INOCULATION OF *MYCOBACTERIUM LEPRAE* TO RATS FED A PROOXIDANT DIET[1]

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It has been held, especially by Bergel ([3]), that the state of prooxidization in experimental animals produced by a suitable diet favors the reproduction of *Mycobacterium leprae* in them. Believing that such investigations should be repeated by workers other than the creators or discoverers themselves, we undertook in 1957 to repeat the experiment with rats fed on prooxidant diets lacking in vitamin E. We concluded ([15]) that in order to reach definite conclusions it would be necessary to use a greater number of animals and to correct the causes of the high mortality rate recorded (64%). We believed then to have anticipated some encouraging results, but we were not able to obtain any statistically valid data. Continuing with this work, we experimented by modifying the causes which we considered detrimental in the previous tests, and using a sufficient number of animals.

MATERIAL AND METHOD

1. *Diet.*—The diet used previously, prepared in quantity,[2] was modified by adding the linseed oil daily to the mixture of other ingredients, to prevent its becoming rancid. The yeast was replaced by a polyvitamin solution, without vitamin E of course, which was also added daily. The smaller mortality rate among the rats we attribute to these modifications, and this has led to useful conclusions; the administration of a prooxidant diet to a sufficient number of animals during 1 year and 9 months is sufficient guarantee.

The advantages of the modified diet can be appreciated by comparing mortality figures. In the previous work we had 64 per cent mortality in Group 1 alone, while under present conditions it has been decreased to 19 per cent in the total of the groups under study, although in Groups 1 and 3 it was somewhat higher, 28 per cent. Silver nitrate, 0.05 per cent, is added to the drinking water, renewing it every day for some of the animal groups. To these conditions, which have caused the decrease in the mortality rate, we must add that the present work has been carried out in quarters provided exclusively for our animals,[3] which has permitted their adequate care.

2. *Animal used, and groups.*—White rats of the *Mus nordelicus albinus* strain[4] were bred in the quarters mentioned, and as careful a selection as possible was made among the males to obtain similar rats before establishing the diet. In this way we were then able to compare the differences in body, fur, etc., which are produced by alimentary

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[2]Starch, 10 kgm.; casein, 4.8 kgm.; linseed oil, 3 kgm.; salt mixture, 0.6 kgm.; yeast, 1.5 kgm.

[3]By the Department of Microbiology of the Medical School of the University of Buenos Aires.

[4]Randon stock, Philadelphia, obtained from the Malbrán Bacteriological Institute in 1957.

changes. Some rats of each group were tested with integral human lepromin, with negative results after 48 hours and 21 days.

The rats thus selected were grouped and treated as follows:

Group 1:	prooxidant diet	inoculated
Group 2:	ordinary diet	inoculated
Group 3:	prooxidant diet	not inoculated
Group 4:	prooxidant diet	inoculated with dead bacteria
Group 5:	prooxidant diet	subinoculated
Group 6:	ordinary diet	subinoculated

These different groups were set up to provide all possible controls, to avoid mistakes which might occur in experiments to which the precaution of using blank tests to verify an assumed mycobacterial development was not used.[5]

As has been pointed out recently by one of us ([12, 14]), it is time for animal experiments made with the leprosy bacillus to adhere strictly to the experimental methods used in biologic science, procedures which are classic and indispensable with respect to the value of an investigation, and to require that those not conforming to such methods be disregarded, as their acceptance has led us to the great confusion we have now in animal research in leprosy. It is also unacceptable to identify acid-fast germs as *M. leprae* simply because they are not cultivable and transmissible, for we cannot base the identification of a germ on negative facts. It may be necessary to revise everything that has been done to date, and to channel the efforts made within the principles of pure biologic research. The only useful datum is from immunology.

3. *Inocula.*—As the material for the inocula we have used lepromas not subjected to any previous treatment whatever, on the assumption that active or virulent germs are to be found in such lesions, taking into account the fact that other bacterial forms are also to be found in lepromas which suggest modified vitality. Untreated patients are considered to be the most contagious, which implies a greater virulence of the bacteria. Other sources of obtaining inoculation material—initial lesions, macules, serum or blood from reaction cases—have been used in previous work, either for seeding cultures or for animal inoculation, as reported by Bordoni-Uffreduzzi ([4]), Ducrey ([5]), Lleras Acosta ([8]), Teich ([11]), Eddy ([6]), Evans ([7]), and Barzizza ([1]) among many other authors.

