

Effect of Immunosuppression on the Multiplication of *Mycobacterium ulcerans* in the Mouse Foot Pad^{1,2}

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Adult animals can be rendered immunologically tolerant by treatment with alkylating agents, purine or pyrimidine analogs, and antagonists of folic acid metabolism⁽¹⁰⁾, provided that antigen is administered along with the immunosuppressive agent^(6, 12). The specificity of the tolerant state has been demonstrated by the fact that the immunologic response to second party antigens remained normal throughout the period the animal was tolerant to further challenges with the original antigen⁽¹¹⁾. Animals remain tolerant for periods from a few weeks to several months, depending on the degree of immunosuppression.

If immune tolerance has a relationship to the multiplication of mycobacterial pathogens in the mouse foot pad, it may be predicted that increased multiplication rates should occur during tolerant states.

This communication reports the results of initial attempts to obtain increased multiplication rates of *Mycobacterium ulcerans*

in the foot pads of mice that had been treated with Imuran⁴ as the immunosuppressive agent. A brief report on this work has been made.⁵

MATERIALS AND METHODS

Animals. Female mice of the CFW strain were used. The animals (18-21 gm. body wt.) were fed *ad libitum* a diet of Rockland mouse pellets and maintained at an ambient temperature of 21-22°C.

Antigen. Homologous-type antigen was prepared by heating *M. ulcerans* at 80°C for 15 minutes in isotonic saline. Heat-killed cells (5×10^6) were injected intravenously 3 weeks prior to foot pad inoculations.

Immunosuppressive agent. The purine antimetabolite Imuran was obtained from Burroughs Wellcome Research Laboratories, Tuckahoe, New York. Imuran was injected intraperitoneally, immediately after injection of antigen, at a concentration of 50 mgm./kgm./day for a period of three weeks prior to foot pad inoculations.

Challenge dose. The two hind foot pads were inoculated with 10^6 cells of the Battista strain of *M. ulcerans* suspended in balanced salt solution. The organism, originally isolated in Australia, was grown in a modified Watson-Reid medium⁽⁷⁾ con-

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⁴Burroughs Wellcome and Co. preparation of azathioprine-(6-(1-methyl-4-nitro-5-imidazolyl) thiopurine).

⁵Data presented at the Leprosy and Tuberculosis Conference, U. S.-Japan Cooperative Medical Science Program, 18-20 May 1966, Tokyo, Japan.

taining 0.1% (v/v) Tween 80 and 0.5% (w/v) serum albumin (fraction V). The medium was prepared without the addition of iron salts and had a trace iron content of 0.16 μ gm./ml.

Bacterial multiplication. At weekly intervals the foot pads were removed surgically from inoculated animals. Individual foot pads were minced with scissors and then ground in a microhomogenizer in 0.1 ml. of balanced salt solution lacking phenol red (⁴). Following centrifugation at 500 g for one minute the numbers of bacilli present in the supernatant fluid were estimated by the microscopic counting method of Hanks, Chatterjee and Lechat (⁵).

Serum agglutinins. During the course of Imuran treatment agglutination reactions were carried out with mouse sera obtained 10, 15 and 20 days after injection of antigen. The tube dilution method, utilizing suspensions of nonclumping mycobacteria, was used (⁶). For the agglutination reaction 0.25 ml. volumes of increasing dilutions of the antiserum were mixed with equal volumes of the bacterial suspension. The mixtures were incubated at 37°C and the results read three to seven hours later and again at 20 hours.

RESULTS

Circulating agglutinins. The agglutinin titers observed on the 10th day after injection of antigen (11 days before challenge) are shown in Table I. The results were obtained with pooled sera from five animals

in each group. Although a complete suppression of circulating agglutinins was not observed, it appears that the differences between animals capable of normal response to antigen and those treated with Imuran plus antigen were of the order of at least four to eight times. The titers for 1+ agglutination differed by four times and those for 2+ agglutination by approximately eight times. In animals that received antigen plus Imuran, no serum dilutions caused a 4+ agglutination. Sera obtained from animals 15 and 20 days after injection of antigen likewise showed a similar range of titers, indicating a decreased agglutination activity due to Imuran treatment.

Multiplication of *M. ulcerans*. Figure 1 shows bacterial multiplication over a three week period in the foot pads of four groups of animals. The results in animals that had received antigen prior to challenge (lower curve) demonstrate that mice are readily immunized by *M. ulcerans* (see also Fenner ^{2,3}). The suppression of acquired resistance by Imuran (top curve) was expressed in two ways: (1) by permitting the bacterial numbers to increase six times higher than in animals treated with antigen alone, and (2) by permitting a doubling of the bacterial numbers in contrast to the two control groups that had not received antigen prior to challenge.

Significant differences were observed in the morphology of stained bacteria recovered from the four groups of animals. Bacilli from the foot pads of animals re-

TABLE I. Effect of Imuran treatment on the agglutination of *M. ulcerans* with mouse sera.

Serum dilution	Nontreated controls	Imuran-treated	Antigen-treated ^a	Antigen plus Imuran-treated
1:20	0	0	4+	2+
1:40	0	0	4+	2+
1:80	0	0	4+	2+
1:160	0	0	4+	1+
1:320	0	0	3+	1+
1:640	0	0	2+	0
1:1280	0	0	1+	0
1:2560	0	0	1+	0
1:5120	0	0	0	0

^a4+ = complete agglutination, 3+ = opalescence in supernatant, 2+ = agglutinated particles remain in supernatant, 1+ = trace agglutination, 0 = no agglutination.

