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THE EFFECT OF CORTISONE ON THE MULTIPLICATION OF *M. TUBERCULOSIS* IN NORMAL AND IMMUNE MICE

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The multiplication of *M. tuberculosis, var. bovis* inoculated into the cornea of mice was studied by staining the whole cornea at various stages after inoculation. Four groups of animals were studied: untreated animals, animals treated with cortisone, animals previously immunized with the same bovine strain, and immunized animals treated with cortisone.

In the untreated immunized group little or no multiplication occurred. In the other three groups multiplication did occur and was about the same for the first week after inoculation. After this stage, cortisone-treated animals, whether previously immunized or not, showed increased multiplication and massive cord formation, as compared with untreated animals in which little further multiplication was seen. The cortisone treatment had thus completely suppressed immunity. The significance of these results is discussed.

When tubercle bacilli of a virulent strain are injected into the cornea of a mouse, progressive multiplication of the organisms occurs until at a certain stage a macroscopic lesion appears which spreads to involve most of the cornea. The infection, however, does not spread systemically, and the corneal lesion, once having reached its maximum, tends to regress somewhat, although the total number of organisms present does not decrease. Following the original inoculation, a relative immunity rapidly develops so that a second inoculation, into the other cornea, no longer produces a lesion and the organisms show no evidence of multiplication. It is believed that this immunity is responsible for the fact that a primary inoculum produces only a limited corneal infection with no systemic spread of the disease (Rees and Robson, 1950; Robson and Sullivan, 1957; Robson, Sullivan and Didcock, 1957).

When a mouse receiving a primary inoculum into the cornea is treated with cortisone from the time of inoculation, the infection no longer remains limited but spreads to involve the whole eye, and also systemically, so that the animal ultimately dies with generalized tuberculosis (Naguib and Robson, 1955). Examination of the cornea of such an animal with the phase contrast microscope shows that the hormone treatment for a time prevents the cellular reaction, so that rapid multiplication and spread of the organisms can occur. When cellular invasion does occur it is no longer effective in dealing with the infection (Robson and Didcock, 1956).

The purpose of the present investigation was to follow quantitatively the multiplication of *M. tuberculosis* inoculated into the cornea of animals treated with cortisone and also to determine the effect of the hormone on the immunity process.

METHODS

Albino mice of the "C" strain were used and were inoculated intracorneally, using the technique of Rees and Robson (1950). The mice were immunized by intracorneal inoculation of *M. tuberculosis, var. bovis* and the challenge infection was given one month later by inoculation into the other cornea, as described by Robson, Sullivan and Didcock (1957).

The immunizing and challenging dose consisted of about 0.01 ml. of a 1/100 dilution of a culture in Dubos medium (approximately 5,000 bacilli). The dilutions were made in normal saline containing 0.05% Tween 80. The animals were divided into four groups:

(1) Animals receiving a primary infection in one cornea.
(2) Animals receiving a primary infection and treated with cortisone.
(3) Animals receiving a reinfection in the second cornea 30 days after the primary infection.
(4) Animals receiving a reinfection and treatment with cortisone.

For convenience, these groups are referred to as "normal," "normal plus cortisone," "immune," and "immune plus cortisone," respectively.

In those groups of animals which received cortisone, treatment of immune animals was started on the day before reinoculation and that of animals receiving only the primary infection on the day before inoculation.

All the animals were given aureomycin (0.5 g./l.) in the drinking water to prevent secondary infection. The adrenal hormone treatment was given both systemically and locally, as cortisone acetate 0.5 mg./day subcutaneously and hydrocortisone eye drops 10 mg./ml. twice daily (Roussel). The animals not receiving cortisone were given the same volumes of suspending fluids by the same routes, namely systemically and locally.

For examination, the animal was killed and the cornea removed and examined by phase contrast microscopy as previously described (Robson and Didcock, 1955). It was then stained as a whole and the number of bacilli present estimated by the method of Robson and Sullivan (1957). This entails comparison of the density of the mass of bacilli in the cornea with a standard scale of densities (the "T" scale), and the results are expressed as "T" units, each of which corresponds approximately to double the number of bacilli of the previous unit.

