

## INFLUENCE OF VARIOUS PRO-OXIDANT NUTRITIONAL CONDITIONS ON THE GROWTH *IN VIVO* OF *M. LEPRAE*

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### Introduction

In an extensive series of papers which we have recently summarized<sup>1, 2, 3</sup>, we published theoretical and experimental findings which permitted us to establish a connection between the autoxidation of lipids and the pathogenesis and therapy of leprosy.

Of all these findings, the growth of *M. leprae* in the testes of rats fed pro-oxidant diets<sup>4, 5, 6</sup> (a semisynthetic diet with a very low content of vitamin E and containing 15% of linseed oil) deserves special attention, particularly in conjunction with results reported in this paper.

In rats fed this diet and inoculated intratesticularly with *M. leprae*, reproduction of this bacillus is sufficient to permit its passage in rats in series, as well as the development of integral lepromins. The observation that such lepromins from rats behave similarly to lepromins of human origin confirms the biological specificity of the bacilli obtained from the inoculated animals<sup>7</sup>.

Using pro-oxidant diets of various types with the object of trying to find the best experimental conditions for the growth of *M. leprae* in rats, we carried out the experiments which are described below.

### Material and Methods

Thirty-six white male rats, raised under the usual nutritional conditions from birth up to the 21st day of age were divided into six groups with six animals per group, and were then submitted to the conditions described below.

The groups were designated by letters A to F; groups A and B were controls, while groups C, D, E and F constituted the experimental groups.

**GROUP A.** This group remained throughout the entire experiment on a complete diet consisting of fresh vegetables, bread and milk, and water *ad libitum*.

**GROUP B.** The animals in this group were fed similarly to those in the previous group, plus twice weekly addition of 40 to 50 mg. of d-l alpha tocopherol acetate given to each rat by mouth.

**GROUP C.** The animals in this group were given the following semi-synthetic diet with a low content of vitamin E and 15% of crude linseed oil:

Dry mix	{	Casein	23.8 gm.
		Yeast powder	8.9 gm.
		Mineral salts	3.0 gm.
		Corn starch	48.9 gm.
		Crude linseed oil	15.5 gm.

The components of this diet, except for the oil, are common to the rest of the groups and from now on will be referred to as the dry mix.

The mixed mineral salts which were used correspond to the formula of Hubbell, Mendel and Wakeman (J. Nutrition 1937, **14**, 273).

The casein employed in these diets was industrial casein used without previous extraction of lipids. The oils were added daily to the rest of the dry mix.

GROUP D. Animals in this group were maintained on the following diet:

Dry mix	84.6 gm.
Cod liver oil	15.5 gm.

GROUP E. The animals in this group were fed as follows:

Dry mix	84.6 gm.
Crude linseed oil	15.5 gm.

This diet, similar to that of group C, was prepared daily and was kept in open cans for six days before being administered. In addition the diet was aerated daily and was heated gently for two hours. The change in colour and odour during the six days period showed that the diet was becoming rancid and it was in a rancid condition when used to feed the animals.

GROUP F. The rats in this group were fed on the following diet:

Dry mix	84.6 gm.
Cod liver oil	15.5 gm.

During the entire experiment, the drinking water consisted of tap water to which was added 0.5 parts per thousand of silver nitrate.

In addition, after 21 days and for 4 months afterwards we gave a once weekly subcutaneous injection of  $\frac{1}{2}$  to 1 cc. of rat blood haemolysate prepared in the following manner. Taking 3 cc. of rat blood and adding 3 cc. of distilled water, the whole was filtered through wet filter paper and the filtrate was used for injection.

On the 36th day, after being placed on the indicated experimental conditions, at which time the rats were 57 days old, an inoculation was given in each testis of all animals of 0.1 cc. of a recently prepared suspension of *M. leprae*. The bacterial suspension was made by grinding a large leproma obtained from an untreated leprosy patient and suspending the ground material in 15 cc. of physiological saline.

Two, five and seven months after the inoculation, two animals were sacrificed from each group, except when animals died, as in the case of group F.

