

Effect of Immunosuppressive Drugs on Infection in Mice by *M. marinum* (balnei), *M. tuberculosis* and *M. leprae*¹

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We have studied the effect of immunosuppressive drugs on the infections in mice produced by three species of mycobacteria, *M. marinum* (balnei), *M. tuberculosis*, and *M. leprae*. Although the ultimate goal of the study, an increase in the growth of *M. leprae* in mouse foot pads, was not achieved, some information was gathered that may prove useful to others, at least in enabling them to avoid repeating these experiments.

The immunosuppressive drugs are known to be able to suppress antibody formation, delayed hypersensitivity, and a homograft rejection (reviewed in ⁽⁶⁾). The drugs are of several types: (a) the alkylating agents or radiomimetic agents, e.g., cyclophosphamide (cytoxan, CTX) and bisulfan (Myleran), (b) the purine antagonists, e.g., 6 mercaptopurine (6MP) and azathioprine (Imuran), (c) pyrimidine antagonists, e.g., 5-fluorouracil and 5-bromodeoxyuridine, and (d) the folic acid analog amethopterin (methotrexate, MTX). In addition there is (e) a group of immunosuppressive antibiotics whose modes of action are to interfere with stages of the translation of the message from DNA to RNA to protein; those whose immunologic action has been especially studied are actinomycin D and chloramphenicol. We have confined our studies to three drugs that have been well studied in mice and shown to have good immunosuppressive activity at a level somewhat removed from the toxic level ^(1, 2). These are MTX, 6MP, and CTX.

MATERIALS AND METHODS

Confusion has arisen in the literature be-

cause of reports of negative experiments that were due in fact to inadequate dosage of drug ⁽⁶⁾. In order to avoid this source of error, it is necessary to administer the drugs at the highest possible sublethal level. Drugs were prepared for injection as follows: MTX was dissolved in 2 per cent NaHCO₃. 6 MP was dissolved in half the final desired volume of water by dropwise addition of 1 N NaOH with constant stirring; 1 N HCl was added in dropwise fashion until a precipitate appeared; 1 drop of 1 N NaOH was then added, and the finally turbid solution made up of 0.75 mgm./ml. CTX was dissolved in 0.85 per cent NaCl. In general the drugs were made up so that the desired per kgm. dose was contained in 10 ml., and the injected volume in ml. was 1 per cent of the body weight in grams. Because of the limited solubility of 6 MP, the top dosage was made up so that the injection was 2 per cent of the body weight. The drugs were stored in brown bottles at 4°C. They were injected subcutaneously.

M. marinum (strain balnei X) and *M. tuberculosis* (strain H37Rv) were young cultures in Tween-albumin medium, stored at -60°C, thawed just before use, and diluted in tryptose broth to the desired concentration. *M. leprae* was fresh passage material.

The mice were of the CFW strain maintained at the Communicable Disease Center. Because the toxicity of the drugs is greater in younger mice, the mice were allowed to grow to at least 80 per cent of their mature weight before the drug injections were started. They were fed commercial pellets.

RESULTS

M. marinum (balnei). In the experiment illustrated in Figure 1, the untreated mice had well-developed redness and swelling by the 13th day, the time of the first harvest; the bacterial population was then between