In preparing the inoculation material, the lepromas were homogenized in saline in a blendor, then centrifuged repeatedly to eliminate all traces of tissue. The bacilli were counted in stained smears, and the suspension was diluted with saline to permit inoculation with the least possible number of bacteria. After further microscopic control, to make sure that there were few bacilli per microscopic field, and planting samples on Petragnani culture medium, which gave only negative results, the suspension was ready for inoculating into animals. Five different suspensions were used in the first two groups of animals.

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[5]According to the list of groups given above, 4 of them were given the prooxidant diet and 2 were given ordinary diet. However, according to the side-heads of the individual groups under Results, it appears that only Groups 1, 3 and 4 were given the prooxidant diet while the diet of Group 5 is described as "lacked." In the text (p. 15), Group 3 is said to have "lacked on vitamin E." Also (on p. 6 and 15) there are statements which suggest that there is a difference between diet lacking in vitamin E and prooxidant diets, suggesting a difference the nature of which is not evident. "Lacked" also appears repeatedly in the later part of the article. Lacking a key to its usage, that term has not been changed.—EDITOR.

4. *Inoculation method.*—Inoculations were made only into the testes, because of previous experience ([12]) and because this organ is the one in which the effect of diets lacking in vitamin E and prooxidant diets is most quickly established. Both testes were inoculated in each animal.

5. *Determination of the prooxidization condition.*—In order to determine when the animals were ready for inoculation we observed the following conditions: The rat is separated from its mother one month after birth at the latest, and placed on the special diet at that time. Evidence of its effect consists in modifications in the fur, diffuse baldness, loss of weight, reduced size, decrease in the number or disappearance of the spermatozoids, and the presence of yellow fat.

Generally, the inoculation was done when the animals had been on the prooxidant diet for one month, i.e., at the age of two months; at that time the changes indicated are present. These changes are much more early when the animals are subjected to more severe dieting conditions, with the addition of silver nitrate, cod liver oil, hemoglobin, etc.

The decrease or disappearance of spermatozoids and the presence of yellow fat, particularly the perigonadal and perirenal fat, or alterations in the liver, kidney, lung, spleen, heart and bone marrow, have been seen in the autopsies. These changes increase with the length of time the animals have been subjected to the diet.

RESULTS, BY EXPERIMENTAL GROUP

Group 1, on the prooxidant diet and inoculated.—Eighty-seven animals were used in this group (174 testicles). Six of the rats were subjected to a severe prooxidant diet; the others received the diet described. Longest period of observation: 1 year and 9 months.

The animals were examined, including smears for acid-fast bacilli, 7 and 15 days after the inoculation, and then once a month for the period of the experiment. Material for study was obtained by unilateral orchidectomy, or by autopsy. Animals were killed beginning from the fourth month after the inoculation in order to observe possible alterations in the other viscera.

Fifty-five animals of this group have been examined to date, i.e., 110 testicles; we mention the number of testicles examined, as that is where changes are most easily observed.

Findings: Acid-fast bacteria with the characteristics of *M. leprae,* and accretions of an acid-fast substance of granular aspect in which it was impossible to find bacterial forms, were observed in 48 of the 55 rats examined. The numbers of bacteria found have in general been small, not enough to permit the assumption that multiplication had occurred. The numbers did not prove to be greater when the testicular tissue was homogenized. There were no interesting changes in the color or the morphologic characteristics of the bacilli.

With reference to the acid-fast substance mentioned, it disappeared when the homogenization method was used, nor was it found in the controls made after homogenization.

In some cases, bacilli were found grouped and sometimes granulated. No well-stained "young forms" were found, such as can be observed in active lepromas.

Histopathology: No important changes were observed in the hematoxylin-eosin sections. Using the Ziehl-Neelsen method, spermatozoids

with fuchsinophil granulations and bodies were found. In general, bacteria were not observed.

Group 2, on ordinary diet and inoculated.—Number of animals inoculated, 20 (40 testicles). The study of this group was carried out in the same way as the previous group. The longest period of observation was 1 year and 4 months.

Results: In the total of 20 animals we found bacteria or acid-fast granulations in 17. Again, as with the animals fed the prooxidant diet, there was no evidence of multiplication. Although this group has only confirmed what is already known about the inoculation of rats with *M. leprae,* it has shown that it is possible to find bacilli with typical color and morphologic characteristics as late as 1 year and 4 months after inoculation.

The Ziehl-Neelsen stain showed generally pink or red spermatozoid tails. In some cases the spermatozoids were frankly acid-fast, but this does not hinder the search for bacilli, nor is there any possibility of confusion.

We have observed that the spermatozoids decrease in number with the age of the rats. In one instance, in which their numbers were very small, we found some acid-fast groups very similar to those we saw in the animals of the first group; apparently the production of this substance is facilitated by old age.

