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THE EFFECT OF TREATMENT WITH A LARGE DOSE OF ISONIAZID ON AN ESTABLISHED TUBERCULOUS INFECTION IN MICE

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Mice were infected intracorneally with *Mycobacterium tuberculosis* var. *bovis* and the infection allowed to develop for a period of 2 weeks. At this stage the animals were divided into two main groups; one received no treatment, the other was treated with large doses of isoniazid (3.0 mg./mouse/day). The effect of treatment on the primary lesion, the viability of the bacilli, the systemic spread of infection, and the production of immunity were observed. Treatment was continued for 11 months, after which the animals were observed for another 8 months. Within a few days of starting isoniazid treatment the primary lesion stopped increasing in size, regressed slightly and stabilized at a size of about one-third of that of the controls. There was little evidence of any clearing of the bacilli from the lesions; they remained strongly acid-fast and morphologically normal for many months after infection, although by 13 months about half of the organisms in both groups had become very granular. The evidence suggests that in control and treated animals the bacilli in the cornea had usually all died within 8 months after infection. In two treated corneas, living virulent bacilli were demonstrated 15 months after inoculation. In the untreated animals, the disease spread systemically to involve the lungs, liver, and spleen, and by one year after inoculation the systemic tuberculous infection was very heavy, though not enough to kill the animals. The lesions in these animals contained many acid-fast bacilli. In the treated group, systemic spread of infection as judged by the development of small macroscopic lung foci was slight; acid-fast bacilli were found in only one animal. In the treated animals, practically no immunity could be detected 5 months after inoculation and had not reappeared 2 months after cessation of treatment; in the untreated animals immunity was present.

Prolonged chemotherapy in tuberculosis frequently fails to eradicate the infection. This has been demonstrated experimentally in animals and in clinical practice. Thus McCune and Tompsett (1956) infected mice intravenously and then treated them with various drugs and combinations of drugs for periods up to about 4 months, but were not able to eradicate the infection. Even with a combination of pyrazinamide and isoniazid which appeared to be most effective under those experimental conditions, there was no complete elimination of the bacilli (McCune, Tompsett, and McDermott, 1956).

In guinea-pigs it has been found that treatment of chronic lesions with streptomycin for 300 days can leave large cyst-like abscesses filled with purulent material in the lungs, liver, and spleen. These contain many viable tubercle bacilli which are still sensitive to streptomycin (Steenken, Wolinsky, Pratt, and Smith, 1952; Bojalil, Perez-Tamayo, and Bastarrachea, 1958).

Tubercle bacilli have also been recovered from healed human lesions after prolonged chemotherapy (Hobby, Auerbach, Lenert, Small, and Comer, 1954). Prolonged prophylactic chemotherapy with isoniazid in children with primary tuberculosis does not always prevent the disease spreading, though this occurs less frequently than in untreated children (Ferebee, Mount, and Anastasiades, 1957). The effect of prolonged chemotherapy of tuberculosis in man is discussed by Canetti (1955).

It seemed of interest to study the fate of a primary tuberculous lesion subjected to prolonged chemotherapy. Primary experimental

tuberculosis of the cornea in the mouse seemed to offer a satisfactory model lesion for this work because the cellular changes and bacterial content can be followed quantitatively by a variety of methods and the degree of immunity present in the animal can also be assessed.

After inoculation of the mouse cornea with *M. tuberculosis* there is rapid multiplication of the organisms and cellular invasion ; after 10 days the number of organisms is such as to elicit the formation of a macroscopically visible lesion. Subsequently, the lesion spreads to involve much of the cornea, although the total number of organisms does not increase much beyond that reached at about two weeks. After reaching a maximum the lesion decreases and reaches a size which remains steady for many months (Rees and Robson, 1950). In the present experiments mice were inoculated intracorneally and a macroscopic lesion allowed to develop. These mice were then treated with large doses of isoniazid over a long period and the development of the local lesion and the systemic spread were compared with untreated controls. Immunity was also investigated and an attempt was made to determine whether viable bacilli were present in the lesions in both groups of animals at various times after starting isoniazid treatment.

METHODS

One hundred and ten albino mice of a mixed strain bred in our laboratories and about 3 months old at the start of the experiment were used. They were infected intracorneally by the method of Rees and Robson (1950) using a 1/100 dilution of a Dubos culture of *M. tuberculosis* var. *bovis* (3rd subculture ; 13th day), originally supplied by Dr. Levinthal, and since then maintained in our laboratory by the methods described by Acharya, Sullivan, and Robson (1958).