The testes, other organs and the depot fat of each animal were investigated. Bacterioscopic studies were made of one testis of each animal and histopathological studies were made of the other one.

The bacterioscopic examination consisted of counting the bacilli and globi present in 50 microscopic fields of 4 impression smears making a total of 200 fields counted. A qualitative study was also made by examining the morphological and acid-fast characteristics of 50 bacilli. Although all these studies yielded only approximate figures, we believe that the results are sufficiently exact to give a reasonably accurate idea of the quantitative and qualitative bacteriological changes occurring in the testes of the inoculated animals.

The bacteriological study of the testes was made by means of impression smears stained by the usual method of Ziehl-Neelsen, and also by histological sections of tissues embedded in paraffin and stained by the usual methods for acid-fast bacilli in tissues.

### Experimental Results

GROUP A. (Complete diet). In this group we noted a progressive decrease in the number of bacilli in both testes from the second through to the seventh month of inoculation, with granulation and loss of acid-fastness of the bacilli.

At the seventh month the histological sections did not show any acid-fast bacilli in the testes, and the impression smears of these organs showed only a small number of granulated bacilli with loss of acid-fast properties.

GROUP B. (Complete diet with vitamin E added by mouth). The bacteriological picture of this group of animals was approximately the same as that of the preceding group A. In fact at the end of the seventh month after inoculation, the histological sections did not show any acid-fast bacilli and impression smears showed only a very small number of bacilli which for the most part were granular with loss of acid-fastness.

GROUP C. (Vitamin E deficient diet with 15% linseed oil). In this group we noted a progressive increase of bacilli in the inoculated testes. At the fifth month there appeared in the inoculated testes a large quantity of thin bacilli which were not very acid-fast, similar to bacilli seen by us in previous work<sup>5</sup> which we have termed "bacilos de regeneración", i.e., new bacilli, of new growth. The sections showed the presence of these bacilli in the intertubular spaces.

The contrast in the bacteriological picture between the second and the fifth month after inoculation was highly significant. In the second month the bacilli were largely acid-fast, granular, and of characteristic diameter, while at the fifth month bacilli of new growth began to appear with characteristics already described.

GROUP D. (Vitamin E deficient diet with 15% cod liver oil).

Bacteriological studies of the inoculated testes of animals in this group showed a progressive increase in the number of bacilli from the second to the seventh month after inoculation. At the seventh month quantities of new bacilli and histological sections showed the presence of these bacilli in the intertubular spaces.

GROUP E. (Vitamin E deficient diet with 15% rancid linseed oil). Bacteriological studies of the inoculated testes showed a very noteworthy increase in the number of bacilli from the second to seventh month after inoculation. Already at the fifth month one would see large numbers of new bacilli which became very frequent at the seventh month when the total number of bacilli was enormous. At this time both new bacilli and bacilli with the usual characteristics were observed. In histological sections both types occurred in the intertubular spaces.

GROUP F. (Vitamin E deficient diet with 15% cod liver oil, with silver nitrate added to the drinking water and with injection of haemolysate). Bacteriological studies of this group, made in almost all of the animals in the fifth month after inoculation, showed at this time an extraordinary number of homogeneous and strongly acid-fast bacilli. New bacilli were also observed. The extreme acid-fastness and homogeneity of these bacilli were very remarkable, as was the number of new bacilli that were present. In addition we observed large red spots composed of thousands of acid-fast bacilli. One animal of this group showed masses of homogeneous and strongly acid-fast bacilli, which formed compact globi in the lungs and spleen, and to a lesser extent in the liver. The bacillary richness of the inoculated testes was extraordinary; histological sections showed enormous numbers of bacilli and globi disseminated throughout the intertubular spaces. The experimental result of this group has been described previously<sup>8</sup>.

## Discussion

From the results of the experiments described above we can draw the following conclusions:

1. In regard to the formation of ceroid pigment in the fat depots, observation of the colour of the fat indicated the absence of this pigment in groups A, B, C and E; and a great quantity of pigment in groups D and F.

