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Immunosuppressant Activity of the Ansamycins

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The immunosuppressive effect of four analogues of rifampin and two streptovaracins on cell-mediated immunity has been determined. Tuberculin hypersensitivity in the footpads of immunized mice was inhibited by three of the rifampin analogues and by both streptovaracins. The observed in vivo immunosuppressive activity of the compounds tested was not correlated with their in vitro activity against mycobacterial growth but was associated with their toxicity in mice. These data indicate that some of the analogues of rifampin and the streptovaracins can significantly suppress cell-mediated immunity and suggest that other ansamycins may have significant immunosuppressant activity.

Rifampin is a widely used antituberculous drug that has been shown to have immunosuppressant properties under certain conditions and to have the availability to inhibit a variety of immune reactions in several species (8, 11, 16, 17). In guinea pigs, for example, rifampin has been shown to suppress the skin reactions to chlor-dinitrobenzine (2) and to purified protein derivative (PPD) (2, 4), and it also suppresses the antibody response to sheep erythrocytes (2). Thus, the antibiotic can inhibit both cell-mediated immunity (CMI) and antibody-mediated immunity.

In humans, conventional antituberculous doses of the drug have been reported to partially suppress the skin reaction to PPD (7, 14) and the antibody response to keyhole limpet hemocyanin and *Salmonella typhi* vaccine (3). It also has been reported that the administration of rifampin to humans produces reversible light-chain proteinuria (3) and that the number of thymus-dependent lymphocytes is reduced in patients who receive the drug (5).

Although rifampin is the only member of the rifamycin group of antibiotics used clinically, it has been possible to synthesize a large number of other rifamycins that vary in antibiotic potency and toxicity. The rifamycins are, in turn, members of a more diverse group of antibiotic substances, the ansamycins, so called because all members of this group contain the ansa ring as a central chemical structure. Included among the ansamycins are the straptovaracins, geldamycin, streptolydigin, and tolypomycin (18). In spite of the fact that some of the ansamycins have been available for years, little has been reported concerning their effect on immunity.

To determine whether some of the analogues of rifampin or whether other ansamycins could suppress CMI, several of the rifamycins and two streptovaracins were investigated, noting their effect on the cell-mediated reaction to PPD in foot pads of mice previously immunized with mycobacteria.

(A preliminary report of some of these data was given at the International Conference on Lung Diseases, Montreal, Quebec, Canada, 18 to 21 May 1975.)

MATERIALS AND METHODS

Immunosuppressant activity. CF_1 female mice (Carworth Farms, Wilmington, Mass.), weighing approximately 20 g were injected intravenously with 10^7 viable units of R_1R_v strain of *Mycobacterium tuberculosis*. One month later the animals were tested for induced delayed-type hypersensitivity by the injection of 0.05 ml of Tween-stabilized PPD (250 U.S. units, Connaught Laboratories, Willowdale, Ont.) intradermally in the hind footpad. The opposite foot was injected with a diluent to serve as a control. The reaction was measured with a caliper (Werner caliper, Scientific Products, Chicago, Ill.) in millimeters at 24 h. Those animals with a positive test (increase in footpad thickness greater than 0.6 mm) were selected and randomized into groups. Separate groups were treated with each of the following compounds: rifampin (Dow Chemical Co., Zionsville, Ind.), AF/ABDP (2,6-dimethyl, 4-benzyl, 4-demethyl rifampin), AF/ABP (3-piperdinoiminomethyl rifamycin SV), and AF/ABP [3-(4-benzylpiparazinoiminomethyl) rifamycin SV] (all provided by Gruppo Lepetit, Milan, Italy). Crude streptovaracin and its purified derivative, streptovaracin C (supplied by Upjohn Laboratories, Kalamazoo, Mich.), were also studied.

The drugs were dissolved in dimethyl sulfoxide

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(Mallinckrodt, St. Louis, Mo.) and fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), in a ratio of 1:10. This solution was further diluted with an appropriate amount of physiological saline to provide the dose of drugs to be used in a constant volume of 0.5 ml. The control animals received 0.5 ml of a solution that contained Me_2SO and fetal calf serum in the highest concentration. The drugs were administered daily by intraperitoneal injection, except the streptovaracins, which were given subcutaneously. The drug doses in milligrams per kilogram of body weight, duration of treatment, and number of animals used are listed in Tables 1, 2, and 3.