The eyes were examined at intervals for about 19 months macroscopically as described by Rees and Robson (1950) and microscopically by phase contrast microscopy and histological methods described by Robson and Sullivan (1957a). This included gross staining of the whole cornea with carbol fuchsin to reveal the localization and density of the bacillary mass, and counterstaining with methylene blue to show the extent and nature of the cellular content.

The disease was allowed to develop for 14 days, by which time lesions had appeared in all inoculated corneas, varying in size from 0.1 (just visible macroscopically) to 2.0 (occupying about two-thirds of the cornea) with an average of 0.6. These animals were divided into three main groups: (1) 13 mice with rather large corneal lesions: range 0.4 to 1.7, mean 0.9 ; (2) 62 mice with smaller lesions: range 0.1 to 1.0, mean 0.6 ; (3) 35 mice with lesions varying from 0.1 to 2.0, mean 0.6.

Groups (1) and (2) were treated with isoniazid (3.0 mg./mouse/day) mixed in M.R.C. diet 41. Group (3) was used as control and left untreated. Group (1), consisting of mice with large lesions, was specifically chosen to demonstrate a marked reduction in lesion size under intensive isoniazid therapy, should this occur. Most of the treated animals were given isoniazid continuously for 11 months. In two subgroups of animals treatment was discontinued 2½ months and 5 months respectively after infection, in the first instance to test for viability of organisms and in the second to test for the presence of immunity.

When used, cortisone was given as cortisone acetate, 0.5 mg./mouse/day subcutaneously.

RESULTS

Effect of Isoniazid on the Corneal Lesion.—In Fig. 1 the average lesion size is plotted against time after infection. In the controls, the lesions appeared after the usual latent period with this size of inoculum (8 days) and gradually increased in size until after three weeks they occupied slightly more than half the cornea. The lesions then gradually regressed, leaving a chronic residual lesion occupying about a third of the cornea. In the treated animals, the lesions did not increase appreciably in size after starting treatment, and, after a latent period of about a week, they began to decrease to a size which was substantially maintained for the rest of the experiment. The decrease in size was rather greater in the group of 13 mice with a higher average lesion size when treatment was started (group (1)), so that both treated groups stabilized eventually at about the same level. In 5 mice which had maximum lesions ranging from 0.4 to 1.7 there was complete regression leaving the cornea clear macroscopically. When isoniazid was discontinued after various intervals from 2 months to 11 months, there was no evidence of breakdown of the lesions.

Microscopic Appearance of the Lesions.—The infected corneas of all groups of mice, treated and untreated, were examined at intervals after inoculation by phase contrast microscopy, gross staining of the eye (Robson and Sullivan, 1957a), and by normal histological techniques.

In the control group 50 days after inoculation, phase contrast microscopy showed a dense cell reaction consisting mainly of macrophages around and among the bacilli. There was also some vascularization. After gross staining, much of the macroscopic lesion was seen to consist of a heavy perifocal inflammation, mainly by macrophages.

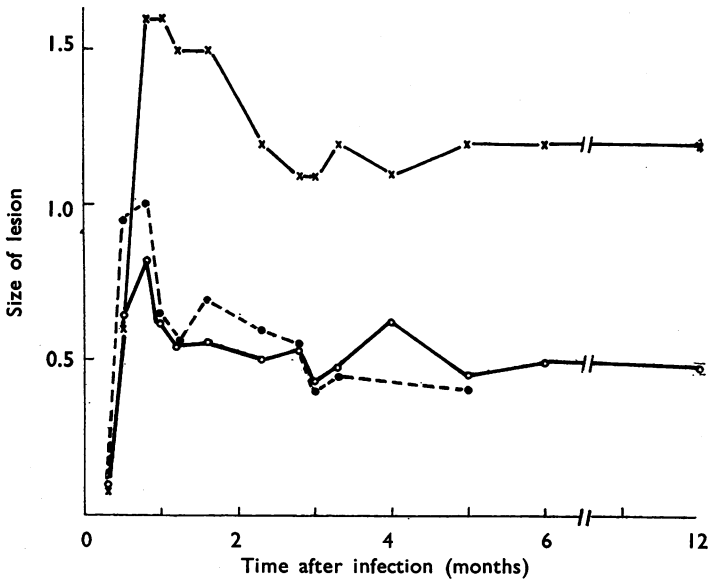

FIG. 1.—The average size of corneal lesion in 3 groups of animals. The experiment was continued for 19 months, but few animals were left after 12 months. Treatment period: 0.5 to 11.5 months. X——X, Untreated mice; O——O, mice treated with isoniazid; ●----●, subgroup of treated mice with large lesions at the beginning of treatment.