The area of the cornea over which the bacilli are originally inoculated is approximately 500 μ in diameter and does not change appreciably during the first fortnight of the experiment, though the density of the bacilli over that area increases with their multiplication. The "T" value for a particular cornea can thus be taken as an index of the number of bacilli present, without taking into account the area over which the bacilli are spread. In the animals on cortisone, small groups of bacilli became detached from the main mass and migrated towards the periphery of the cornea, but their number was quite small in comparison with the main mass and thus did not affect the validity of the "T" value as a criterion of the total number of bacilli present in the cornea.

RESULTS

Macroscopically, the corneas of the "normal" animals remained clear until the appearance of a tiny lesion after about 7 days. The lesions gradually increased in size during the following weeks, reaching a maximum at about one month after inoculation. Blebs were only occasionally seen in these eyes.

In the "normal plus cortisone" groups the appearance of lesions was delayed until about the 11th day after inoculation. The formation of blebs also began at this time, and by the 15th day after inoculation more than half of the animals had blebs visible in the cornea. Both the blebs and the surrounding lesions increased in size until the end of the experiment on the 18th day.

In the "immune" group no lesions appeared in the challenged eye throughout the period of observation.

The macroscopic appearance of the "immune plus cortisone" group was almost exactly the same as of the "normal plus cortisone" group, with the development of lesions and blebs, so that the immunity was completely suppressed.

Microscopic examination of the corneas in the four groups showed that in the "normal" groups there was a rapid cell invasion with phagocytosis of the bacilli as previously described (Robson and Didcock, 1955). In the "immune" group there was less invasion of the challenged eye by phagocytes and bacillary multiplication was inhibited (Robson, Sullivan and Didcock, 1957).

The appearances in the "normal plus cortisone" and "immune plus cortisone" groups were exactly the same. There was almost complete inhibition of cell invasion at first and the bacilli multiplied,

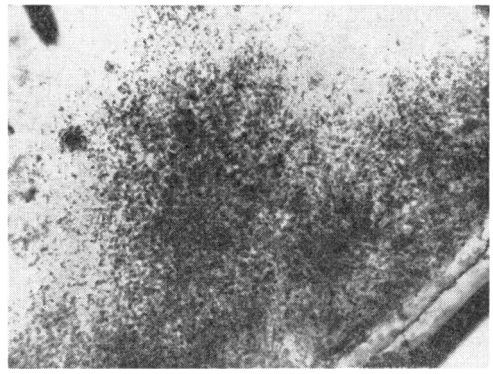

FIG. 1.—Unsectioned mouse cornea 10 days after inoculation with *M. tuberculosis, var. bovis*. Stained with carbol fuchsin and counterstained with methylene blue. Note cell invasion. The bacilli, stained red in the original preparation, were seen dispersed in and among the cells, but are not obvious in this photograph. (×240.)

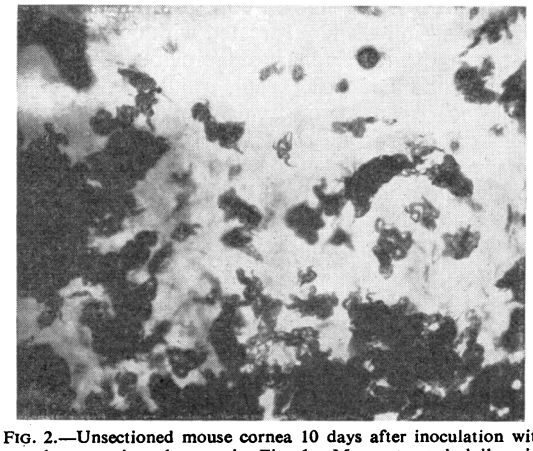

FIG. 2.—Unsectioned mouse cornea 10 days after inoculation with the same inoculum as in Fig. 1. Mouse treated daily with 0.5 mg. of cortisone. Stained with carbol fuchsin and counterstained with methylene blue. Note massive cords of bacilli and complete absence of cell invasion. (×240.)