Animals were tested with PPD prior to the initiation of the study, at 7-day intervals during drug administration, and 1 week after the drugs were discontinued.

The in vitro antibacterial activity of the compounds was determined in a liquid Tween-albumin medium with the R_1R_v strain of *M. tuberculosis* as the test organism. The growth of the cultures, as determined by nephelometric assay, was allowed to enter early log phase (40 nephlos units), and the drugs were added in amounts sufficient to give final concentrations of 100, 10, 0.1, and 0.01 μg per ml.

RESULTS AND DISCUSSION

Tables 1, 2, and 3 list the effects of the administration of rifampin, rifampin analogues, and the streptovaracins on CMI in mice. As can be seen, rifampin, AF/API, AF/ABDP, AF/ABP, and the streptovaracins suppressed the PPD footpad reaction in mice immunized with attenuated mycobacteria.

The dose of rifampin required to suppress the footpad reaction to PPD in this animal species was large when compared with the dose required to produce a similar effect in guinea pigs (2). In guinea pigs 20 mg of oral rifampin per kg partially suppressed the intradermal reaction to PPD in 14 days, but in mice 200 mg of parenteral rifampin per kg per day was required to produce significant suppression of that reaction (Table 1).

The reasons for this difference in sensitivity to rifampin by these two species of rodents are not clear. Serum rifampin levels in mice and guinea pigs have been reported by others (10, 15). In general, it appears from these previously published data that the concentrations of rifampin in mice, after oral administration, are higher than those obtained in comparably treated guinea pigs. In addition, rifampin was eliminated from blood in mice more slowly than in guinea pigs (15). Thus, it appears that differences in the rates of metabolism of rifampin in the two animal species are unlikely explanations for the observed difference in sensitivity to rifampin.

In mice, as in guinea pigs, this effect of rifampin on CMI was reversible. Cessation of therapy was usually followed by a return of PPD hypersensitivity, as indicated by an increased reaction to PPD when the animals were tested 7 days after the drug was discontinued. Rifampin administered in a dose of 300 or 200 mg/kg, but not 150 mg/kg, suppressed the footpad reaction, and the dose of 300 mg/kg appeared to result in some delay in the return of the reaction to normal (Tables 1 and 2).

Three of the four analogues of rifampin tested had an immunosuppressive effect (Tables 1 and 2) in mice. These analogues suppressed the mouse footpad test at a dose that was approximately one-fourth the dose of rifampin required to produce the same effect. Compounds AF/API, AF/ABDP, and AF/ABP inhibited CMI when given in a daily dose of 50

TABLE 1. *Effect of rifamycins on PPD hypersensitivity in mice*

Treatment groups[a]	Mean footpad diameter ± standard deviation (mm)		
	Days of treatment		Posttreatment[b]
	7	14	
Control	2.8 ± 0.2	2.8 ± 0.1	2.7 ± 0.1
Rifampin 300[c]	2.4 ± 0.1[d]	2.3 ± 0.1[e]	2.5 ± 0.3
Rifampin 150	2.5 ± 0.2	2.7 ± 0.1	2.7 ± 0.1
Rifampin 50	2.7 ± 0.1	2.6 ± 0.2	2.7 ± 0.1
Rifamycin SV 300	2.4 ± 0.2	2.6 ± 0.2	2.6 ± 0.2
Rifamycin SV 150	2.6 ± 0.1	2.6 ± 0.1	2.7 ± 0.1
AF/ABDP 50	2.5 ± 0.1	2.2 ± 0.1[e]	2.3 ± 0.1[e]
AF/API 50	2.3 ± 0.1[e]	2.4 ± 0.1[d]	2.6 ± 0.2
AF/API 25	2.6 ± 0.2	2.4 ± 0.2	2.7 ± 0.4
AF/ABP 50	2.3 ± 0.1[e]	2.3 ± 0.1[e]	2.6 ± 0.2
AF/ABP 25	2.5 ± 0.2	2.5 ± 0.2	2.5 ± 0.2

[a] Eight animals in each group.
[b] Drugs discontinued for 7 days.
[c] Milligrams per kilogram per day.
[d] $P < 0.05$, based on t statistic.
[e] $P < 0.01$, based on t statistic.

