

Effect of B.1912, A New Riminophenazine Derivative In Murine Leprosy¹

Y. T. Chang²

B.663, a riminophenazine derivative of Barry's phenazine series, has been reported to have marked suppressive activity in experimental infections of *Mycobacterium tuberculosis* (2), *M. lepraemurium* (8, 9, 10) and *M. leprae* (14). Clinical trials in the treatment of leprosy made throughout the world in a total of 718 cases in 22 countries have proved that B.663 is as effective as the standard drug, diaminodiphenyl sulfone or DDS (3, 4, 16). It is also effective in cases resistant to DDS (13). In addition, B.663 exhibits an anti-inflammatory effect in patients suffering from lepra reaction, a severe complication which frequently aggravates the infection (5). One disadvantage of B.663 is a red to dark brown coloration of the skin due to drug deposition (4), which has caused some patients to refuse continued treatment (12). Search for less cumulative phenazines has led Barry to synthesize a new compound, 2-anilino-3 cyclohexylimino-5-phenyl-8-chloro-3, 5-dihydrophenazine, or B.1912, which shows a lower tissue level but a higher serum level than B.663 (3). The present report deals with a comparative study on the effect of B.663 and B.1912 in murine leprosy.

MATERIALS AND METHODS

Female mice (Swiss albino; weight 16-20 gm.) of the general purpose strain of the National Institutes of Health were used. Each mouse was inoculated with 0.5 ml. of a 1:30 (weight:volume) suspension in normal saline of the omenta and pelvic fat pads from mice which had been infected with the Hawaiian strain of *M. lepraemurium* five months previously. B.663 and

B.1912 were mixed in powdered Purina rat chow and administered orally. Two doses of each drug were used. A total of four experiments was performed. Treatment was begun on the day of inoculation and continued for three weeks in one experiment and three months in the other. A third experiment involved delayed treatment in which drugs were given two months after infection and continued for three months thereafter. In experiment four, animals were treated for the first five months of infection, then observed for another five months without treatment to see if there was any bactericidal activity. The number of animals used for each group was 20 in the three-month and delayed treatment experiments and 10 in the three-week and bactericidal (fourth) experiment. Autopsy was performed on animals killed at the terminations of the experiments and on those which died during the long period of observation.

The technic of the chemotherapeutic assay employed has been reported elsewhere (6, 7). Bacillary enumeration of spleen and liver and a "leprosy index," which is an average evaluation of the gross lesions in various sites and organs, was the basis of the assay. For bacillary enumeration, organs were homogenized for two minutes in a Waring blender using a semimicro Monel metal container (Cenco No. 17246B). Four ml. of ice water were used for each spleen and 7 ml. for each liver minus the estimated volume of the organ, taking grams of weight as ml. (7). Smears of tissue homogenates, each comprising a volume of 10 μ l., were prepared and fixed in a delineated 1 cm.² (11.3 mm. in diameter) circle of an antibody slide (Bellco Glass, Inc., No. 1956), according to the technic described by Shepard and McRae (15). Organisms were stained according to Nyka's periodic acid technic (11) with the follow-

¹ Received for publication 21 July 1970.

² Y. T. Chang, M.D., Research Pharmacologist, Laboratory of Biochemical Pharmacology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014.

ing modification. The smears were flooded with 2 ml. of 1 per cent periodic acid, heated gently until bubbles began to arise, and allowed to stand for one minute. The slides were then washed and subjected to regular Ziehl-Neelsen staining. This technic gives more solidly stained organisms than the classic Ziehl-Neelsen method. Bacillary enumeration was made by using a 5 mm. square grill in the ocular. The total number of bacilli in the organ will be the number of bacilli per square field multiplied by the factor of:

$$\frac{\text{Area of smear in mm.}^2}{\text{Area of square grill in oil immersion, in mm.}^2} \times 100 \times \frac{\text{Volume of suspension in ml.}}{\text{Volume of smear in ml.}}$$

For example, the factor for each spleen will be $\frac{100}{0.003364} \times 100 \times 4$ or approximately

1.2×10^7 , and the factor for each liver will

be $\frac{100}{0.003364} \times 100 \times 7$, or approximately 2.1×10^7 .

RESULTS

Results of all four experiments are noted in Table 1. B.663 and B.1912 showed marked suppressive activity in both the smaller and larger doses in the three-week and three-month experiments. All drugs revealed equal activity except the smaller dose of B.1912, which was slightly less effective.

In the delayed treatment experiment, treatment was commenced two months after infection and continued for three months. The smaller dose of both drugs showed only a slight suppressive activity. The larger dose of the drugs exhibited marked activity with the leprosy indices remaining at approximately the same level as observed before drug treatment.

