Adjuvant Disease-Prevention with Complete Freund's Adjuvant and with Mineral Oil Alone (37358)

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In a previous paper (1), this laboratory reported that adjuvant disease can be prevented by subcutaneous injections of heat-killed *Mycobacterium butyricum* in oil two weeks prior to routine induction. Pretreatment with 50 μg of killed organism in mineral oil provided optimal protection. Contrary to the report of Zahiri *et al.* (2), we observed no prevention of the disease by pretreatment with mineral oil alone at 14 days. One major difference between the work of Zahiri's group and ours was in timing. They induced the disease four weeks after pretreatment; our waiting period was two weeks. The experiment reported here was designed to test the suppressive effects of various doses of killed mycobacteria in mineral oil, and of mineral oil alone, administered to rats four weeks prior to the usual induction procedure.

Materials and Methods. Rats. Forty-eight, nine-week-old, 170–200 g, female, random-bred, Holtzman rats were numbered consecutively, housed two to a cage, and fed Purina Rodent Lab Chow *ad libitum*. Six pretreatment groups of eight animals each were randomly selected from this pool.

Pretreatment. On Day 0, animals in Groups 3–6 were treated with 0.1 ml of sterile mineral oil (Paraffin oil, white, USP, viscos 340–355, M C & B, Cincinnati, OH) containing 12.5, 50, 200, or 800 μg of heat-killed *Mycobacterium butyricum* (Difco), respectively. Group 2 received 0.1 ml of sterile mineral oil alone, and Group 1 was injected with 0.1 ml of sterile saline. All injections were given subcutaneously, three inches distal to the base of the tail.

Induction. Twenty-eight days after a single pretreatment, each test animal was given a subcutaneous injection containing 500 μg of heat-killed *Mycobacterium butyricum* in 0.1 ml of sterile mineral oil, one inch distal to the base of the tail.

Evaluation of arthritis. The visual scoring system, published earlier (1), was a modification of that used by several investigators (3–7). Scores in this system range from 24 for a disease-free animal to 82 for the most severely affected rat. An animal scoring 40 or more in our system is moderately to severely diseased. Animals were scored without knowledge of group category or designation.

Results. Control animals pretreated with saline followed the usual course of disease when induced 28 days later; the mean peak score was 58.1 (Fig. 1). Animals pretreated with mineral oil alone followed the same course, but with a mean peak score of 39.1, which was significantly lower ($p < 0.01$) than the saline controls. The low and intermediate dose groups developed lesions on the same time course as the controls but never exceeded a mean peak score of 29.3; significantly lower than the mineral oil group ($p < 0.05$), and still lower than the saline controls ($p < 0.001$).

Both of the high dose pretreatment groups developed disease two weeks prior to the inductive challenge. The mean peak score of 33.8 for the 200 μg group was significantly lower ($p < 0.05$) than the 49.0 for the 800 μg group. A protective effect from the latter was suggested, since the mean peak score was 9 points lower than for the saline controls at the corresponding post-induction day (Day 56, Fig. 1). However, this difference was not

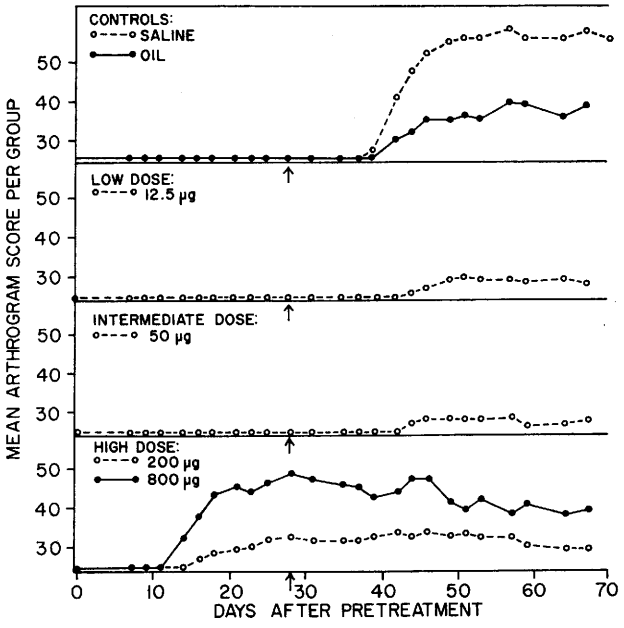

FIG. 1. Influence of mineral oil or various doses of killed *M. butyricum* in oil on adjuvant disease induced 28 days later. Six groups of 8 female, random-bred Holtzman rats each received subcutaneous, pre-induction injections of 0.1 ml sterile saline, mineral oil, or oil containing indicated doses of killed *M. butyricum*, on Day 0 in the tail. Induction (↑) on day 28 was with 500 μg of the same killed organism given in mineral oil as above.

significant at the 95% level.