In the treated groups, the lesion centre was qualitatively similar to that of the control group, with many macrophages containing bacilli and also vascularization. The cornea surrounding the lesion was relatively acellular, and after gross staining very little perifocal inflammation was seen.

The same appearance was found in eyes from the treated group examined five and six months after infection. After gross staining, bacilli and also acid-fast debris corresponding exactly in their distribution to the shape of the macroscopic lesion was seen and there was very little perifocal inflammation. Most of the bacilli were normally stained, but others consisted of a number of dots; occasionally acid-fast globules were all that remained of a bacillus. Up to this time there was no evidence of any decrease in the number of bacilli. By 7 months the presence of granular bacilli and acid-fast globules became more marked, though many organisms of normal shape were still present in most of the eyes.

Eight months after infection, an animal from the treated groups was killed and its cornea examined. When treatment was started, this animal had had a corneal lesion occupying one-fifth of the eye. After treatment for three weeks, this had regressed completely and the eye had been macroscopically clear for the following seven months. However, there was microscopic vascularization and a small lesion 300 μ in diameter consisting mainly of macrophages (some with intracellular bacilli) and a few granulocytes. When stained many morphologically normal and a few very granular bacilli were seen. There were relatively few cells and these were confined to the site of the bacilli.

By 13 months after inoculation, there was no essential change in the histological appearance. Bacilli were still intracellular in macrophages and by this time were roughly divided so that half were morphologically normal and half granular. There was, however, still no evidence of any decrease in the total number of bacilli present in the eyes. At this time there were more invading cells in the corneas of the control than of the treated groups, but the morphology of the bacilli was identical in both. During the whole of this time there was no detectable difference in the acid-fastness of the bacilli in treated and untreated mice.

Fifteen months after infection, corneas from the control and treated groups showed no qualitative differences. In both there was corneal fibrosis with vascularization and large foci of inflammatory cells, almost exclusively macrophages, but also some lymphocytes and very occasional polymorphs. Bacilli, many morphologically degenerate, were also seen in the cell foci.

Effect on Viability of Organisms.—When mice inoculated in the cornea were treated with 0.3 mg. of isoniazid/mouse/day for 36 days and treatment was then stopped, lesions appeared after a latent period of about 11 days. When the daily dose of isoniazid was 3.0 mg. the latent period was considerably longer (Robson and Didcock, 1956). Furthermore, living and fully virulent tubercle bacilli could be recovered from such a cornea after 50 days of treatment. In other experiments, cessation of treatment with the higher dose of isoniazid after 28 days was not followed by breakdown during a subsequent observation period of three months (Goulding, 1956). The present experiment allowed the viability of

bacilli to be investigated after longer periods of treatment.

In one subgroup of six mice, treatment was stopped after 62 days of treatment, and observations continued for about four months. During this period there was no change in the size of the macroscopic lesions. To determine whether any live organisms were present in these corneas, three of the animals were treated with cortisone during the last five weeks of the period, since it has been shown that cortisone can reactivate a chronic corneal lesion in the mouse (Robson and Sullivan, 1957b). No such reactivation was seen in the present experiment; in one animal there was an appreciable decrease in the size of the lesion. There was no evidence of multiplication of bacilli or of cord formation.

Eight months after the beginning of the experiment another attempt was made to determine whether viable organisms were present. This was done in two subgroups of animals, one treated with isoniazid and the other controls. The animals were killed and the corneas dissected, washed four times with sterile saline and put into Dubos medium containing penicillin (50 units/ml.). In corresponding eyes examined microscopically at this time many bacilli were present within macrophages; the majority of the bacilli were morphologically normal although there were many granular bacilli present. None of the six treated animals killed at this time showed any evidence of systemic infection, either macroscopically or microscopically; of the four control animals, two had heavily infected lungs containing many acid-fast bacilli, the majority of which were granular or polar.