TABLE 2. *Effect of rifamycins on PPD hypersensitivity in mice*

Treatment groups[a]	Mean footpad diameter ± standard deviation (mm)			
	Days of treatment			Posttreatment[b]
	7	14	21	
Control	2.7 ± 0.1	2.7 ± 0.2	2.6 ± 0.2	2.7 ± 0.2
Rifampin 300[c]	2.4 ± 0.1[d]	2.2 ± 0.1[e]	2.2 ± 0.1[e]	2.4 ± 0.1[d]
Rifampin 200	2.4 ± 0.2[d]	2.4 ± 0.2[d]	2.4 ± 0.2[d]	2.6 ± 0.2
Rifampin 100	2.7 ± 0.2	2.6 ± 0.2	2.5 ± 0.2	2.7 ± 0.1
ABDP 50	2.8 ± 0.1	2.4 ± 0.2[d]	2.3 ± 0.1[e]	2.5 ± 0.3
ABDP 25	2.8 ± 0.1	2.6 ± 0.2	2.5 ± 0.3	2.6 ± 0.2

[a] Nine animals in each group.
[b] Drugs discontinued for 7 days.
[c] Milligrams per kilogram per day.
[d] $P < 0.05$, based on t statistic.
[e] $P < 0.01$, based on t statistic.

mg/kg and produced a suppressant effect similar to that produced by the daily administration of 300 mg of rifampin per kg. As with rifampin, the effect of the analogues was reversible and delayed-type hypersensitivity usually returned after the administered drug was stopped. Administration of compound AF/ABDP was followed by some delay in the return of CMI when given for either 14 or 21 days (Tables 1 and 2), but in the case of the other compounds the PPD reaction returned to pretreatment levels after their administration had been stopped for 7 days.

To see whether the administration of some of these compounds could be effective at a smaller dose if administered for a longer period of time, rifampin and compound AF/ABDP were given for a total of 21 days in another experiment (Table 2). As can be seen, the additional 7 days of therapy did not alter the results obtained. In most instances, when suppression occurred, it was noted between 7 and 14 days.

Rifamycin SV had no effect on delayed-type hypersensitivity in this system. Although mean footpad swelling appeared to be smaller in animals receiving this compound than in the controls, statistical analysis, using the paired *t* test (6), failed to demonstrate a significant difference. It appears that, under the conditions employed, rifamycin SV had less effect on CMI than rifampin or the other rifamycins tested.

Thus, it appears that several analogues of rifampin are immunosuppressant when administered to mice in a high dose. There has been a report of similar studies by another investigator who used a different assay system.

Osoba (9) has studied the effect of rifampin and several rifamycins on immunity using the in vitro plaque-forming cell assay. In this system he found that rifampin and 4-demethyl rifampin at concentrations of 40 μg/ml inhibited the reaction, but two of the compounds found to be active by us in mice, AF/ABDP and AF/ABP, did not have immunosuppressive activity in vitro using the plaque assay system.

Table 3 lists the results of the administration of crude streptovaracin or streptovaracin C in PPD hypersensitivity in mice. Both compounds were immunosuppressive. The dose of streptovaracin required to produce an effect was less than that of rifampin, although the difference was not large. As with the rifamycins, the suppression of immunity associated with streptovaracin administration was usually reversible when the drugs were stopped.

These findings must be interpreted with caution. Crude streptovaracin contains a nonspecific cytotoxic factor that might have an effect on immunity (personal communication, Upjohn Laboratories). This cytotoxic factor has been shown to be eliminated from the purified compound streptovaracin C and, since streptovaracin C suppressed the footpad test, it would imply that the immunosuppressive activity noticed with both streptovaracins was the result of inherent activity and not due to contamination.

