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Further Studies on B.663 in Murine Leprosy

Absence of Resistance of *M. lepraemurium* to B.663 and Delay in Development of Resistance to Isoniazid[1]

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B.663, a rimino compound of Barry's phenazine series (1) (Fig. 1), has been reported from this laboratory to possess an unusual long-lasting activity in the suppression of murine leprosy (7). Among various antituberculosis drugs that have been tested, B.663 has been the only one to hold the infection in animals in check for as long as 816 days. An additive action was observed when the animals were treated concurrently with isoniazid. Mice inoculated with organisms obtained from animals treated with the combination of B.663 and isoniazid for a period of 240 days showed a favorable response to B.663, equal to that of mice infected with organisms from untreated animals. The response to isoniazid alone in mice previously treated with a combination of B.663 and isoniazid, was marked but not as effective as in previous experiments in which organisms from untreated animals were used as the inocula. This finding indicates that *Mycobacterium lepraemurium* has acquired some degree of resistance to isoniazid, but not to B.663 (7).

Browne and Hogerzeil (3) have reported effectiveness of B.663 in the treatment of human leprosy. However, development of resistance of *M. leprae* to this drug (judged by the appearance of the bacilli), was observed after treatment for one year.

In a recent publication Browne (2) stated that the reappearance of solid-staining bacilli after 12 months' treatment with B.663 alone, which had led him to conclude that resistance of *M. leprae* to B.663 occurred, was a transient phenomenon, the solid-staining rods gradually disappearing in the course of three to four months. The eventual bacterial and clinical improvement in these patients seemed unaffected by this episode. Therefore, true resistance to B.663 was not observed in his clinical trial.

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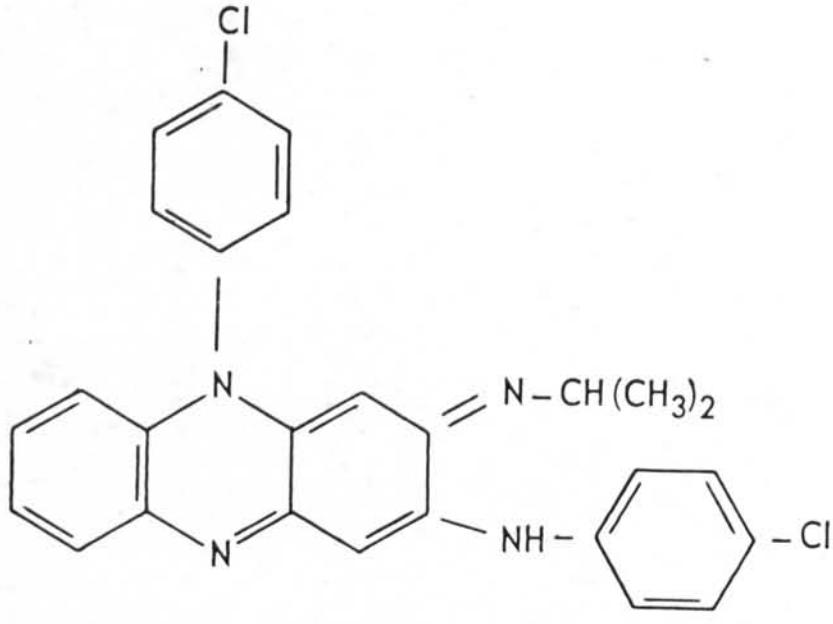

FIG. 1. Chemical structure of B.663, 2-(4-chloranilino)-3-isopropylimino-5-(4'-chlorophenyl)-3, 5-dihydrophenazine.

Since emergence of resistance of *M. lepraemurium* to B.663 has not been observed with the combined treatment of B.663 and isoniazid for a short period of 8 months, it would be interesting to know if resistance will develop when treatment has been given for a much longer time. This paper deals with the results of such a study following combined drug therapy for a period of 816 days.

METHOD

As reported previously ([7]), studies have been undertaken to measure the survival time of mice that were infected with murine leprosy and treated with different doses of B.663 and isoniazid, given either singly or in various combinations. One group of animals was treated with a combination of B.663 (0.005% in the diet) and isoniazid (0.01% in the diet) continuously until the deaths of all the animals (Group 6 of Table 2 ([7])). On the 816th day the last mouse of this group was sacrificed, although still in good condition. Macroscopic leprosy lesions were not observed; short acid-fast organisms, however, were found in smears made from the liver and omentum. A tissue homogenate was prepared from the omentum and pelvic fat. It was slightly yellowish-red and contained a few acid-fast bacilli. Subcutaneous inoculations of 0.5 ml. of the homogenate were made in three mice. Palpable subcutaneous lesions were noticed in 6 months, and generalized extensive lesions developed later. Animals were sacrificed on the 343rd day of the infection. Material from two of the animals was used to infect 5 groups (20 mice per group) of NIH general purpose female mice, weighing 16 to 20 gm. each. The groups were placed under treatment, with two different doses of B.663 and isoniazid, separately, to determine if resistance to either of these doses would develop. Autopsy was performed on all animals after 3 months of treatment. The technic of the chemotherapeutic assay has been reported elsewhere ([5]). The average weight of the omenta and pelvic fatty pads, and a "leprosy index," which is an average evaluation of the gross lesions in various sites and organs, formed the bases of the assay.