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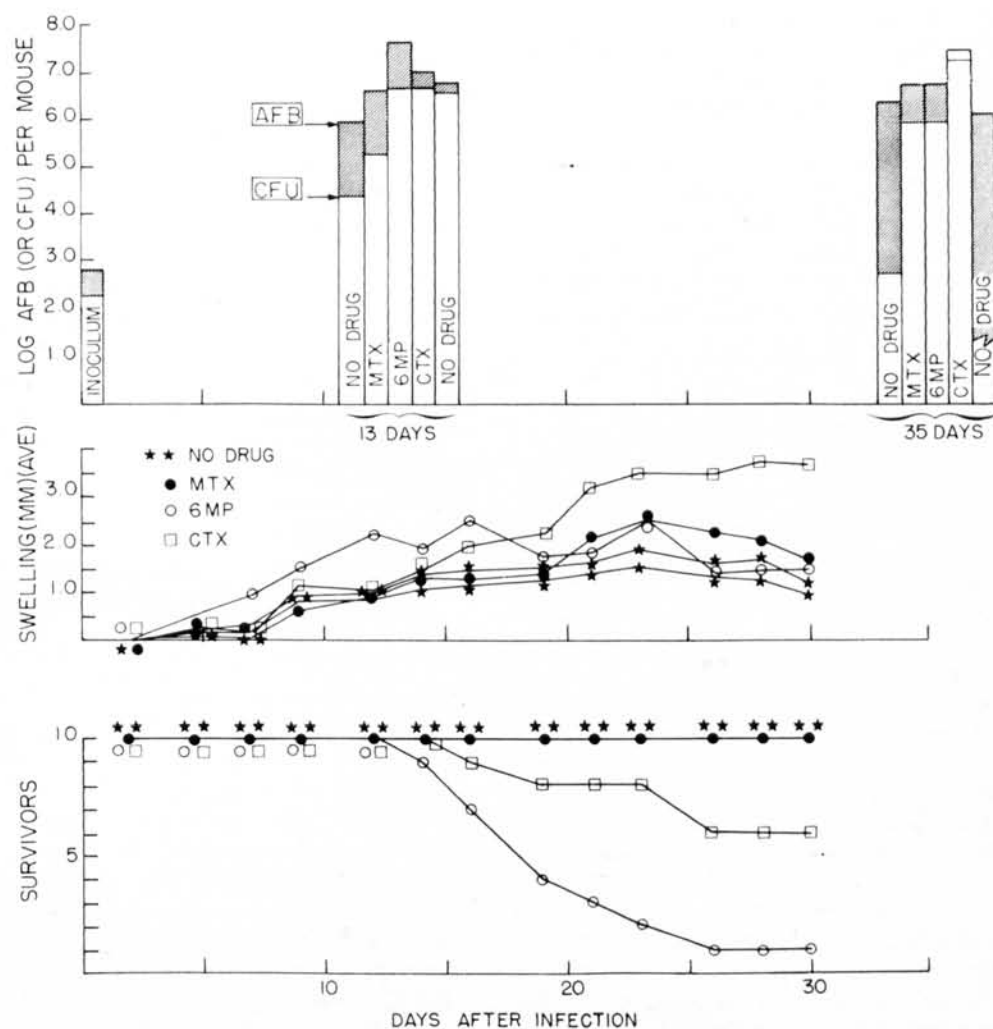


FIG. 1. Effect on *M. marinum* (balnei) infections of MTX, 6MP, and CTX. Two days before infection the drugs were started in the following dosages: MTX, 6 mgm./kgm., three times weekly; 6MP, 75 mgm./kgm., three times weekly; CTX, 300 mgm./kgm., once weekly. MTX was given for 32 days, 6MP for 19 days, and CTX for 28 days. *M. marinum* (balnei) was given in the right hind foot pad. The amount of swelling was measured with calipers (comparison of infected to uninfected foot). On the 13th and 35th day after infection, four mice were killed, and their right hind foot pad tissues pooled for counts of acid-fast bacteria (AFB) and colony-forming units (CFU) on 7H9 agar (at 35 days, however, only one 6MP mouse remained for counts). (In the counts on CTX mice at 35 days, observed CFU were slightly higher than observed AFB.) Of the 20 mice started for each group, 10 were saved for the bacterial counts and 10 for observations of infected feet and mortality.

$10^{6.0}$ and $10^{7.0}$, the normal plateau level for this strain of mouse. The mice treated with 6 MP had more severe tissue response to the infection and somewhat higher acid-fast bacillus (AFB) counts. By the 35th day in the untreated mice, the number of viable bacilli had dropped characteristically to low levels, without much change in the number of stainable bacilli; in the second group of controls no colonies at all were seen in the lowest dilution. In the treated mice, the number of viable bacilli remained high, especially in the CTX-treated mice. The CTX-treated animals had distinctly more redness and swelling late in the infection also.

Very similar results were obtained in another experiment in which the doses of the drugs were varied in two-fold steps. The early tissue reaction was increased only in the mice treated with the highest dose of 6 MP (150 mgm./kgm.), and the late tissue reaction only with CTX (300 mgm./kgm.). Bacillary counts were carried out only on the 28th day and only in drug groups receiving the dosages of the experiment in Figure 1. The number of AFB was within the expected limits in all groups except the CTX, where it was $10^{8.8}$. The number of colony forming units (CFU) was below detectable limits in all the controls, but was $10^{4.6}$, $10^{5.2}$, and $10^{7.5}$ in the MTX, 6MP, and CTX groups, respectively.

Thus CTX had the most pronounced effect among the three drugs. Since this was in agreement with reported results for these drugs on suppression of homograft response⁽²⁾ and antibody response⁽¹⁾, and since CTX is more convenient to administer, subsequent work was limited to this drug.