TABLE 3. *Effect of streptovaracin on PPD hypersensitivity in mice*

Treatment groups[a]	Mean footpad diameter ± standard deviation (mm)			
	Days of treatment			
	7	14	21	28
Control	2.7 ± 0.2	2.7 ± 0.2	2.6 ± 0.1	2.7 ± 0.1
Crude streptovaracin 150[b]	2.4 ± 0.2[c]	2.2 ± 0.1[d,e]	2.3 ± 0.1[d]	2.4 ± 0.1[c]
Crude streptovaracin 50	2.6 ± 0.2	2.4 ± 0.2[c]	2.6 ± 0.2[e]	2.6 ± 0.1
Streptovaracin C 150	2.5 ± 0.3	2.3 ± 0.1[d,e]	2.3 ± 0.1[d]	2.6 ± 0.2
Streptovaracin C 50	2.6 ± 0.2	2.6 ± 0.2	2.5 ± 0.1[e]	2.7 ± 0.2

[a] Eight animals in each group.
[b] Milligrams per kilogram per day.
[c] $P < 0.05$, based on *t* statistic.
[d] $P < 0.01$, based on *t* statistic.
[e] Drugs discontinued.

The studies of the in vitro antibacterial potency of the compounds used were initiated to see whether there was a correlation between in vitro antimycobacterial activity and in vivo immunosuppressive effect. All of the compounds assayed were found to inhibit mycobacterial growth, confirming similar previous studies that had been reported by others (10, 13, 15). Under the conditions employed, compound AF/ABDP and both streptovaracins inhibited mycobacterial growth of R_1R_v strain of *M. tuberculosis* at a concentration of 0.1 μg/ml. Rifampin, rifamycin SV, and the other AF compounds were more potent than AF/ABDP and inhibited growth of the R_1R_v strain of *M. tuberculosis* at 0.01 μg/ml.

It appeared from these studies that there was no correlation between in vitro antimycobacterial activity and in vivo immunosuppression. For example, rifamycin SV inhibited in vitro mycobacterial growth at a lower concentration than compound AF/ABDP, but rifamycin SV had no significant effect on in vivo CMI. AF/ABDP inhibited CMI in mice and was more active than rifampin in that respect.

To determine the toxicity of these compounds, the experiments with the rifamycins and streptovaracins were designed to permit demonstration of the effect of a wide range of

doses in immunized animals and at the same time to show suppression of CMI. It was clear from these studies that toxicity and immunosuppression were correlated. Daily injections of various doses of rifampin, its analogues, and the streptovaracins in mice indicated that rifampin and rifamycin SV were the least toxic of the substances tested. Streptovaracin C and crude streptovaracin were more toxic than rifampin. The AF compounds, found to be the most potent immunosuppressives, were also the most toxic drugs used. In general, significant (>20%) numbers of deaths occurred among the mice when any of the compounds were given in doses two to three times as great as the dose needed to produce immunosuppression (Tables 1, 2, or 3). The doses of compounds found to be immunosuppressive, but not lethal, were associated with some obvious signs of distress in the animals, such as loss of body weight, ruffing of the fur, and ascites, but recovery occurred when the administration of the drugs was stopped. The cause of death in those animals receiving toxic doses of drugs was not ascertained, and as a result it cannot be stated whether their death was related to suppression of immunity or to a more direct toxic effect of the drugs.

Thus, it appears that the property of immunosuppression may occur among different rifamycins and the streptovaracins. Five of the six rifamycins tested suppressed footpad reaction to PPD in mice in doses that appeared to be tolerated by the mice over the period of study.

The observed immunosuppressive effect of the streptovaracins leads one to speculate that other ansamycins may also have immunosuppressive activity. The ansamycins have many common properties. Bacterial cross-resistance between the rifamycins and several other ansamycins has been reported, and it seems clear that the antibacterial mechanism of action of the rifamycins, the streptovaracins, and certain other ansamycins is the result of the inhibition of deoxyribonucleic acid-dependent ribonucleic acid polymerase (18). In the cases of the rifamycins and the streptovaracins, it is now possible to add inhibition of cell-mediated immune reactions to the list of properties shared by these compounds.

It is tempting to compare the potency of the compounds reported here. Unfortunately, this comparison would fail to consider such factors as absorption, protein binding, and the rates of excretion, all of which might affect blood levels of free drug and, in turn, suppression of immunity in the intact animal. It can be stated only that the dose required to suppress in vivo immunity was variable and that the difference was considerable on occasion.

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