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THE EFFECT OF ISONIAZID IN EXPERIMENTAL CORNEAL TUBERCULOSIS

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It has been shown previously that when mice are inoculated intracorneally with *M. tuberculosis* there is a latent period of about 10 days before lesions begin to appear. When such mice are treated with isoniazid (0.3 mg./mouse/day), starting on the day of inoculation, no macroscopic lesions appear during the period of treatment. When the treatment is stopped at the end of 28 days there is again a latent period of some 10 days during which the cornea remains clear; lesions then begin to appear and progress rapidly (Rees and Robson, 1950; Goulding and Robson, 1952).

It would thus seem that the treatment keeps the infection in abeyance but does not eradicate it, and that the development of a macroscopic lesion requires a certain latent period which is of the same order whether it occurs from the time of inoculation or from the cessation of isoniazid treatment.

When, however, larger doses of isoniazid are given during the period of treatment (3.0 mg./mouse/day) withdrawal of the drug is not followed by the development of lesions after a short latent period, and the cornea remains macroscopically clear for a longer time; in some mice lesions then begin to appear and develop to involve much of the cornea. Hence, the more intensive treatment has produced, in some way, an effect which keeps the infection in abeyance for a longer period.

These explanations are, of course, largely descriptive and we do not know what happens in the cornea during the period of isoniazid treatment, or in the latent period preceding the appearance of a macroscopic lesion. We have recently described a technique (Robson and Didcock, 1955) with which it is possible to make detailed observations of cells and bacilli within the cornea at a stage when this tissue appears macroscopically normal. We have used this method to study the phenomena discussed above.

METHODS

Inoculation of the cornea and its examination with the phase contrast microscope have been described previously (Rees and Robson, 1950; Robson and Didcock, 1955). In the present experiments the inoculum consisted of about 5,000 organisms (i.e., a 1/100 dilution of a culture in Dubos medium) of the same strain used in the previous work, unless stated otherwise. Isoniazid was given in the diet, the daily dose being 0.3 mg. or 3.0 mg. in 5 g. of M.R.C. diet 41.

In some experiments an attempt was made to recover the bacilli from the cornea by inoculating it into Kirschner's medium and incubating at 37° C. The details of the procedures are given in the text.

RESULTS

Two experiments were performed. The first investigated the effect of 0.3 mg. of isoniazid, and the second the effect of 3.0 mg.

First Experiment.—This consisted of two main groups of mice—a control group receiving no treatment and a group receiving 0.3 mg. of isoniazid/day from the day of inoculation. In some of these mice, treatment was continued for 16 days and in others for 24 days, in order to study the effect of various periods of treatment.

In a third group of animals isoniazid treatment was started two days before inoculation in order to determine whether pretreatment would affect the course of the ocular lesion. No difference was found between this group and that in which treatment was started on the day of inoculation, and no further reference will be made to this experiment.

In the control group the lesions developed rapidly with the stages previously described: early cellular invasion of the site of inoculation, forming a dense core in which no details could be made out microscopically; and the appearance of organisms in macrophages around the core, with the organisms rapidly multiplying within the

macrophages and destroying them; this coincided with the appearance of a macroscopic lesion in some animals about 5 days after inoculation.

Treatment with isoniazid did not suppress the initial cellular reaction leading to the formation of a dense core, or the appearance of a few bacilli within macrophages around this core. However, as long as treatment was continued (16 or 24 days) the lesion progressed no further; no multiplication of bacilli and no destruction of macrophages were observed. Until the end of treatment, therefore, the lesion presented a relatively quiescent picture, with the bacilli short and almost coccoid in form, in contradistinction to the long, almost filiform organisms present in overgrown macrophages in the absence of treatment. In animals in which treatment was continued for some time we believe that the central core was becoming less dense so that individual cells could now be identified.

In the animals in which treatment was stopped at 16 days, a new macrophage invasion was beginning to occur two days later; in another three days overgrowth and destruction of macrophages by bacilli were seen, and 8 days after cessation of treatment there was complete breakdown with the appearance of a macroscopic lesion. The sequence of events after cessation of treatment was therefore the same as in untreated animals following inoculation.

Second Experiment.—This was on four groups of mice—untreated controls and groups of animals treated respectively for 16, 30, and 50 days with 3.0 mg. of isoniazid/day.

