blastogenesis in cell culture by the five rifamycins was good, with the possible exception of rifampin. Rifampin in vitro was less active than rifamycin SV, but slightly more active than rifamide. Rifamide was the least active substance studied, particularly when PHA was used as a mitogen. As compared with its effect on DTH in the mouse, rifampin seemed to be about as active as 8 methyl-rifamycin or rifamycin SV. This difference between the in vivo and in vitro antiimmune activity of rifamycin cannot be explained with the data available.

On the basis of the data presented here, screening of the ansamycins for immunosuppressive activity might be accomplished by determining their ability to inhibit mitogen-induced blastogenesis in cultures of human peripheral blood leukocytes, since this assay is reliable, simple, and uses only small amounts of drugs. Whereas rifampin was found to be relatively less active in vitro as a suppressor of Con A-induced blastogenesis than in its effect on DTH in vivo, it was clearly more active than rifamide when PHA was used as a mitogen. Nevertheless, the in vivo and in vitro results with rifampin indicate that determination of suppression of mitogen-induced blastogenesis is not a perfect predictor of in vivo activity.

It has been stated that the immunosuppressive activity of rifampin is the result of the inhibition of protein synthesis in mammalian cells (1, 2); the data presented here are compatible with that proposal since it is possible that the rifamycin analogs tested could have different activities in this regard.

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