RESULTS

B.663 showed a marked suppressive activity on murine leprosy in the present

TABLE 1. *Development of drug resistance in murine leprosy. Animals previously treated with a combination of B.663 and isoniazid for a period of 816 days.*

Drug	Dose in diet %	No. mice died/ used	Body wt. gm.	Wt. of omentum gm.	Wt. of pelvic fat gm.	Leprosy index
Leprosy control untreated		0/20	26.8	0.12	1.32	5.5
B.663	0.005	0/20	27.5	0.05	0.92	0.2
B.663	0.01	1/20	27.6	0.10	0.78	0.1
Isoniazid	0.01	0/20	27.1	0.09	0.91	3.4
Isoniazid	0.02	1/20	26.7	0.09	0.98	4.4

study. As shown in Table 1, the weights of omenta and pelvic fatty pads of the two groups of B.663-treated animals were less than those of the untreated leprosy controls. The leprosy indices of the two treated groups were 0.2 and 0.1 respectively, while that of the controls was 5.5. Isoniazid showed only slight suppressive activity. The leprosy indices of the two isoniazid-treated groups were 3.4 and 4.4 respectively.

For studies of the development of resistance of *M. lepraemurium* in the long-term experiments, it is necessary to compare the findings of the present study with those observed previously. A summary of the leprosy indices of various related groups of animals of both the present and previous experiments is shown in Table 2.

The suppressive activity of B.663 remained the same in both the present and previous studies, as shown by the leprosy indices of B.663-treated animals of 0.2 and 0.1 for the former (Table 2, Expt. 5) and 0.4 and 0.5 for the latter (Table 2, Expt. 2). This indicated that there was no development of resistance to B.663 by *M. lepraemurium* that had been treated previously with the combination of B.663 and isoniazid for a period of 816 days.

Differences were observed, however, in the findings of isoniazid-treated animals between the present and the previous experiments. In the experiment in which the animals were infected with *M. lepraemurium* from untreated mice, isoniazid showed marked activity, as indicated by low leprosy indices, 1.0 and 0.7, in comparison with the respective indices 8.4 and 9.7 for the controls (Table 2, Expt. 1). When murine leprosy infection was treated with isoniazid alone for a period of 407 days, *M. lepraemurium* became completely resistant to the drug. Animals infected with organisms obtained from such animals revealed a leprosy index of 10.4 for the isoniazid-treated group and 9.2 for the controls (Table 2, Expt. 3).

Development of slight resistance to isoniazid was observed in animals infected with murine leprosy and previously treated with a combination of B.663 and isoniazid for a short period of 8 months. Animals infected with organisms obtained from such animals showed a leprosy index of 2.8 for the isoniazid-treated and 14.9 for the controls (Table 2, Expt. 4). Marked resistance to isoniazid was observed when the previous treatment of the combination of B.663 and isoniazid was extended to a total of 816 days. Animals infected with organisms obtained from such animals revealed leprosy indices of 3.4 and 4.4 for the isoniazid-treated, in comparison with 5.5 for the controls (Table 2, Expt. 5).

It is interesting to note that although marked resistance to isoniazid had developed at this time, the resistance was, nevertheless, not complete. The antimicrobial activity of isoniazid, although less, was still evident at this stage; this picture was quite different from the findings observed in animals infected with organisms from animals that had been treated with isoniazid alone for a period of only 407 days (i.e., about half the time used for combined therapy). Furthermore, development of resistance to isoniazid has been observed by other investigators in murine leprosy in rats following a shorter period of time of administration of the drug, e.g., 6 months by Bushby and

TABLE 2. *Summary of related experiments on drug resistance in murine leprosy*

Experiment No.	Drug in diet %		Average survival time days	Time of transfer after infection days	Time, new animals infected after transfer days	Treated again with same drug singly 3 months	Leprosy index: treated / control
	B.663	Isoniazid					
1[a]		0.01					1.0 / 8.4 ; 0.7 / 9.7
2[b]	0.005						0.4 / 8.4 ; 0.5 / 9.7
3[c]		0.01	279	407[f]		Isoniazid, 0.01%	10.4 / 9.2
4[d]	0.005	0.01	509	816	158	B.663, 0.01% Isoniazid, 0.01%	0.7 / 14.9 2.8 / 14.9
5[e]	0.005	0.01	581	816	393	B.663, 0.005% B.663, 0.01% Isoniazid, 0.01% Isoniazid, 0.02%	0.2 / 5.5 0.1 / 5.5 3.4 / 5.5 4.4 / 5.5

[a] 3-month experiment (7).
[b] 3-month experiment (7).
[c] Treatment continued until death of all animals (6).
[d] Treated for the first 8 months and the animals were observed until their deaths (7).
[e] Treatment continued until death of all animals. Ref. 7 and the present study.
[f] At this stage the animal had lesions extensive enough to inoculate two groups of animals for the second 3-month treatment.