In the untreated controls, lesions developed typically, and by 6 days after inoculation overgrowth of macrophages was occurring, with the appearance of macroscopic lesions in some animals.

When treatment was continued for 50 days, intracellular bacilli (in macrophages) remained visible for the whole period. These organisms rapidly became thin and rather polar, and sometimes almost coccoid in appearance. Occasionally, however, some large forms of bacilli were also seen. Throughout the whole period macrophages and leucocytes were present in the cornea, but at no time were there more than a few bacilli present in any one macrophage. The size and density of the microscopic lesion also gradually decreased. Thus, 48 hr. after inoculation there was a thick core, several hundred micra in diameter, in which no detail could be made out. This core consisted of densely packed cells several layers in thickness. Four days later it was thinning so that some individual cells could be made out, and later still further reduction of the lesion occurred. Thus in one animal, examined 43 days after inoculation, there was only a patch of cells, covering an area roughly 50×25 μ and consisting mainly of macrophages. In another animal, examined 50 days after inoculation, the lesion consisted of a smear of macrophages over an area some 200 μ in diameter. The decrease in the lesion thus involves a reduction in its density as well as in its dimensions. It would thus appear that during the continued treatment with isoniazid many macrophages containing bacilli disappear from the cornea. What happens to the bacteria contained in these macrophages, and particularly whether they leave the cornea dead or alive, is at present unknown. Living bacteria do, however, remain in the cornea throughout the period of treatment, as shown by cultures in Kirschner's medium (containing 1 unit/ml. of penicillin) of corneas removed 33 and 50 days after inoculation. In both experiments the bacteria so obtained were fully sensitive to isoniazid (i.e., to 0.0128 μg./ml.).

When treatment was stopped, either at 16 or 33 days after inoculation, none of the eyes developed macroscopic lesions up to 17 days after cessation of treatment in the first group, and 20 days after cessation of treatment in the second group, when the experiment was terminated. However, changes visible under the microscope did occur, indicating both new cell invasion and some bacterial multiplication.

When treatment was stopped after 16 days, some overgrowth of macrophages by bacilli was beginning 4 days after cessation of treatment, and when treatment had been stopped for 17 days an active-looking lesion was seen consisting of macrophages and leucocytes, though the cornea was still macroscopically clear. The macrophages contained long, almost filamentous bacilli. This cornea was cultured in Kirschner and gave a heavy growth of bacilli fully sensitive to isoniazid.

When treatment was continued for 30 days and then stopped, examination at 13 and 20 days after cessation of treatment showed cell invasion which was particularly striking at the latter period. The cornea, which was macroscopically clear, then contained a dense core 450 μ in diameter, with several satellite micro-lesions around it, consisting of collections of macrophages. Some cellular invasion of the remainder of the cornea was also seen. Small intracellular bacilli were present, but there was at this stage no obvious multiplication, as compared with the eye of an animal in which treatment had been continued for the whole period.

This cornea, too, was cultured and gave a heavy growth of organisms fully sensitive to isoniazid.

When organisms were seen they were mostly in macrophages, as described above. Occasionally a few extracellular organisms were seen in the cornea, both during the period of treatment and after cessation of isoniazid administration.

Difference Between These Two Experiments.—When mice inoculated intracorneally with tuberculosis are treated with 0.3 mg. isoniazid/day and treatment is discontinued, macroscopic lesions appear after a latent period of some 10 days. When the dose of isoniazid is increased to 3.0 mg./day, no macroscopic lesions appear for a longer period after cessation of treatment, though some new cellular invasion can be seen on microscopic examination. There are three possible explanations for the more prolonged suppression of macroscopic lesions with the larger doses of isoniazid:

1. That the number of organisms present in the cornea of animals treated at the higher dose level was depleted during the course of continued isoniazid administration to a greater extent than with the lower dose, so that those left, though undoubtedly alive and capable of multiplication *in vitro*, were no longer sufficiently numerous to initiate rapidly a macroscopic lesion. The experimental data strongly suggest that the number of bacteria in the cornea was markedly decreased.