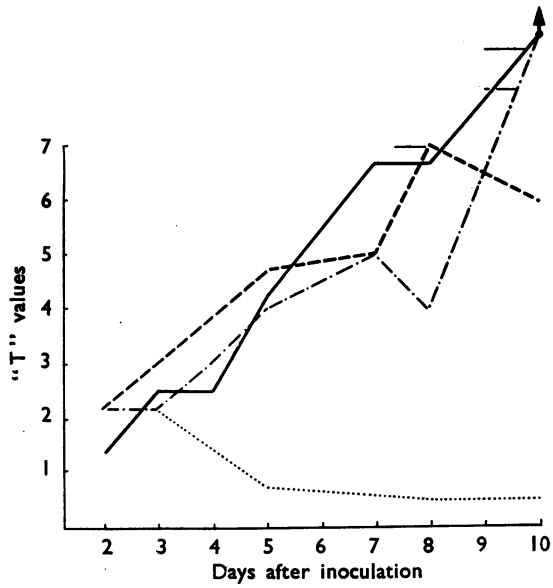

FIG. 3.—Rate of multiplication of *M. tuberculosis* in the corneas of various groups of mice. ------ Normal animals (untreated). ..... Immune animals. ——— Normal animals + cortisone. -.-.-. Immune animals + cortisone. Arrow indicates a value well above the T7 level to which no accurate figure can be given.

forming cords and palisades. In a few animals, where some leucocytes and macrophages had invaded the cornea, there seemed to be little or no attempt at phagocytosis, so that macrophages not containing any bacilli were often seen close to small groups of organisms. Furthermore, there was no aggregation of corneal corpuscles around the site of the bacilli, as is seen in untreated animals. The appearances of the stained cornea in an untreated animal and in an animal treated with cortisone are shown in Figs. 1 and 2. As previously reported (Robson and Didcock, 1956), cellular invasion of the cornea is not permanently inhibited under the influence of cortisone and this was strikingly seen in the present experiment. On the 15th day after inoculation only a few intracellular bacilli were seen, but 2 days later there was marked cellular invasion, forming an impenetrable core of cells and bacilli.

The increase in the number of bacilli in the corneas of the various groups, as estimated by the " T " scale, is shown in Fig. 3. Animals were killed at various times after inoculation and each value on the graph represents the average of the " T " values of three corneas. No bacillary multiplication was observed in the " immune " group, which is in agreement with previous observations (Robson, Sullivan and Didcock, 1957).

There was rapid multiplication in the other groups, and, during the first 8 days or so after inoculation, the rate of multiplication was the same in all of the three groups. After this period, however, multiplication in the " normal " group slowed down, whereas in the cortisone-treated animals the multiplication continued unimpeded until the end of the experiment, by which time the cornea was packed with massive palisades of bacilli.

Systemic Disease.—On the 18th day after inoculation, all the remaining animals were killed and post-mortems carried out to search for evidence of systemic disease. The animals were examined macroscopically and smears of the liver and lung were stained with Ziehl-Neelsen stain. No sections were cut except in one case of a lung nodule which proved to be a typical tuberculous focus. In the " normal " and " normal plus cortisone " groups no evidence of systemic disease was found and no acid-fast bacilli were found.

In one animal in the " immune " group acid-fast organisms were found in a hard yellow nodule attached to the under-surface of the liver.

In the " immune plus cortisone " group 7 of 8 animals had macroscopic evidence of infection, and in 5 of these acid-fast organisms were found in smears of the lungs.

Effect of Cortisone on an Established Lesion.—The right (immunizing) eyes of the two immune groups were removed and examined when the experiment was terminated, namely, 7 weeks after infection (18 days after reinfection of the second eye, and after 19 days of cortisone treatment). In corneas from 5 " immune " animals there were large masses of bacilli corresponding to the large macroscopic lesions. Nearly all the bacilli were intracellular and there was little evidence of spread into the sclera. No cord formation was seen in any of these animals. In all 8 animals of the " immune plus cortisone " groups examined, extracellular bacilli were seen, and clear evidence of cord formation and spread to the sclera was seen in 5 of these animals.