Histopathology: Testicles and epididymis were without apparent lesions. The Ziehl-Neelsen sections show the heads of the acid-fast spermatozoids.

Group 3, on the prooxidant diet without inoculation.—Number of animals examined: 20 (40 testicles). Longest period of observation: 1 year and 4 months. Some of these animals were submitted to a more severe prooxidant diet.

Results: The most important thing in this group was finding, in almost all of the testicles examined as well as in other organs, accretions of an acid-fast substance very similar in aspect to numerous grains of *M. leprae.* In some cases they had an elongated shape, with a pseudobacillary morphology. These pictures were found most frequently and in greatest number in animals which had been longest on the lacked diet, and in those subjected to the more severe prooxidant diet. This fact has been pointed out by Quartrup *et al.* ([10]), Mason *et al.* ([9]), Bergel ([2]), and other investigators, who have reported the presence of an acid-fast substance, relating it to the yellow fat which is found in hibernating animals, especially in the young "mistela mink." This picture appears in similar form in animals fed experimentally on special diets.

Histopathology: Abolition of spermatogenesis. Fuchsinophil bodies which cannot be identified as bacilli were observed after Ziehl-Neelsen staining.

Group 4, on the prooxidant diet and inoculated with dead bacilli.—Number of animals, 10 (20 testicles). Longest period of observation, 1 year and 4 months. For the dead bacilli used in this group, lepromin was used as the inoculum.

Findings: Acid-fast bacilli were found in 4 of the animals. There were also observed groups of an acid-fast substance with the same characteristics as those found in Groups 1, 3 and 5.

The value of this control group lies in the fact that, in addition to accretions of an acid-fast substance similar to those found in all the other animals submitted to prooxidant diets, there could be found acid-fast bacilli, although few in number, as late as 1 year and 4 months after inoculation.

Group 5, lacked and subinoculated.—Number of animals, 23 (46 testicles). Longest period of observation, 7 months and 20 days. The inocula for this group were prepared from rat testicles of a previous experiment which contained few bacilli per microscopic field.

Results: In 17 of the animals, accretions of acid-fast substance were found and, very rarely, isolated acid-fast bacilli.

Group 6, on ordinary diet and subinoculated.—Number of animals, 10 (20 testicles). Longest period of observation, 7 months. The same inocula were used in this group as in Group 5.

Results: Isolated bacilli were found very rarely.

DISCUSSION

The total of the groups examined consists of 118 rats in inoculated Groups 1, 2, 4, 5 and 6, which means the examination of 236 testicles. To this number must be added the 20 rats (40 testicles) of Group 3, lacked on vitamin E and not inoculated. In total, therefore, we have studied 138 rats, examining 276 testicles.

Before summarizing the general results, it is desired to draw particular attention to those obtained in Group 3, the rats on the prooxidant diet but not inoculated.

(*a*) In view of the presence of accretions of acid-fast substance having an appearance similar to bacillary groups or bacillary granulations;

(*b*) In view of the fact that these acid-fast accretions were also found in Groups 1, 4 and 5, animals on the prooxidant diet but inoculated;

(*c*) In view of the error to which this may lead when one has not taken the precaution of including lacked group controls or controls of a prooxidant diet but *without inoculation* (Group 3 of our experiment);

(*d*) In view of the need of always using the homogenization method for the tissues of all animals subjected to special diets and inoculated, in order to avoid the mistake of supposing, on the basis of the microscopic impression alone, that a bacillary development exists;

For all these reasons, the blank group is the most important control to verify whether an inoculation has been positive or not.

The general results may be summarized as follows:

(1) Bacilli were found in proportions which varied from 83 to 85 per cent in inoculated rats, in 30 and 74 per cent in subinoculated rats, and 40 per cent in rats inoculated with dead bacilli.

(2) It was impossible to prove multiplication of the leprosy bacillus since, in spite of the high percentage of positive findings, in no single case was the number of bacilli found greater than that of the inoculum.

(3) Groups of an acid-fast substance were found in 86 per cent of the lacked animals or in those subjected to the prooxidant diet, whether inoculated or not. It was established that this acid-fast substance is in no way related to *M. leprae* for the following reasons:

(*a*) Its presence in the control group that was not inoculated (Group 3);

(*b*) Its absence in the smears made after homogenization for the purpose of eliminating all traces of tissue;

(*c*) The discrepancy between its microscopic appearance before and after homogenization.

(4) Our attention was drawn to the presence of bacilli and cocci, not acid-fast and without the characteristics of *M. leprae*, in rats in apparent good health a year or more after inoculation with *M. leprae*, in some animals of the various groups.