2. That isoniazid treatment at the higher dose produced an effect on the virulence of the bacilli rendering them unable to produce the usual type of lesion. There is a fair amount of evidence (rather controversial) that bacilli made resistant to isoniazid by being subjected to the prolonged action of the drug become less virulent. The bacilli which failed to produce lesions in the present experiments were indeed not resistant to isoniazid, and there is no suggestion that isoniazid lowers the virulence of the bacilli if it does not increase resistance to the drug.

3. That the presence of bacilli in the cornea during the course of isoniazid treatment produced a condition of immunity so that, after cessation of treatment, their further multiplication with the production of a macroscopic lesion was prevented or slowed down by the immune process. It would then have to be assumed in addition that no such immune process occurred when the treatment was with 0.3 mg. isoniazid/day, but that only bacilli subjected to the higher concentration of the drug were able to initiate the immune reaction. This is not a very likely assumption. Bloch (1955) has given evidence suggesting that BCG bacilli, whose multiplication was arrested by isoniazid administration, were nevertheless capable of conferring immunity in mice.

The Effect of Prolonged Treatment with Isoniazid on the Virulence of Bacilli in the Cornea

One possible explanation for the failure of eyes treated with the larger dose of isoniazid (3 mg./day) to break down soon after cessation of treatment, as compared with eyes treated with a smaller dose of isoniazid (0.3 mg./day), is that the prolonged treatment has reduced the virulence of the organisms. This possibility was investigated in the following way:

The eye of an animal inoculated with a 1 in 100 dilution of a culture of *M. tuberculosis*, and treated for 50 days with 3 mg. isoniazid/day, was removed immediately after cessation of treatment and put into Kirschner's medium containing penicillin (1 unit/ml.). Growth in the tube was first detected some 26 days later and was quite definite in another 7 days. The virulence of this culture was compared with that of the original culture with which the eye had been inoculated, in the following way:

To make the comparison as valid as possible, the two cultures were inoculated at the same time in the same batch of medium and the period of subculture was the same. Viable counts of the two cultures were made by the method of Knox (1955), in order to ensure that the same number of organisms was inoculated, and that any difference in virulence that might appear was not due to difference in bacterial content. Both cultures—the original and the "recovered"—were subcultured in Dubos medium, and various dilutions of the Dubos cultures were then made. These were inoculated intracorneally into mice.

The results of the viable counts were quite clear. The two cultures each contained approximately 50,000,000 organisms/ml. Both cultures were fully and equally sensitive to isoniazid.

The various groups of animals inoculated (each of 6 mice) were observed and lesions noted at various times after inoculation. The average size of the lesions in each group is shown in Table I. The lesions developed at nearly equal rates for the various dilutions in the two groups, strongly suggesting that there was no difference in the virulence of the two cultures, at least as shown by this method. It would thus seem that the failure of the eyes in animals treated with a large dose of isoniazid to break down soon after inoculation is not due to reduced virulence of the organism.

TABLE I

THE DEVELOPMENT OF LESIONS IN MICE INOCULATED WITH VARIOUS DILUTIONS OF ORIGINAL CULTURE (BOVINE) AND BACILLI RECOVERED FROM MICE TREATED WITH ISONIAZID

The values shown represent the averages for the corneal lesions in each group of animals. The size of the lesions was assessed in a semi-quantitative manner as described by Rees and Robson (1950)

	Dilution	No. of Organisms Inoculated	Time After Inoculation (Days)						
			6	7	10	13	17	21	41
Recovered bacilli	1 in 10,000	50	Nil	Nil	Nil	0·4	1·8	1·9	1·7
	1 ,, 1,000	500	,,	,,	,,	0·4	0·7	0·8	0·9
	1 ,, 100	5,000	,,	? Lesions	0·7	1·4	1·8	2·2	2·1
	1 ,, 10	50,000	Lesions	0·5	1·8	2·3	2·5	2·7	2·5
	1 ,, 1	500,000	,,	1·0	2·3	2·4	2·5	2·8	2·7
Original culture	1 in 10,000	50	Nil	Nil	Nil	0·4	1·6	2·1	2·0
	1 ,, 1,000	500	,,	,,	,,	0·3	1·0	1·4	1·3
	1 ,, 100	5,000	,,	0·4	1·4	1·9	2·0	2·1	1·8
	1 ,, 10	50,000	Lesions	0·5	2·1	2·3	2·6	2·7	2·7

The full data on the development of lesions with various dilutions are given in Table I, since such data have not been previously recorded. It is clear that increase in the size of the inoculum can appreciably reduce the latent period preceding the appearance of a macroscopic lesion.