Barnett (4) and 2 to 4 months by Nishimura and Masuda (9). Thus, previous treatment with the combination of B.663 and isoniazid markedly delayed the emergence of resistance of *M. lepraemurium* to isoniazid.

DISCUSSION

It is clear that the activity of isoniazid was markedly prolonged by the concurrent use of B.663 in the animals. Advantages of the extended activity are obvious, since isoniazid has been reported by many investigators to have a favorable effect in the clinical treatment of leprosy, despite the development of resistance by *M. leprae* to the drug following only a few months of treatment. With the concurrent use of B.663, it seems possible that the effect of isoniazid will be extended to a longer period of time in the treatment of human leprosy.

B.663 maintained its potency for 816 days with the concurrent use of isoniazid. It is

of interest to know if its potency will remain unchanged without the concurrent treatment with isoniazid. Unfortunately, data are not available to clarify this point in the present study, since an animal transfer was not made in the group of animals treated with B.663 alone for 816 days in the previous study; however, studies of mycobacterial infections other than murine leprosy have indicated that development of resistance to B.663 has been observed in both experimental and clinical infections in which the drug was given alone. Grumbach ([8]) reported that emergence of resistance of *M. tuberculosis*, H37Rv, to B.663 has been observed in mice following previous treatment with B.663 for only 3 to 4 months. Browne and Hogerzeil ([3]) have claimed that *M. leprae* developed resistance to B.663 in human leprosy after one year of treatment. It is likely, therefore, that the concurrent use of isoniazid may have protected *M. lepraemurium* from the development of resistance to B.663 in the present study.

As reported previously, eradication of mouse leprosy is observed in animals treated with both B.663 and isoniazid, but not with either drug given alone. The extent of skin pigmentation, resulting from accumulation of B.663 in the tissues, is reduced somewhat by concurrent treatment with isoniazid. These findings suggest that isoniazid has mobilized the stored B.663 in some way by turning it into a more active form, or perhaps by altering its solubility and thereby increasing its blood concentration. With such an interaction between B.663 and isoniazid in the animals, a mutual protection against the development of resistance was obtained when a combined therapy was administered.

Thus, by combination of B.663 and isoniazid, the full antimycobacterial activity of each agent can be maintained for a long period of time. This combination seems to offer an unusual opportunity for the treatment of leprosy.

SUMMARY

Continued study of the development of resistance of *Mycobacterium lepraemurium* to B.663, a phenazine derivative, and isoniazid, was made in mice in a long-term experiment.

Murine leprosy was induced in mice with bacilli from animals previously treated with a combination of B.663 and isoniazid for an 816 day period. These newly infected mice were then treated with either B.663 or isoniazid. The preconditioning of the murine leprosy bacilli by a combination of the two drugs appeared to prevent the development of resistance of *M. lepraemurium* to B.663, and to cause a marked delay in the development of resistance to isoniazid.

RESUMEN

Fueron realizados en un experimento de larga duración, estudios continuados del desarrollo de la resistencia del *Mycobacterium lepraemurium* al B.663, un derivado de la fenazina, y la isoniazida.

La lepra murina fué inducida en las ratas con bacilos provenientes de animales previamente tratados con una combinación de B.663 e isoniazida por un período de 816 días. Estos animales infectados a nuevo, fueron entonces tratados sea con B.663 o con isoniazida. El preacondicionamiento del bacilo murino leproso por una combinación de las dos drogas, parece prevenir el desarrollo de la resistencia del *M. lepraemurium* al B.663, y causa un marcado retardo en el desarrollo de la resistencia a la isoniazida.

RÉSUMÉ

La suite d'une étude sur le dévelopement de la résistance de *Mycobacterium lepraemurium* au B.663, un dérivé de la phenazine, ainsi qu'à l'isoniazide, a été mené chez des souris au cours d'une expérience de longue haleine.

La lèpre murine a été transmise aux souris en utilisant des bacilles obtenus chez des animaux traités auparavant par une combinaison de B.663 et d'isoniazide durant une période de 816 jours. Ces souris récemment infectées ont alors été traitées soit par le B.663 soit par l'isoniazide. L'exposition préalable des bacilles de la lèpre murine à une combinaison de ces deux produits semble empêcher le dévelopment de la résistance au B.663 chez *M. lepraemurium*, et causer un retard marquê dans le développment de la résistance à l'isoniazide.

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