After 23 days, one of the corneas removed from the treated subgroup was taken from the Dubos medium and examined by phase contrast microscopy and then stained with carbol fuchsin. Practically all the epithelium had fallen from the cornea, but a few invading cells in the stroma were still intact and some intracellular bacilli were easily seen. After staining, many extracellular and intracellular bacilli were visible and of normal appearance, but there was no sign of multiplication or cord formation. At the same time all the other eyes were transferred into fresh Dubos medium without penicillin. The Dubos medium in which the eyes had been incubated for three weeks was transferred to centrifuge tubes and spun at 3,000 rev./min. for 20 min. The deposit was stained by the Ziehl-Neelsen method, but no bacilli were found in any of the tubes. After incubation for a further two weeks, all the eyes were removed from the Dubos medium and examined by phase contrast microscopy and stained. By phase contrast, many bacilli were seen to be present in all the eyes of both subgroups. These were easily seen, but cell structure was difficult to define by this time, although some morphologically intact macrophages were still visible. After staining, many bacilli were found in both subgroups of eyes with rather more in the controls than in the treated group. The controls all had larger lesions than the treated group and this difference in numbers of bacilli would be expected. There was no evidence of cord formation or of multiplication of the bacilli. At the same time as the viability was being tested, two control animals and two treated animals were killed and the corneas removed and implanted subcutaneously in the groin of four guinea-pigs. Ten months later these guinea-pigs were killed and examined. All animals were completely normal with no evidence of tuberculosis.

Treatment with isoniazid was stopped in all animals after 11 months. Two months later another viability test was carried out. Four animals from groups (2) and (3) were taken, the corneas removed and digested in a collagenase: hyalase solution for 18 hr. at 37° (Robson and Smith, 1959) to release the bacilli. The digest was then transferred to Kirchner medium and incubated at 37°. After culture for seven weeks there was no evidence of growth in any of the tubes. One tube from each group was subcultured into Dubos medium; after three weeks there was still no sign of growth.

One month later (14 months after the beginning of the experiment) another 4 animals were taken from the same 2 groups and the corneas removed and digested in collagenase: hyalase as before. In addition a piece of lung and spleen from each animal was similarly digested. A drop of each of these digests was placed on to Löwenstein-Jensen and Dorset egg-slopes, and into Dubos medium. Four guinea-pigs were also given injections into the lung and spleen, 2 with digests of a cornea from the control and treated groups, and 2 with digests of lung and spleen from the treated group only. Six months later these guinea-pigs were killed and showed no evidence of tuberculosis. In the cultures there was no growth from the digests from the treated animals, but tuberculous colonies grew on 2 of the Löwenstein-Jensen slopes from the lung and spleen digests from control animals.

One month later (15 months after the beginning of the experiment) another 2 mice from the same groups were killed, and their corneas were

removed and implanted subcutaneously in the groin of 4 guinea-pigs. The 2 guinea-pigs implanted with the treated corneas died from generalized tuberculosis, one after 3 months and the other after 4 months. Organisms from the liver of the animal which died after 3 months were cultured on Löwenstein-Jensen slopes and thereafter kindly tested by Professor Knox for sensitivity to isoniazid. They were sensitive to 0.08 μg./ml. of isoniazid as was the original parent strain used for inoculation. The guinea-pigs implanted with the untreated corneas survived and appeared normal until they were killed for examination 5 to 12 months later: there was no evidence of tuberculosis.

Nineteen months after infection (8 months after cessation of treatment) two treated animals survived which had shown no breakdown of corneal infection. Their corneas, as well as that of a control mouse, were implanted into guinea-pigs. One of the treated eyes which had no macroscopic lesion showed a small lesion under phase contrast. This consisted of an aggregate of cells containing a number of clearly seen bacilli. These guinea-pigs were killed after 4.5 months and there was no evidence of tuberculosis in any of them.

Effect of Treatment on the Systemic Spread of the Disease.—Previously it has been shown that very little systemic spread of infection occurs in mice after intracorneal inoculation with virulent bovine tubercle bacilli. This has encouraged the use of the intracorneal method for testing anti-tuberculous drugs, since the lesion in the eye is a closed one and there is little risk to man in handling the animals. This lack of spread is certainly true if the animals are kept for only the few weeks necessary to perform a screening test, but we have found that if animals are kept for many months systemic spread occurs and extensive lesions are produced, mainly in the lungs. We were therefore interested in studying, as part of this investigation, the effect of isoniazid treatment on the systemic spread from a corneal lesion.