These data also make it possible to calculate the rate of multiplication of the organisms within the cornea, provided the following two assumptions are made: That macroscopic lesions appear when the number of organisms in the cornea has reached a certain level; and that the rate of multiplication of organisms within the cornea is largely independent of the number of organisms present. With an inoculum of 50 organisms there was a latent period of some 13 days whereas with an inoculum 1,000 times larger the latent period was about 6 days. Hence in 7 days the number of organisms had become 1,000 times larger, giving a period of generation of about 17 hr.

Immunity in Mice Following Intracorneal Inoculation

When mice are inoculated intracorneally with *M. tuberculosis* the resulting lesion reaches a maximum at about 30 days, after which it tends to regress somewhat. If at that time the second eye of such animals is inoculated with the same dose of the organism, a second lesion does not develop, even though control eyes develop lesions in the usual way. The inoculation in the first eye has thus led to some type of immunity reaction which protects the animal against a similar second inoculum. This process is now being studied in detail and the results will be published separately. The method has been used to investigate whether mice inoculated intracorneally with *M. tuberculosis* and treated with isoniazid (at 0.3 mg. or 3.0 mg./day) develop any immunity.

The experiment was performed on 6 groups of mice (totalling 90). The groups were those shown in Table II. Groups 1 to 5 were inoculated on day 1 with a 1 in 1,000 dilution of *M. tuberculosis*, i.e., with approximately 500 organisms. Groups 2 and 3 received 0.3 mg. of isoniazid/day from the day of the first inoculation for 36 days, i.e., 4 days before the second inoculum. This interval was to ensure that no isoniazid should be present in the body at the time of the second inoculum. Groups 4 and 5 received 3 mg. of isoniazid/day for 36 days. Forty days after the first inoculum groups 1, 3, 5, and 6 received the second inoculum in the left eyes. This consisted of a 1 in 1,000 dilution of a culture of the same strain of *M. tuberculosis*.

TABLE II

THE EFFECT OF ISONIAZID ON THE DEVELOPMENT OF IMMUNITY TO *M. TUBERCULOSIS* IN MICE

Isoniazid was administered daily to groups 2, 3, 4, and 5 for 36 days; groups 1 and 6 had none. I, intracorneal inoculation with *M. tuberculosis* (about 500 organisms). L, macroscopic lesion. Total no. of mice, 90.

Group	Day 1 (Right Eyes)	Dose of Isoniazid (mg.)	Day 40 (Left Eyes)	Development of Lesions	
				Right Eye	Left Eye
1	I	—	I	L	None
2	I	0·3	Nil	L after treatment	—
3	I	0·3	I	L after treatment	L
4	I	3·0	Nil	L delayed or nil	—
5	I	3·0	I	None	L
6	Nil	—	I	—	L

The right eyes of the animals in group 1 all developed lesions, which started after the usual latent period of 10–12 days, and progressed to reach a maximum around 30 days. The left eyes of these animals were inoculated 40 days after the

right eyes, and none of them developed a macroscopic lesion (up to 7 weeks after inoculation, i.e., for the total period of observation). Control eyes (group 6) inoculated with the same culture all developed the typical lesions, with the usual latent period. These experiments show quite clearly that inoculation of the right eyes conferred upon the animals a certain degree of immunity which was sufficient to prevent the development of macroscopic lesions in the left eyes. When the second inoculum consisted of the same number of dead bacilli macroscopic lesions also failed to develop, except in one eye in which a tiny spot visible to the naked eye appeared. The microscopic findings in these animals will be described elsewhere.

Animals of groups 2 and 3 received the smaller dose of isoniazid after the right eye inoculation and none had any macroscopic lesions during the period of treatment. After cessation of treatment these eyes broke down, as previously described. The left eyes of the animals in group 3, inoculated 40 days after the right eyes, developed lesions, as did the controls (group 7). Hence the bacilli in the right eyes of these animals, whose multiplication was inhibited by isoniazid, failed to confer any detectable immunity.