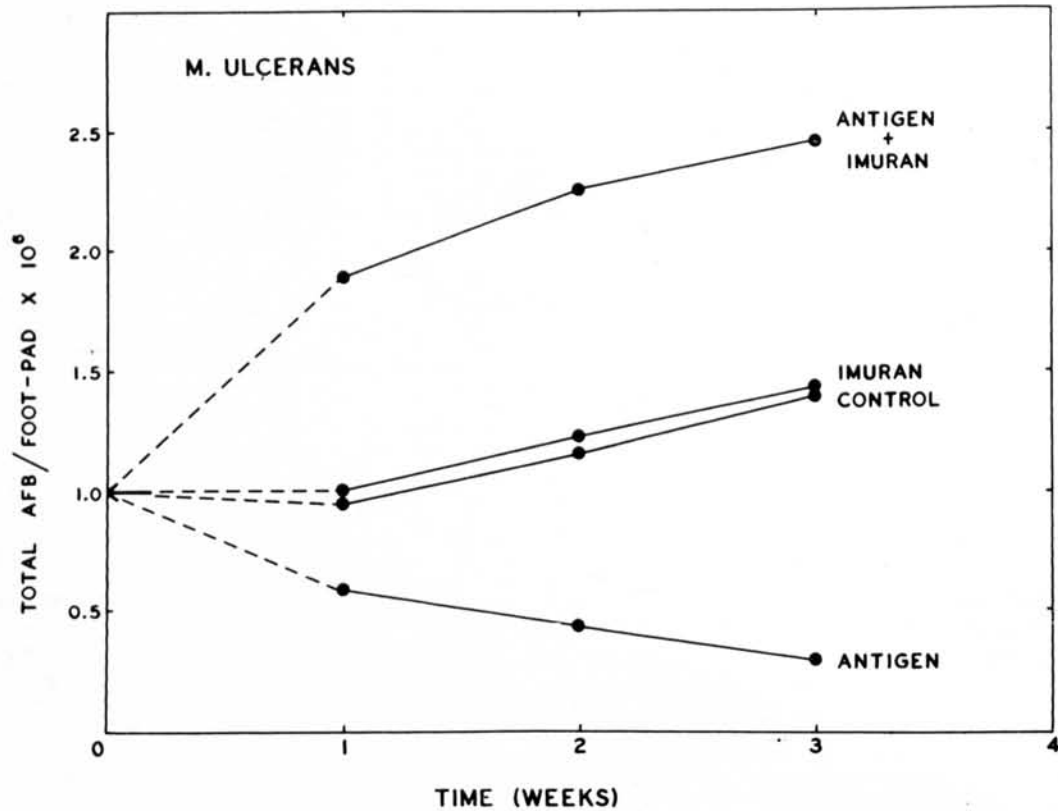


FIG. 1. Multiplication of *M. ulcerans* in the four groups of experimental animals. Each point represents the average count from paired foot pads of five animals.

ceiving antigen alone were mostly in the form of unevenly stained short rods, while bacilli from animals that had received antigen plus Imuran showed a large proportion of elongated forms indicative of cell multiplication.

DISCUSSION

This preliminary study demonstrates that immunosuppression by means of well tolerated doses of Imuran interferes with both the serologic and bactericidal modes of immune response. Since it is unlikely that optimal conditions were obtained in respect to Imuran dosage or the saturation of immunocytes with antigen, the results appear to justify further work with this compound and with other recently available agents⁽¹⁾. Optimal conditions are of critical importance in obtaining a more nearly perma-

nent and complete suppression of immunologic responses⁽¹¹⁾. It is probable that Imuran functions as an antagonist of nucleic acid metabolism necessary for antibody synthesis.

The convenient model described should facilitate investigation of the conditions required for obtaining maximal suppression of immune responses during the essentially self-limiting micro-infection caused by *M. leprae* in mouse foot pads. An increase of 2 logs in bacterial numbers as a result of thymectomy and irradiation⁽⁸⁾ indicates clearly that immunologic processes are in part responsible for the limitation in bacterial multiplication.

SUMMARY

Treatment of adult mice with the immunosuppressive agent, Imuran, resulted in

(1) a decreased level of circulating agglutinins and (2) a two to three fold increased multiplication of *M. ulcerans* in the foot pad. Imuran effectively produced a suppression of acquired resistance for a period of three weeks. The results are consistent with the view that immunologic processes are in part responsible for the limitation in growth of mycobacterial pathogens in the mouse foot pad.

RESUMEN

El tratamiento de ratones adultos con el agente inmunosupresivo, Imuran, resultó en (1) una disminución del nivel de aglutininas circulantes y (2) en 2 a 6 veces la multiplicación de *M. ulcerans* en el colchón plantar. Imuran produjo efectivamente una supresión de la resistencia adquirida por un período de tres semanas. Los resultados son consistentes con la observación de que el proceso inmunológico es en parte responsable por la limitación en el crecimiento de mycobacterias pathogenas en el colchón plantar del raton.

RÉSUMÉ

Le traitement de souris adultes par l'Imuran, un agent immunosuppressif, a entraîné (1) une diminution du niveau des agglutinines circulantes, et (2) une augmentation de deux à six fois de la multiplication de *M. ulcerans* dans la sole plantaire. L'Imuran a réellement produit une suppression de la résistance acquise durant une période de 3 semaines. Les résultats sont en accord avec l'opinion qui veut que les mécanismes immunologiques soient en partie responsables pour la limitation de la croissance des organismes mycobactériens pathogènes dans la sole plantaire de la souris.

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