In the bactericidal experiment, animals were treated for the first five months and observed for another five months without treatment. Increase in both leprosy indices and bacillary counts was observed in animals treated with the smaller dose of the

drugs as compared with the results observed in the three week and three month experiments. With the larger dose of the drugs, increase in leprosy growth was observed in the bacillary counts but not in leprosy indices. These results indicated that both drugs exhibited no bactericidal activity. This is in agreement with the previously noted observation that organisms obtained from animals which were treated with B.663 for a period as long as 816 days still showed typical growth of murine leprosy (⁹).

Body weight of all treated animals was slightly lower than the controls at the end of the three week experiment. Animals receiving the larger dose of the drugs showed less weight gain than those receiving the smaller dose. At the end of the three-month experiment, body weight of all treated animals approached that of the normal mouse controls. It seemed likely that neither of the phenazine compounds was very palatable and food intake was less in the beginning.

Drug accumulation in various tissues of animals was a characteristic phenomenon of both B.663 and B.1912. Yellowish coloration of the exposed areas of skin, such as ears, feet, and tail, were observed at the end of three weeks and became more intense later. Subcutaneous fat and the fatty tissue within the abdominal cavity, such as omentum, pelvic fat, and areas along the intestines, appeared yellow at three weeks and orange at three months and thereafter. At three months, spleen and liver were dark red. Intestines appeared pink. Skin could be seen to be pinkish-red after wetting with water. Urine appeared pink. Coloration was generally less in animals receiving the smaller dose than those receiving the larger dose of the drugs.

Difference in the degree of coloration between the two phenazines was not observed until the end of the three month experiment. At this time, coloration of animals receiving the larger dose of B. 1912 was more marked than those receiving the same dose of B.663. Chains of tiny dye spots were observed in the omentum and pelvic fat in animals receiving the larger dose of B.663.

TABLE 1. Comparative study on the activity of B.663 and B.1912 in murine leprosy.

Drug	Dose, % in diet	3-week experiment		3-month experi- ment	Delayed- treatment experiment	Bactericidal experiment		
		No. bacteria per organ $\times 10^6$		Leprosy index ^a	Treatment commenced on 60th day and terminated on 147th day	Animals treated for 5 months, then observed for another 5 months without treatment		
		Spleen	Liver			Leprosy index	No. bacteria per organ $\times 10^6$	
				Spleen	Liver		Spleen	Liver
Leprosy control untreated		38	169	13.0 ^b	4.6 ^c 15.7	15.0 ^d		
B.663	0.005	6	14	1.2	11.3	3.2	2,075	9,612
B.663	0.01	3	17	1.2	6.3	1.0	31	676
B.1912	0.005	8	97	2.4	14.5	8.8	12,711	25,292
B.1912	0.01	3	32	1.1	4.8	1.0	201	2,390

^a Leprosy index is a measurement of the gross leprous lesions of various sites and organs with their maximal scores as follows: site of inoculation, 2; omentum, 4; mesentery, 2; pelvic fat, 4; spleen, 4; liver, 4; diaphragm, retrosternal region, thymus and pericardium, each 1, the total maximal score being 24.

^b One animal died before termination.

^c There are two leprosy indices in this experiment. The smaller one was obtained on the day before drug treatment. The larger one was obtained at the deaths of animals, all of which died before termination.

^d Eighteen animals died before termination.

Difference in drug coloration between the two compounds was clearly observed in the bactericidal test experiment at the end of the five-month period of drug discontinuation. Nearly complete disappearance of coloration was observed in animals receiving both the smaller and larger dose of B.1912. On the other hand, bright yellow coloration was observed in the subcutaneous and internal fat of animals receiving the larger dose of B.663 and a slight but definite yellowish tint in those receiving the smaller dose. This indicated that the rate of elimination of B.1912 was faster than B.663.

Coloration differences were also observed in the delayed treatment experiment. At termination, only 16 animals of the two groups that received the smaller dose of both drugs were surviving. Nine of these animals showed extensive lesions with no visible drug coloration, while the other seven animals with less lesions exhibited drug coloration.

DISCUSSION

Both B.663 and B.1912 showed marked drug coloration. Since coloration depends on drug accumulation, the extent of coloration should bear a direct relationship to the drug level in tissues. However, this does not seem to apply to phenazine compounds. The color of B.663 is medium orange in comparison to the orange-red B.1912. An equal coloration in the animals should indicate that the tissue concentration of the lighter dye is higher than the deeper one. A deeper coloration in the orange-red dye-treated animals might represent a tissue level less than or approaching that in the lighter dye-treated animals.