The animals of groups 4 and 5 received a larger dose of isoniazid (3 mg./day). Forty days after inoculation the left eyes of group 5 received the second inoculation. The right eyes of group 4 remained clear for 2 months. They were not examined for the subsequent 4 weeks, but then—practically 3 months after inoculation—4 out of 6 had moderate or large lesions of the cornea whereas the other two were still completely clear, and remained clear for the next 2 months—the full period of observation. The right eyes of group 5 remained clear throughout the period of observation, some 3 months after inoculation. The left eyes of group 5, inoculated 40 days after the right ones, developed typical lesions at the usual time, thus showing that here too no immunity detectable by the present method had been conferred by the bacilli in the right eyes.

An interesting result is the difference between the development of lesions in the right eyes (i.e., the eyes receiving the first inoculation) of groups 4 and 5. Whereas no lesions developed in group 5 (in which the left eyes had been inoculated subsequently), 4/6 of the right eyes of group 4 did develop lesions. It would thus appear that the second inoculum in group 5 conferred immunity on these animals which was effective in preventing the breakdown of the right eyes.

Investigation of the Cornea, after Prolonged Treatment with Isoniazid, by Transference to Medium in Vitro

When the cornea is inoculated with some 50 organisms there is a latent period of some 13 days before a macroscopic lesion appears. There is also evidence that under these conditions organisms divide once every 17 hr., and this is, in fact, in agreement with other observations on the rate of multiplication of *M. tuberculosis*. If it be assumed then that only one live organism remains at the end of isoniazid treatment, it would only take about 5 days for this to produce 50 organisms, so that a macroscopic lesion ought to appear some 18 days after cessation of isoniazid action. All this assumes that after cessation of the action of isoniazid the organisms are capable of normal multiplication. In fact we have found that the latent period is more than 24 days and in some animals lesions did not develop even after some two months. This suggests that under these conditions the organisms are not capable of normal multiplication. This does not necessarily imply that their failure to multiply is due to the action of isoniazid, since it has not yet been determined whether a very small number of organisms put into the cornea in the absence of any treatment is capable of initiating a lesion. To throw further light on this question, corneas have been removed from animals at the end of a period of treatment with isoniazid and put into Kirschner's medium to follow the fate of the organisms. The following results have so far been obtained:

1. The cornea of a mouse, treated for 33 days with 0.3 mg. isoniazid/day, was removed under sterile precautions, washed with sterile saline to remove any isoniazid present in it, and examined microscopically; it was then transferred to Kirschner's medium containing 20 units/ml. of penicillin and 25 μg./ml. of chloramphenicol. These antibiotics were added, as in some previous experiments secondary infection was troublesome.

After 2 days in Kirschner the cornea was removed and examined under the phase contrast microscope. There appeared to be no change. After another 3 days in Kirschner it was examined again and was found to contain large numbers of bacilli with signs of early cord formation. The bacilli were associated with cells or cellular debris and large numbers were also found in nerves, either free or in cells. It is obvious, therefore, that under these conditions a tremendous bacterial multiplication had occurred within 5 days.

In another experiment two mice were treated for 57 days with 0.3 mg. of isoniazid/day and the

corneas were then removed, and transferred to Kirschner's medium with 20 units of penicillin/ml. and no chloramphenicol, since chloramphenicol has some tuberculostatic action. Before transferring to Kirschner, both these corneas (C and D) were examined under the phase contrast microscope. Both were clear on naked eye examination. C contained an impenetrable lesion some 200 μ in diameter, with a few macrophages and corneal corpuscles around it. No bacilli were seen. D contained a core consisting of a smear of macrophages and corneal corpuscles. A few bacilli were seen both extracellularly and intracellularly.

After 4 days in Kirschner, D was examined again ; it contained large numbers of bacilli in the area of the lesion, which had not extended. There was no cord formation. C was examined after 6 days in Kirschner ; it contained a few bacilli. C was again examined after 8 and after 12 days in Kirschner, and again a few bacilli were found, perhaps fewer than after 6 days. Hence marked development had occurred in D and some growth, though not marked, in C.

2. A cornea removed from a mouse after 33 days' treatment with isoniazid (3 mg./day) was washed to remove the isoniazid and then put into Kirschner with penicillin and chloramphenicol. The cornea was examined 2, 4, 6, 10, 16, and 23 days later. Organisms were seen at these various stages, but no evidence of multiplication was obtained. Several organisms forming a definite pattern were seen and photographed ; 4 days later the same field was accurately identified and again photographed—there was no evidence that the organisms had changed or multiplied.