(5) In one of the experimental groups (Group 2), a very few spermatozoids and acid-fast groups with the characteristics of yellow fat were found in the testicles of a nonlacked rat 1 year and 8 months old. This finding is interpreted as a change connected with age.

CONCLUSIONS

After intensive experimental work carried on during almost four years, the following conclusions have been arrived at:

1. No multiplication of *M. leprae* occurs in rats subjected to a prooxidant diet.

2. It is easy to find accretions of an acid-fast substance in animals on a lacked diet, which may lead to confusion if those accretions are considered to be groups of granulations or leprosy bacilli.

3. This acid-fast substance often increases with the time the animal is subjected to a lacked diet, and is greater in animals subjected to severe prooxidant diets.

4. These acid-fast accretions are not related to the *M. leprae* as is shown by the control Group 3 (lacked and not inoculated).

5. Subinoculation was not successful. Using the controls established in the present paper, i.e., control groups of animals and homogenization method, we found that the number of bacilli was not greater than in the inoculum.

6. Both in the subinoculated and in the lacked animals we found groups of acid-fast substance, a substance which is exclusively connected with their state of prooxidization and in no way related to the supposed multiplication of the bacilli. This is shown by the control groups composed of lacked rats which have not been inoculated and by search for *M. leprae* after homogenization.

7. It is necessary to demand that the experimental work in leprosy be carried out strictly according to the classical rules accepted in biologic investigation; otherwise erroneous conclusions may be reached.

8. The failure of all attempts to date to obtain infection by inoculation of animals with *M. leprae,* obliges us to reconsider our knowledge of this bacillus which refuses to fulfill the postulates of Koch, and to try to understand better its behavior in its only host, man, in order to provide it, outside man, with the necessary conditions for its multiplication.

SUMMARY

In order to study the possibility of multiplication of *M. leprae* in rats fed on a diet lacking in vitamin E, and continuing previous experiments, we proceeded to make intratesticular inoculations of *M. leprae* obtained from patients who had never received antileprosy treatment, to a group of rats fed a prooxidant diet, using another three groups as controls. One of the controls was a group of rats which were inoculated but not lacked on vitamin E; the second group consisted of rats which were not inoculated but lacked on vitamin E, and the third group were lacked on vitamin E and inoculated with dead bacilli.

The facts established by the test group and the control groups at the end of periods of observation which varied from 1 year and 9 months (test group) to 1 year and 4 months (control group) do not demonstrate that there occurred any multiplication of *M. leprae* in these animals.

RESUMEN

A fin de estudiar la posibilidad de la multiplicación del *M. leprae* en las ratas alimentadas con un régimen carente en vitamina E y, en continuación de experimentos anteriores, se procedió a verificar inoculaciones intratesticulares de *M. leprae* obtenidos de enfermos que jamás habían recibido tratamiento antileproso, a un grupo de ratas a las que se había suministrado una alimentación prooxidante, usando otros tres grupos como testigos. Uno de los testigos era un grupo de ratas, que fueron inoculadas, pero que no carecían de vitamina E; el segundo grupo costaba de ratas que habían sido inoculadas, pero que carecían de vitamina E, y el tercer grupo era carente en vitamina E y había sido inoculado con bacilos muertos.

Los hechos establecidos por el grupo de ensayo y los grupos de testigos al final de períodos de observación que variaron de 1 año y 9 meses (grupo de ensayo) a 1 año y 4 meses (grupo testigo) no muestran que haya habido multiplicación del *M. leprae* en estos animales.

RESUMÉ

Afin d'étudier la possibilité d'une multiplication de *M. leprae* chez des rats nourris par un régime manquant de vitamine E, et dans la ligne d'expérimentations antérieures, nous avons procédé, chez un groupe de rats soumis à une diète pro-oxydante, à des inoculations intratesticulaires de *M. leprae* obtenus de malades qui n'avaient jamais reçu aucun traitement contre la lèpre. Trois autres groupes de rats ont été pris comme témoins. Un des groupes de contrôle consistait en groupe de rats qui furent inoculés mais n'étaient pas soumis à une carence en vitamine E; le deuxième groupe était composé de rats soumis à une carence en vitamine E mais non inoculés; le troisième groupe était soumis à une carence en vitamine E mais fut inoculé avec des bacilles morts.

La période d'observation a varié de 1 an et 9 mois (groupe testé) à 1 an et 4 mois (groupes témoins). Les faits établis à la fin de l'observation par l'analyse du groupe étudié et des groupes témoins ne démontrent pas qu'il se soit produit une multiplication de *M. leprae* chez ces animaux.

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