In respect to the atrophic degenerative change in the testes, such changes were absent in the control groups but were manifested in the rest of the groups, especially in animals sacrificed in the fifth and seventh months of inoculation.

2. The weight of the animals of all the groups was essentially the same except in the case of group F in which some of the animals suffered an appreciable diminution in weight as compared with the animals in other groups.

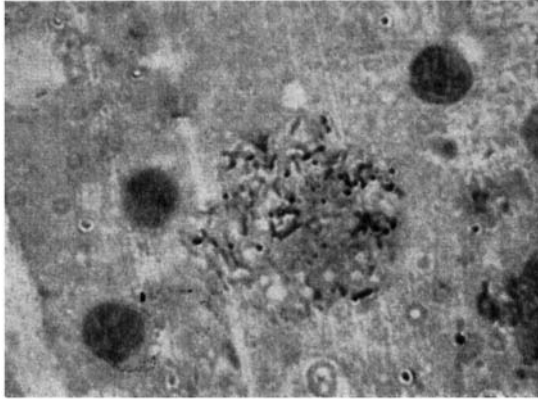


FIG. 1.—*Impression smear of testis of rat Group C sacrificed at the fifth month after inoculation showing group of new bacilli. (1000x)*

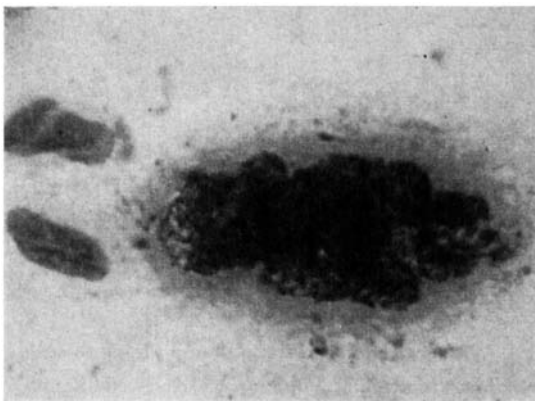


FIG. 2.—*Impression smears of testis of rat of Group F which died in the fifth month after inoculation showing large red spot composed of acid-fast bacilli. (1000x)*

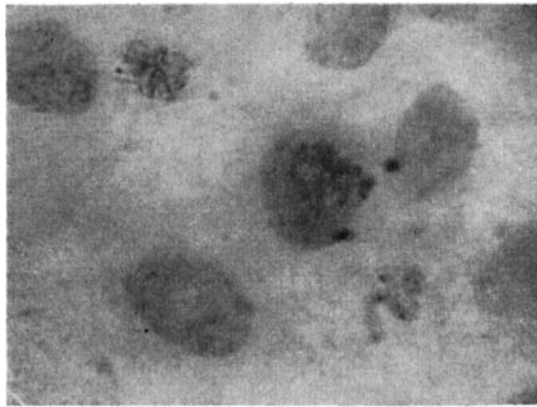


FIG. 3.—*Impression smears of lung of a rat of Group F found dead in the fifth month after inoculation, showing large globi, one of which is intracellular. (1000x)*

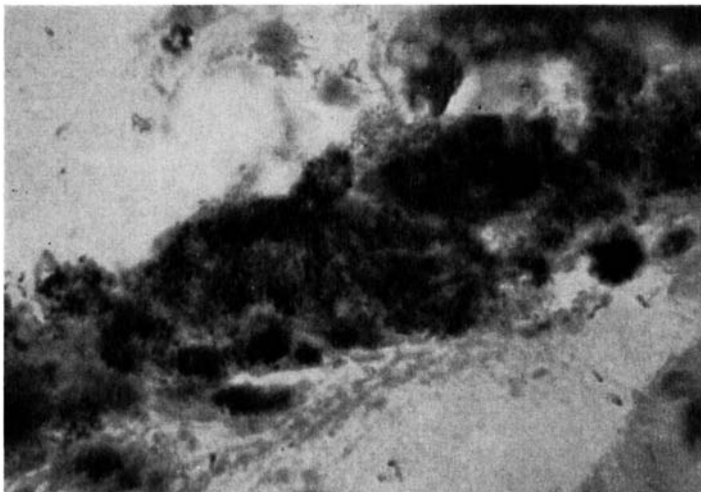


FIG. 4.—*Section of a rat testis of Group F found dead in the fifth month after inoculation showing a great quantity of agglutinated bacilli in the subcapsular zone. (1000x)*

It must be emphasised that in none of the inoculated animals could there be observed any type of macroscopic lesions, nor hypertrophy of any organ attributable to the growth of the Hansen bacilli.