Eight months after infection, 6 treated animals and 4 controls were killed and examined. In the treated group, 5 animals had many small macroscopic foci of infection on the lung surface; apart from the cornea, they had no other macroscopic evidence of disease. Smears of the lung, liver, and spleen, stained by the Ziehl-Neelsen method, revealed no bacilli despite intensive search. All 4 of the control group had lung lesions of which 2 were considerably more extensive than in any of the treated animals. There was no other macroscopic evidence of disease. Many acid-fast bacilli were found in the lung smears from the 2 animals with extensive lung lesions, but not in the other animals. No acid-fast bacilli were found in smears of liver and spleen.

By 13 months after infection, there was evidence that the anterior chamber had become affected in some of the animals (5 of 15 controls and 3 of 21 treated animals). Five control and 5 isoniazid treated animals were examined after killing at that time (8 of these were also used for the viability tests). In the control group 4 out of the 5 had a heavy lung infection with many coalescing foci over the whole surface of the lung. The spleen of one had macroscopic lesions, but the livers and spleens of the others were macroscopically normal. The fifth animal also had a heavy lung infection with many discrete foci over the whole surface of the lung. The liver and spleen of this animal were normal. Many bacilli were found in smears from the lung of all 5 animals, from the spleen in 2, and from the liver in 2. In the treated group 2 of the 5 had very few (3 to 6) small lesions in the lungs, but the livers and spleen were normal. In the other 3 there was no macroscopic evidence of infection. Smears were made of the lungs, liver, and spleen and stained, but no acid-fast bacilli were found.

Fourteen months after infection one animal from each group was killed and various organs removed for histological examination. In the control animals the lung showed alveoli packed with large foamy macrophages and a few polymorphs; cholesterol clefts were also seen. In the lung substance there were multiple foci of chronic inflammatory cells, mainly macrophages and lymphocytes, but few polymorphs. There was no evidence of necrosis. In Ziehl-Neelsen stained sections there were many bacilli, particularly in the foamy macrophages in the alveoli. The appearance of the lung was that of a typical segmental tuberculous broncho-pneumonia possibly suggestive of a blood-borne infection. In the liver there was much infiltration of the portal tracts by macrophages and a few plasma cells. No tubercle bacilli were seen. Tiny foci were also found in the kidney cortex and the heart; the spleen was normal.

In the treated animal there was little evidence of disease in the lungs; there was some emphysema, but there were no large cell masses of infiltration, no foamy macrophages, and no

cholesterol clefts. No bacilli were found in the sections, and there was little to suggest tuberculous disease. In the liver there was infiltration around the portal tracts, though less than in the control. This consisted mainly of macrophages with a few plasma cells, polymorphs, and eosinophils. Again there were tiny foci in the kidney and heart consisting of macrophages and a few plasma cells. The spleen was normal. No bacilli were found in the sections from this animal. At the same time that animals were taken for the histological examination, 4 from each group were killed and parts of various organs were cultured for viable bacilli. In smears of the lungs, liver, and spleen stained with carbol fuchsin, organisms were found only in the lungs of 3 control animals and no acid-fast bacilli were found in any of the smears from the treated group. There was definite evidence of spread of viable bacilli to the lungs of some control animals, but not in the treated group. Two weeks after these animals were killed 2 treated animals died from an intercurrent infection and acid-fast bacilli were found in a smear of the lungs of one of them. This is the only time that we have found acid-fast bacilli in the organs (apart from the cornea) of a treated animal.

The Effect of Treatment with Isoniazid on Immunity.—It has previously been shown by Acharya *et al.* (1958) that tuberculous infection of one cornea in the mouse rapidly leads to the development of immunity which will prevent the development of a lesion if the animal is reinfected in the other eye. These authors showed that this immunity persists for at least 13 months after primary infection. By one week after infection the immunity was sufficient to prevent lesions developing in animals reinfected in the other eye with a 1:1,000 dilution of a Dubos culture of *M. tuberculosis* (about 500 bacilli). In the present experiment, treatment with isoniazid was started 2 weeks after infection, so that by then sufficient immunity would be present to inhibit completely the formation of lesions in a group challenged with the size of inoculum used. Five months after infection (4.5 months after the commencement of treatment) 2 groups of 6 mice, from groups (2) and (3), were reinfected in the other eye with a 1:1,000 dilution of *M. tuberculosis* var. *bovis*. In the group of mice which had received no isoniazid, no lesions had developed in any of the challenged eyes one month after challenge and after 9 weeks a small lesion had developed in only one animal. Of the treated group in which isoniazid was discontinued before challenge, 4 animals had developed lesions by 3 weeks after reinfection, and by 9 weeks another had developed a lesion. The eye of the remaining animal remained clear up to 9 months later. Thus, after treatment with isoniazid lasting for 4.5 months, there was a marked difference in immunity between the control and treated groups.