M. tuberculosis. In the experiment of Figure 2, mice were infected intravenously with a dose of tubercle bacilli that caused slow mortality between the 20th and 90th day. A similar mortality curve was seen in uninfected mice receiving CTX. Infected mice receiving CTX died rapidly between the fifth and 15th days. The two remaining mice were taken for bacterial counts on the 15th day. Bacterial counts were a little higher in the CTX-treated mice, especially in the spleens. The proportion of bacteria that were viable was also usually higher.

In another experiment the dosage of ba-

cilli was varied and in another the dosage of drug was varied, but the effect on mortality was not any more clearly discernible. When CTX was given in completely sublethal amounts, the effect on mortality was not distinct.

M. leprae. Little if any effect on bacterial growth was noted (Table 1). In experiments A, B, and C, CTX was not started until multiplication had carried the bacterial population to countable levels. In A and B, CTX was given in a large dose and after 60 days only three mice remained for counts. Because of the excessive mortality, the dosage of CTX was lowered in experiments C and D. A marked weight loss had been observed to precede the deaths by a few weeks; so an average weight loss of a few grams per mouse was set as a criterion for withholding drug. In experiment D, the drug was started three days before infection.

In an earlier experiment with cortisone it had been observed that the most distinct effect of the drug was an increase in the proportion of solidly staining bacilli⁽⁷⁾. However, no such effect was noted in any of these experiments with CTX.

Histologic observations. Tissues of selected organs of many mice, including most of those killed for bacterial counts, were saved for histologic examination. The spleens and lymph nodes of mice receiving 300 mgm. CTX/kgm. in repeated (3-5) injections were devoid of follicles and relatively acellular. However, in the experiments C and D of Table 1, where the dose of CTX was lowered to avoid excessive mortality, follicles were observed regularly in spleen and lymph node.

Some observations were made also of the feet infected with *M. marinum* (balnei). No differences between treated and untreated mice were observed, and both groups had the expected necrotic lesions, frequently liquefactive, with a peripheral cellular response that was predominantly polymorphonuclear.

DISCUSSION

MTX, 6 MP, and especially CTX had distinct promoting effects on the *M. marinum* (balnei) infections. However, the effect of CTX on *M. tuberculosis* infections