3. The animals of the two control groups showed practically identical bacteriological pictures of progressive diminution in the number of bacilli in the inoculated testes. In addition, the bacilli suffered alterations in morphology and staining ability, consisting in granulation and loss of the acid-fastness.

At the seventh month after inoculation, impression smears showed only a very low number of granulated and non-acid-fast bacilli. This can be taken as evidence that conditions were unfavourable to the growth of *M. leprae*.

4. All the experimental groups showed an evident and progressive increase in the number of bacilli from the second to the seventh month after inoculation; it must be emphasised however that the bacteriological picture in each group was distinct. Groups C and E showed the appearance of new bacilli at the fifth month after inoculation; group D showed them at the seventh month. On the other hand, group F at the fifth month showed an enormous number of strongly acid-fast bacilli, whose degree of acid-fastness was not observed in any of the other groups.

5. In regard to the richness of bacilli, groups E and F showed an enormously increasing growth of *M. leprae*. On the other hand the growth rate of the bacilli was of a lesser degree in groups C and D.

6. From the results of these experiments one deduces that there is no direct relation between ceroidogenesis and the growth of *M. leprae*. Although it is certain that with diets such as diet F which is strongly ceroidogenic there was obtained an enormous development of bacilli, such development also was seen with diet D which is very weakly ceroidogenic.

7. For reasons which escape us, but which may be connected with genetics, not all of the animals reacted in the same way, and although the results in general were more or less uniform, some animals within a given group showed less development of the bacilli than others. Likewise it appeared that in some animals the growth rate of the bacilli slowed soon after reaching a high level, and in these cases the bacilli seemed even to deteriorate in regard to morphology and acid-fastness.

8. The group of animals in which conditions were the most favourable for the growth of *M. leprae* was group F. The diet of this group is highly ceroidogenic and pro-oxidant, and in addition to favouring the growth of the bacilli caused marked renal and hepatic changes which led to the premature death of a large number of the experimental animals.

9. From the point of view of importance in the pathogenesis of human leprosy the diet which can be most plausibly connected with

leprosy in humans under natural conditions is diet E. This diet containing 15% of rancid linseed oil caused an extraordinary growth of *M. leprae*. The relationship between the consumption of this diet by rats in which *M. leprae* would multiply and the ingestion of large quantities of rancid foods by populations living where leprosy is endemic is highly significant and merits future investigation in the field of the prevention, pathogenesis and treatment of leprosy.

Taking into account the high variety of pro-oxidant factors and the possible combinations of two or more of these, such as for example pro-oxidant metallic catalysis, peroxides, oxygen, polyunsaturated oils, rancid fats, irradiation, absence of anti-oxidant factors, etc., it is easy to realize that there are many possibilities for finding optimal nutritional and pharmacological conditions for the growth *in vivo* of *M. leprae*.

### Summary

A comparative study has been made of the growth of *M. leprae* inoculated intratesticularly in white rats submitted to various pro-oxidant nutritional conditions. With the pro-oxidant diet employed (Vitamin E deficient diet with linseed oil, with rancid linseed oil, with cod liver oil, with or without the addition of silver nitrate in the drinking water and injection of haemolysates) there was a notable growth of *M. leprae* in relation to their growth in control animals fed on ordinary diets. The great development of *M. leprae* in the group of animals which received the diet containing the rancid linseed oil may be connected with the pathogenic mechanism of human leprosy. It is indicated that the search for new nutritional and pharmacological pro-oxidant factors may lead to the finding of optimal conditions for growth *in vivo* of *M. leprae*.

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