Since tissue coloration at the end of three weeks was similar with both drugs, this seems to indicate that the rate of accumulation of B.1912, the deeper dye, was less than B.663, the lighter one. In other words, it appears that B.1912 actually exhibited a lesser tissue concentration than B. 663. It

should be noted that this conclusion is based chiefly on the appearance of tissue coloration instead of an actual dye determination. It is known that intracellular B.663 crystals appear in various tissues of mice and patients treated with the drug ^(1, 2). Efforts to uncover metabolites of B.663 have been unsuccessful. It is believed that B.663 is stored in the body and excreted in the urine almost exclusively as the unchanged substance ⁽¹⁷⁾. This does not exclude the possibility that a difference in color intensity might exist between the two compounds after their binding to the tissue constituents. However, this does not seem likely, since the present conclusion is in agreement with Barry's finding that B.1912 exhibited a lower tissue concentration level than B.663.

In the delayed treatment experiment, poor absorption of phenazine compounds was observed in some animals receiving the smaller dose of the drugs but not in those receiving the larger dose. This may be a result of disturbed intestinal function caused by leprosy growth in the alimentary tract shortly after commencement of the treatment. Since extensive growth of murine leprosy has been observed within the intestinal villi (unpublished data) an influence on drug absorption is likely. With the smaller dose, a slow rate of drug accumulation was apparently inadequate to suppress the infection which rapidly progressed to an extent that caused impaired intestinal function. With the larger dose, a sufficient drug level was rapidly built up to suppress further leprosy growth which would have interfered with intestinal function. This suggests that the full dose of drugs should be employed in chemotherapy studies with murine leprosy.

Although the mouse foot pad-*M. leprae* model is now being used frequently for chemotherapeutic studies, murine leprosy still has potential for furnishing valuable information as revealed in the present study. Murine leprosy is a "malignant" infection in mice. All animals infected with heavy inocula are overcome by extensive growth of murine leprosy in a manner similar to the lepromatous type of human leprosy. On the other hand, *M. leprae* in

mouse foot pads exhibits a "benign" type of infection. Growth of organisms gradually reaches a plateau and the organisms eventually die off, resembling the tuberculoid type of human leprosy. In the "benign" infection, the self-limiting nature of the disease itself tends to inhibit the bacterial growth, in which case a less active drug might exhibit suppressive activity; while in the "malignant" infection only potent agents would be expected to show noticeable activity. Observations obtained from both infections can provide equally important information for a more thorough understanding of drug activity.

SUMMARY

The effect of a new riminophenazine derivative, B.1912, was compared with B.663 in murine leprosy in mice. The activity was compared in three-week, three-month, delayed treatment, and bactericidal test experiments. Both drugs caused marked coloration in the animals. B. 1912 showed a lesser rate of drug accumulation and a faster rate of drug elimination than B.663. With proper dosage, the suppressive activity of B.663 and B.1912 was similar. Neither drug exhibited bactericidal activity.

The usefulness of murine leprosy as a model for chemotherapeutic studies is emphasized.

RESUMEN

Se comparó el efecto de un nuevo derivado rimino-fenazina, B.1912, con el del B.663 en la lepra murina del ratón. La actividad fue comparada en tres semanas, tres meses, tratamiento tardío y pruebas de poder bactericida. Ambas drogas colorearon en forma marcada a los animales. El B.1912 mostró un índice menor de acumulación de droga y un índice mayor de eliminación de droga que el B.663. Con una dosificación adecuada, la actividad supresora del B.663 y del B.1912 fue similar. Ninguna de las dos drogas demostró actividad bactericida.

Se hace énfasis en la utilidad de la lepra murina como modelo para estudios terapéuticos.

RÉSUMÉ

On a comparé l'effet d'un nouveau dérivé de la rimino-phénazine, le B.1912, à celui du B.663, dans la lèpre murine chez la souris.

Cette activité a été comparée au cours d'essai portant sur 3 semaines, sur 3 mois, lors d'un traitement différé, et par des expériences bactéricides. L'un et l'autre de ces produits ont entraîné une coloration marquée des animaux. Le B.1912 a témoigné d'un taux d'accumulation du médicament inférieur, et d'un taux d'élimination médicamenteuse plus rapide, que le B.663. A des doses adéquates, les activités suppressives du B.663 et du B.1912 étaient similaires. Aucun des ces deux médicaments n'a témoigné d'une activité bactéricide.

On souligne la grande utilité de la lèpre murine en tant que modèle pour des études de chimiothérapie.

Acknowledgment. Grateful acknowledgment is made to R. W. Scaggs for his technical assistance.

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