Thirteen months after infection (2 months after stopping treatment) the presence of immunity was again investigated in the treated animals, the second eye being challenged with a 1:1,000 inoculum. Three of the 6 animals had developed lesions in the challenged eye by the 14th day; one month after reinfection, all 6 animals had developed lesions. Thus in this group there had been practically complete suppression of the immunity by the treatment. Control animals, challenged in the same way, were unfortunately killed prematurely.

In conclusion, therefore, it may be said that after 5 months of treatment, while control animals still showed almost complete immunity, the immunity of the treated group had almost all gone, and after 13 months, while the controls were still probably immune (Acharya *et al.*, 1958), in the treated group all the immunity had disappeared.

Discussion

Intensive therapy with isoniazid produced marked effects on a progressive, established tuberculous lesion in the mouse. Treatment started 14 days after infection prevented further increase in size of the lesions; after about a week the lesions decreased somewhat in size and then stabilized. Prolonged treatment did not produce any further decrease in lesion size. In untreated mice the lesions increased in size during the first month after infection, then regressed slightly and stabilized at a size more than twice that of treated animals. This final difference in size can be explained by two effects of the treatment. Firstly, the further increase in the main core of the lesion (the mass of cells containing bacilli) which occurred in the control animals after the first fortnight was inhibited in the treated group; secondly, perifocal inflammation disappeared completely under the impact of therapy, but was maintained indefinitely in the controls. On the other hand, it is clear that prolonged treatment had no significant effect on the established focal lesion, all the bacilli remaining intracellular within a core of cells.

Within a few months of inoculation few viable bacilli remained in the corneal lesions of either

the treated or the control groups. Treatment had no effect on the acid-fastness, morphological appearance, degeneration or removal of the bacilli from the lesion, although it arrested their multiplication. The puzzling fact remains, however, that in the control eyes the perifocal inflammation persisted, whereas it cleared up quickly under treatment. This raises the highly speculative suspicion that the untreated bacilli, though nonviable when tested by methods involving culture or inoculation into guinea-pigs, nevertheless still retain some biochemical activity which maintains the perifocal inflammation and that this activity is suppressed by isoniazid. It is possible, of course, that the effect of isoniazid on the perifocal inflammation is not due to an action of the drug on the tubercle bacilli only, but also to an anti-inflammatory effect, since there is evidence that isoniazid can diminish inflammation (Spain, 1953; Theobald, 1955). It must, moreover, be emphasized that two corneas removed from animals three months after stopping isoniazid treatment (14 months after infection) produced tuberculosis when inoculated into guinea-pigs. This finding supports the results of other investigators (see Canetti, 1955) that tubercle bacilli can survive very prolonged chemotherapeutic action.

The observed inhibition of systemic spread must represent an important aspect of the action of isoniazid. There is little reason to suppose that isoniazid would inhibit the migration of bacilli from the primary focus, but it would effectively suppress any multiplication if these bacilli were deposited at other sites. It is probable, therefore, that bacilli in small numbers did reach the lungs and other organs of the treated animals, but failed to multiply. Further, they must have been rendered incapable of multiplication within the host because there was no evidence of breakdown in the 8 months of observation after the end of treatment.

The gradual loss of immunity in the treated animals must presumably be due to removal of the antigenic stimulus by the treatment. The total effect, however, is beneficial, since in spite of the disappearance of immunity there was no recrudescence of the disease. These experimental results in mice are in agreement with the clinical findings of Ferebee *et al.* (1957), who found that prophylactic isoniazid therapy in children with asymptomatic primary tuberculosis decreased the incidence of serious extrapulmonary tuberculous complications. Here too the therapy with isoniazid must have prevented the spread of infection from the primary focus.

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