The percent of animals with moderate to severe disease in each group at the mean peak of disease was as follows: saline, 87.5%; mineral oil, 50%; 12.5 μg, 12.5%; 50 μg, 12.5%; 200 μg, 25%; and 800 μg, 75%. By Day 67, a 12.5% decrease (improvement) was seen in the 200 and 800 μg groups in contrast to a 12.5% increase in the saline controls.

Discussion. In this study, low and intermediate doses of killed *Mycobacterium butyricum* in mineral oil did not induce visible signs of disease, but gave effective protection against induction of the disease 28 days later. The high doses (200 and 800 μg) both induced disease prior to challenge at 28 days, but the disease following 200 μg was mild and the challenging dose had no noticeable effects. Protection by mycobacterial pretreatment has been reported previously (1, 2, 7–13), and may be explained on the basis of specific tolerance induction.

Protection without induction of disease was also noted following treatment with mineral oil alone. This was previously reported by Zahiri *et al.* (2), and is difficult to explain. Perhaps mineral oil, known to be essential for the induction of adjuvant disease (12), alters certain cell receptor sites prior to the attachment of mycobacterial Wax D. This alteration may be great enough that the receptors are recognized as nonself, and "enhancing antibodies" are produced making induction at 28 days impossible. Alternatively, mineral oil injections may stimulate production of "suppressor T lymphocytes" (13), which would inhibit later induction of the disease. Regardless, the mineral oil protective effect is provocative. It might explain the reported inhibition of adjuvant disease induction by bacterial lipoprotein pretreatment (9, 10), since those lipoproteins were suspended in mineral oil.

One of the most interesting aspects of this study is the difference between these results and those obtained after a 14-day waiting period (Fig. 2) (1). Pretreatment with

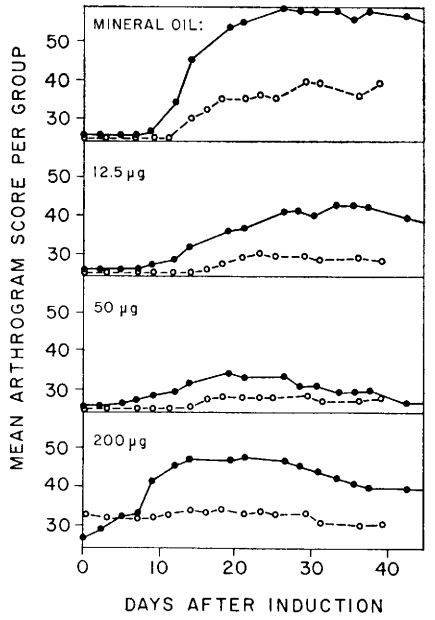

FIG. 2. Adjuvant disease following 14 (●—●) or 28 (O- -O) day pause between pretreatment and induction. Eight groups of 8 female, random-bred, Holtzman rats each receved subcutaneous, pre-induction injections in the tail of 0.1 ml sterile mineral oil, or oil containing indicated doses of killed *M. butyricum*. Induction on day 0 was with 500 μg of the same killed organism given as above.

mineral oil failed to protect at 14 days, but there was significant protection at 28 days ($p < 0.01$). This was also true in the groups receiving 12.5 μg and 200 μg ($p < 0.05$). The 50 μg group had lower mean scores at 28 days, but the difference was not significant at the 95% level. The extra two weeks of waiting after 200 μg seemed to eliminate the appearance of an "anamnestic response" such as that reported by Pearson and Wood in 1959 (3). The cells responsible for increased disease severity after 14-day challenge appear to have "functionally disappeared" by the 28th day. The activation of "suppressor T lymphocytes" (13) late in the progress of adjuvant disease could account for this disapperance.

Summary. Rats pretreated with various doses of *Mycobacterium butyricum* in mineral oil were protected against the induction of adjuvant disease 28 days later. Single 12.5 and 50 μg doses provided almost total protection. A 200 μg dose induced mild disease by itself, but prevented an anamnestic response following inductive challenge on the 28th day. Pretreatment with mineral oil alone afforded moderate, but significant protection. There were significant differences between these results and those obtained following inductive challenge on the 14th day.

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