The cornea was put back into Kirschner without chloramphenicol, but secondary infection soon appeared and the preparation was discarded.

In another experiment two mice were treated with 3 mg. isoniazid/day for 57 days and the corneas (C and D) were then removed and examined under the phase contrast microscope. The lesion in C consisted essentially of a smear of macrophages, corneal corpuscles, and some dots, but no bacilli were definitely identified, though a few coccoid objects might have been bacilli. D had a dense lesion in which some cells could still be made out and also contained blood vessels in which rouleaux of red blood cells could be seen. There were macrophages and corneal corpuscles around the lesion. No organisms were identified. C was again examined after 4 days in Kirschner containing penicillin but no chloramphenicol. No obvious change had occurred. D was examined after 6 and 8 days in Kirschner, and both C and D were examined after 12 days in Kirschner. At none of these examinations were any bacilli seen. Hence in the corneas of these animals no growth had occurred in Kirschner's medium.

Discussion

One of the most important problems in the chemotherapeutic treatment of tuberculosis is the frequent failure of drugs to eradicate the organisms from the body. Though such treatment is, as a rule, strikingly successful in the control of signs and symptoms, the disease relapses in many patients at various times after cessation of drug administration. The present experiments help to throw some light on what is happening in such lesions, though they are not concerned with drug resistance, which is an important factor in the control of human disease.

In the first place they show that prolonged treatment with isoniazid, though it checks the multiplication of the bacilli, does not kill all of them. They remain visible in macrophages, mostly as small, thin bacilli, presumably a resting form ; and on cessation of treatment they can start multiplying again, as is demonstrated by growth in Kirschner's medium and also by the subsequent development of typical lesions in the cornea. Another point of interest is that, as the duration of isoniazid therapy increases, the extent of the microscopic corneal lesion gradually decreases ; this involves not only a decrease in the diameter and thickness of the aggregate of cells which make up the lesion but also a decrease in the density of the cell population, so that as time elapses cells become individually visible, whereas at the beginning of treatment the dense packing of the cells within the lesion made it impossible to see any detail under the phase contrast microscope. What happens to the cells which disappear from the lesion and, more important, to the bacilli which they presumably contain ? The duration of life of such cells is in all probability quite limited and thus they must break down, liberating the bacilli which they contain. Such bacilli may then (1) die and disintegrate and this may of course also happen while the bacilli are still within cells ; (2) be carried away to other parts of the body where they may be dealt with ; or (3) be taken up by macrophages within the lesion, thus increasing the population of bacilli per cell. This third possibility is not supported by what we have seen by the examination of lesions under the phase contrast microscope, for there was no suggestion of any increase in the number of bacilli per cell.

We realize that the maximum period for which we have treated the animals in these experiments—50 days—is short as compared with the many months of chemotherapy now usual in human tuberculosis. It would obviously be of interest to determine what happens to corneal lesions subjected to more prolonged action of drugs. It may well be that the gradual attrition of a lesion, with progressive diminution of cell population and bacterial content, represents the mechanism by which the body conquers the disease under the protection of chemotherapy.

The delayed breakdown, or sometimes even the absence of breakdown, of macroscopic lesions after prolonged chemotherapy with large doses of isoniazid, as compared with the regular breakdown after smaller doses of isoniazid, represents another problem. Similar findings have previously been observed with combined chemotherapy of corneal infections as, for example, with isoniazid and streptomycin (Goulding and Robson, 1952) and with streptomycin and a thiosemicarbazone (Rees and Robson, 1951); in these experiments breakdown sometimes occurred many weeks after cessation of treatment. These findings too have their clinical counterpart in the relapse which may occur in human tuberculosis after cessation of treatment with combinations of drugs.

A possible explanation for such a delayed breakdown is that the prolonged treatment has decreased the virulence of the organisms within the lesions. The present experiments have shown that this is not so within the limits of the very special conditions under which they were carried out: they do show that organisms recovered from the cornea of an animal subjected to prolonged administration of isoniazid in large doses are not less capable of producing corneal lesions than are the organisms originally inoculated.