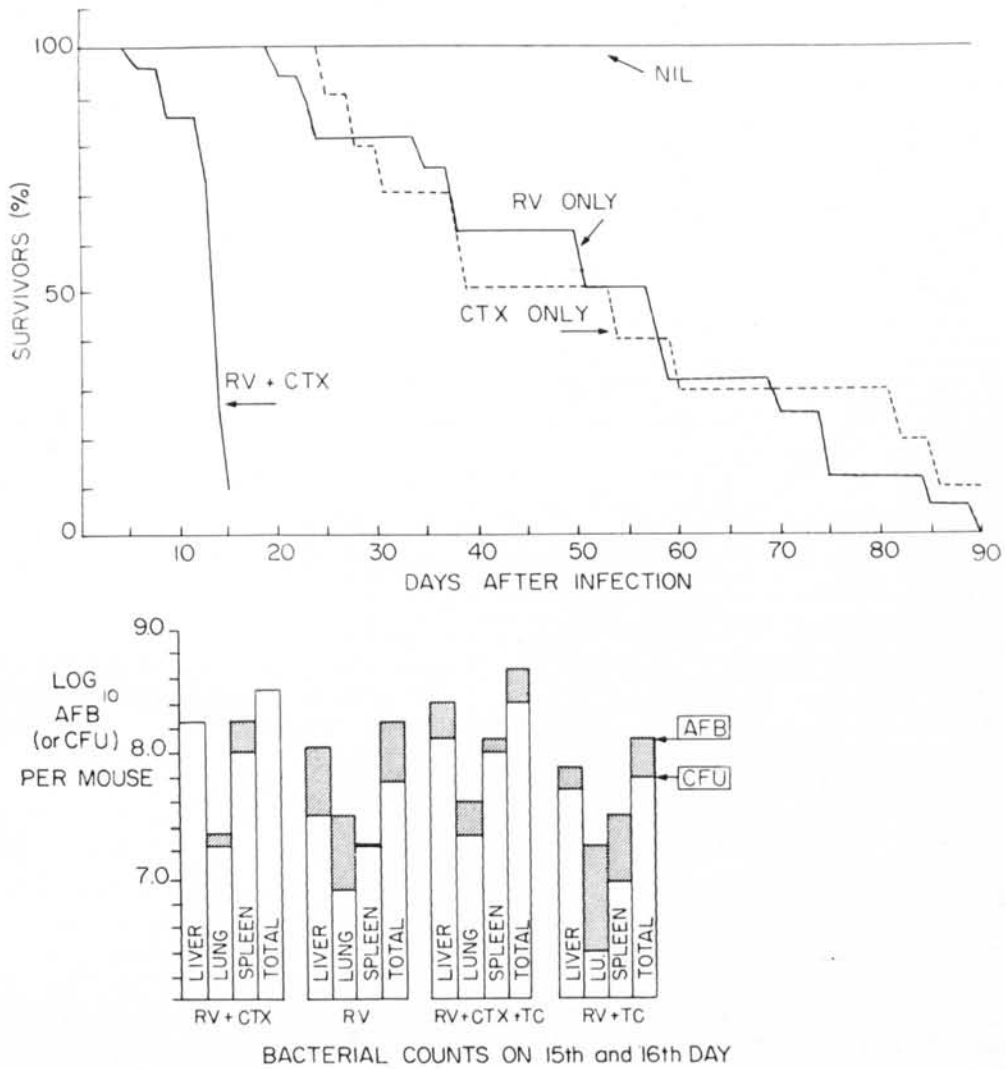


FIG. 2. Effect of CTX on *M. tuberculosis* infections. CTX (300 mgm./kgm.) was given five days before and two and nine days after intravenous injection of 0.2 ml. H37Rv strain of human tubercle bacilli (4.0×10^7 AFB, 2.4×10^7 CFU, 0.02 mgm. [dry weight], 0.1 μ l (Hopkins tube)). The "Rv + CTX" and "Rv only" groups contained 25 mice; the CTX and uninoculated control (Nil) groups contained 10 mice. Parallel groups with the same number of mice received 0.03 gm. tetracycline/1 drinking water.

Upper half: Mortality curves for the mice not receiving tetracycline. Excluded from consideration are mice dying on day 1, and eight "Rv only" mice killed for bacterial counts on day 16. For the tetracycline mice the mortality was very similar, except that in the "Rv only" and "CTX only" groups the survivors levelled off at 40 to 50 per cent after 50 days.

Lower half: Bacterial counts on pools of tissues. "TC" refers to mice receiving tetracycline. The pools consisted of the two survivors in each of the CTX-treated groups, and 8 mice from each of the groups not treated with CTX; these were taken on the 15th and 16th days, respectively.

TABLE 1. *Effect of CTX on M. leprae infections.*^a

Exper.	CTX treatment	Harvests (Day: AFB/mouse, S/(N + S))	
A	Nil 300 mgm./kgm./wk. for 5 wks. (191-226 d).	190 d: 1.2×10^5 , 17/50.	252 d: 3.7×10^5 , 4/50. ^b 252 d: 2.2×10^5 , 0/20.
B	Nil 300 mgm./kgm./wk. for 5 wks. (147-182 d).	146 d: 2.5×10^6 , 27/50.	208 d: 1.2×10^6 , 5/50. ^b 208 d: 5.7×10^6 , 2/50.
C	Nil 200 mgm./kgm., 119, 140 d; 150 mgm./kgm. 126, 161, 175, 196, 214, 238 d, depending on weight loss.	^c 115 d: 4.8×10^5 . ^b 182 d: 2.9×10^6 , 3/50. ^b 182 d: 6.0×10^6 , 2/50.	^d 175 d: 3.4×10^6 , 6/50. 242 d: 1.9×10^6 , 3/50. 175 d: 1.7×10^6 , 18/50. 242 d: 3.5×10^6 , 1/50.
D	Nil 150 mgm./kgm., -3, +4, 18, 32, 46, 59, 67, 74, 87, 93, 123, 129, 143, 157, 186, 200, 222 d, depending on weight loss.	126 d: 3.8×10^6 , 6/50. 126 d: 1.8×10^6 , 6/50.	235 d: 1.9×10^6 , 0/50. 235 d: 1.7×10^6 , 1/50.

^a Mice were inoculated with 4 to 5 thousand *M. leprae* with a high proportion of solidly staining bacilli, and part of them were treated with CTX. The effect of drug was judged by its effect on the number of, bacilli harvested (AFB/mouse), and the proportion of solidly staining bacilli (S/(N+S)). The numbers of mice in the treated groups at the start of treatment were 24, 32, 30 and 40 in the four experiments, respectively.

^b Three mice only.

^c One mouse only.

^d Two mice only.

was less distinct, and that on *M. leprae* infections was not clearly discernible.