Thus the administration of cortisone to animals with established lesions caused a reactivation of the infection with free extracellular multiplication of the bacilli and spread of the infection.

DISCUSSION

These experiments show that treatment with cortisone does not affect the initial rate of multiplication of tubercle bacilli in the cornea of mice. For the first 7 days after inoculation the number of organisms increased at the same rate in the control and cortisone-treated animals. Since in the control

mice the bacilli rapidly became intracellular, whereas in the treated animals they remained extracellular, multiplying in cords, throughout this period, it can be concluded that, under the conditions of these experiments, they multiply at the same rate inside leucocytes as in an extracellular environment. These results are also in agreement with the finding that cortisone does not enhance the multiplication of *M. tuberculosis in vitro* (Lurie, Zappasodi, Dannenberg, and Cardona-Lynch, 1953).

After the 7th day the bacilli in the control corneas show little further multiplication, presumably because of the development of immunity. It has been shown that, if at this stage the second cornea of such an animal is challenged, little or no bacillary multiplication occurs (Robson, Sullivan and Didcock, 1957). In the cortisone-treated animals, on the other hand, multiplication continues, leading to the formation of very thick cords of bacilli with destruction of the eye and ultimate systemic spread and death, as shown by Naguib and Robson (1955). This shows that massive cord formation can occur not only *in vitro* but also in living tissues. It seems likely that these changes occur because the immunity, which checks the further multiplication in the control animals, is not effective in the presence of cortisone. There are two possible explanations for this: (1) The treatment with cortisone has prevented the development of immunity. There is indeed some evidence that cortisone inhibits the formation of antibodies during active immunization (Fischel, 1953), though the effect is by no means a complete one (Germuth, 1956). In any case, the question as to whether antibodies play any part in immunity to tuberculosis is by no means settled. (2) Immunity has developed but is unable to exert its action. If immunity in tuberculosis is dependent on the action of cells on the bacilli, as has been suggested, then the inhibition of cellular invasion which is observed would explain the failure of the immune process to check the bacillary multiplication.

Furthermore, it has been observed that there is a positive correlation between cord formation *in vitro* and virulence (Middlebrook, Dubos and Pierce, 1947). It may well be that the massive cord formation seen in the corneas of cortisone-treated animals allows the development of an increased virulence of the bacilli, and this may also be responsible, to some extent, for the eventual spread and fatal outcome of the disease.

Solotorovsky, Gregory and Stoerk (1951) have shown that cortisone overcomes the beneficial effects of vaccination with heat-killed tubercle bacilli in mice inoculated intravenously with a highly virulent strain of *M. tuberculosis*. These results are in agreement with the previous findings of Hart and Rees (1950), who found that cortisone exacerbated a chronic tuberculous infection in mice, presumably overcoming the immunity present in these mice. In the present experiments, an immunity sufficient to inhibit completely the increase in the number of organisms in the challenged cornea was overcome so that the organisms multiplied at the same rate as in an eye not protected by previous immunization. Since cellular invasion was almost completely inhibited in the cortisone-treated animals, these results could be explained on the basis of a cellular mechanism of immunity in tuberculosis. It must, however, be remembered that cortisone not only inhibits the production of antibodies during active immunization, but also after immunization is well established (Fischel, 1953), so that these results do not exclude the possibility that the effect of cortisone involves some interference with antibodies.

The effect of cortisone on the immunizing infection in the first cornea also requires consideration. In a number of these animals there was clear extracellular bacillary multiplication, with cord formation and spread of bacilli to the sclera, and the development of systemic disease. This is not seen in corresponding animals not treated with cortisone, in which no evidence of bacillary multiplication is observed. This difference may be dependent on the comparatively short life of macrophages. When such cells break down in untreated animals, the bacilli released are taken up by new macrophages and, in the presence of immunity, no further bacterial multiplication occurs. In the cortisone-treated animals, new macrophages are not available to take up the released bacilli, which multiply, invade the sclera and spread systemically. This seems to be the most reasonable explanation for the phenomena observed.

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