The question as to whether organisms in the cornea subjected to the action of isoniazid (at both doses) can lead to the development of an immunity reaction was also investigated. It was shown quite clearly that no immunity could be demonstrated in the other eye of such animals, even though a striking immunity can be shown in the second eye when the lesion in the first eye is allowed to develop unhampered by any drug action. This question too is of practical importance, and contradictory results have been obtained by different investigations (see Bloch, 1955). More recently Palmer and Ferebee (1955) have found that, when guinea-pigs are infected with *M. tuberculosis* and treated with isoniazid in doses which protect the animals, such a controlled infection does confer some degree of resistance to a subsequent challenge. It may well be that the size of the original inoculum and the exact extent to which isoniazid controls its multiplication determine whether any immunity reaction occurs. This problem requires further investigation, particularly since it has been suggested that isoniazid should be used prophylactically in man, and that such use would not interfere with the development of immunity to tuberculosis.

The likeliest explanation for the delayed breakdown after prolonged treatment with large doses of isoniazid is that the number of organisms in the cornea has been so depleted that they no longer represent a sufficient inoculum to start immediate multiplication, or, in some cases, even to lead to the development of a lesion. This question will not be further discussed at present since it is proposed to study the effect of inocula smaller than 50 organisms (the smallest used in the present experiments) to determine whether they are capable of initiating a lesion in the cornea, and, if so, with what latent period. It is interesting, however, that the experiments in which the cornea was transferred to an artificial medium *in vitro* (Kirschner's medium) revealed differences between the corneas of animals treated respectively with 0.3 mg. and 3.0 mg. of isoniazid/day, similar to those demonstrated in the corneas which remained in the living animals. There are a number of snags in the interpretation of such *in vitro* experiments which are discussed in the masterly review by Brieger (1951). We are performing further experiments on the fate of organisms in the cornea transferred to various media outside the body.

Summary

1. The changes which occur in the corneas of mice inoculated intracorneally with *M. tuberculosis* and treated orally with isoniazid have been studied by means of the phase contrast microscope. The treatment does not interfere with the initial cellular invasion of the cornea, but organisms do not multiply appreciably within the cells during the period of drug administration. When the dose of isoniazid is 0.3 mg./mouse/day, cessation of treatment is followed by bacterial multiplication and the appearance of a macroscopic lesion within a period of some 10 days, i.e., the same as the latent period before the appearance of a lesion in the untreated animals. When the dose of isoniazid is 3.0 mg./day the lesion appears a good deal longer after cessation of treatment.

2. Possible explanations for this difference have been investigated. It has been shown: (*a*) That treatment with the larger dose of isoniazid for

50 days has not affected the virulence of the organisms remaining in the cornea after this time. (*b*) That when the development of a lesion is prevented by the administration of isoniazid (in either dose), inoculation of the second eye leads to the development of a normal lesion. Such a lesion does not develop in the second eye when the first infection remains untreated. Hence the isoniazid-controlled infection of the first eye failed to confer any systemic immunity. (*c*) That when the cornea of a mouse treated with 0.3 mg. isoniazid/day is removed from the animal and put into Kirschner's medium rapid multiplication of the organisms *in situ* can be demonstrated. When a similar experiment is performed with a cornea of an animal treated with the larger dose of isoniazid such rapid multiplication is not observed.

3. The relevance of these findings to the chemotherapy of human tuberculosis, and in particular to the reappearance of active lesions after prolonged chemotherapy, is discussed.

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REFERENCES

Bloch, H. (1955). *Experimental Tuberculosis*, p. 237. London: Churchill.

Brieger, E. M. (1951). *Bibl. tuberc.*, **5**, 238.

Goulding, R., and Robson, J. M. (1952). *Lancet*, **2**, 849.

Knox, R. (1955). Ibid., **2**, 110.

Palmer, C. E., and Ferebee, S. H. (1955). *Publ. Hlth Rep., Wash.*, **70**, 759.

Rees, R. J. W., and Robson, J. M. (1950). *Brit. J. Pharmacol.*, **5**, 77.

—— —— (1951). Ibid., **6**, 83.

Robson, J. M., and Didcock, K. A. (1955). *Brit. J. exp. Path.*, **36**, 560.