In the *M. marinum* (balnei) infections the chief effect of all three drugs was that the bacteria remained viable in the plateau phase of the infection. In the untreated controls the proportion of stainable bacteria capable of forming colonies decreased to about 1 in 10^4 . The period of observation was about 30 days after infection and a little over two weeks after the onset of the plateau phase.

In the *M. tuberculosis* infections the most obvious effect of CTX was a hastened mortality. However, this was seen only with a dosage that eventually killed many of the mice that were not experimentally infected. The CTX-treated mice also had somewhat higher bacterial counts. It is possible that two technical factors interfered with the manifestation of any CTX effect on the bac-

terial counts: one, that the mice selected for counts were the survivors and may therefore have had less bacterial infections than the group average; the other, that the infections observed were probably in the early plateau phase rather than the late stage when the *M. marinum* infections manifested the greatest effect. However, it has been reported by Rees and Hart (⁵) that the proportion of viable bacilli in chronic pulmonary infections of mice remained constant over extended periods of observation and did not fall, as it did here in the *M. marinum* (balnei) infections.

Prichard and Hayes (³) carried out an experiment with MTX in guinea-pigs infected with *M. tuberculosis*. They also observed an accelerated mortality in the 42-day period of observation; the dosage of MTX was sufficient to kill two of four uninfected animals, and the inoculum of ba-

cilli was enough to kill three of 11 animals not given drug. Histologic observations suggested that there were many more bacilli in the treated guinea-pigs.

In our *M. leprae* experiments the mortality due to drug itself necessitated a reduction in its dosage to a level where there were enough surviving animals after the time period required by this very slow infection. The reduced dosage of drug no longer suppressed lymphoid follicle activity in spleen and lymph node. No clear effect was noted on the growth of *M. leprae*.

Since this work was begun, Rees (⁴) has reported that *M. leprae* grows to higher plateau values in adult mice that have been thymectomized and x-irradiated. The dose of radiation, 850 r, was heavy, and the mice were protected from death by transplantation of isogenetic bone marrow cells. It might be speculated that such treatment is successful because it is directed especially toward the lymphoid cell population. A similar explanation might be raised for the potentiating effect of cortisone on the plateau level of solidly staining *M. leprae* in mice (⁷). On the other hand the immunosuppressive drugs probably exert their effect on all rapidly multiplying cells in the body.

SUMMARY

The immunosuppressive drugs amethopterin (MTX), 6-mercaptopurine (6MP), and cyclophosphamide (CTX) were studied for their effect on infections of mice with *M. marinum* (balnei). The principal effect noted was a marked increase in the proportion of the stainable bacilli that were viable (platable).

In *M. tuberculosis* infections of mice the chief effect noted for CTX was an acceleration in mortality.

In *M. leprae* infections of mice the dosage of CTX had to be reduced to avoid the excessive drug-associated mortality that appeared during the extended time periods required for observation in this infection. No promotion of the growth of *M. leprae* was observed.

RESUMEN

Las drogas inmunosupresivas amethopterin (MTX), 6-mercaptopurine (6MP), y cyclophosphamide (CTX) fueron estudiadas por

sus efectos en infecciones de ratones con *M. marinum* (balnei). El efecto principal notado fué un marcado aumento en la proporción de los bacilos teñibles que estaban vivos (platable).

En infecciones de ratones con *M. tuberculosis* el efecto mayor notado con CTX fué una aceleración en la mortalidad.

En infecciones de ratones con *M. leprae* la dosis de CTX tuvo que ser reducida para evitar la excesiva mortalidad asociada con la droga que apareció durante los prolongados periodos de tiempo requeridos para la observación en esta infección. No se observó aceleración del aumento en *M. leprae*.

RÉSUMÉ

On a étudié l'action, sur les infections de la souris par *M. marinum* (balnei), des produits immuno-suppresseurs suivants: l'amethopterin (MTX), la 6-mercaptopurine (6MP) et la cyclophosphamide (CTX). L'action la plus importante qui ait été notée a consisté en une augmentation notable dans la proportion des bacilles colorables qui étaient en même temps viables (qui pouvaient être ensemencés sur boîtes de Petri).

Dans les infections de la souris par *M. tuberculosis*, l'action principale observée pour le CTX a été une accélération de la mortalité.

Au cours des infections de la souris par *M. leprae*, les doses de CTX ont dû être réduites, afin d'éviter la mortalité excessive associée à l'administration du produit, qui est apparue durant la période prolongée requise pour permettre l'observation chez des animaux infectés de la sorte. On n'a observé aucune stimulation de la croissance de *M. leprae*.

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