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Letters to the Editor

Are There Regional Differences Regarding Secondary Amyloidosis in Leprosy?

If we consider patients with Hansen's disease from different environments, we may be concerned with similar populations from the statistical point of view, but this is not necessarily the case. The relative prevalence of the different clinical forms of leprosy varies between one country and another. In comparative studies of this subject, mistakes may be avoided if properly randomized samples are taken from each country, but if such care is not taken, conclusions can be applied with safety only to the samples on which work was performed.

For some time emphasis has been laid on the fact that the incidence of amyloidosis in leprosy in oriental countries differs from that registered in occidental countries (Satyanarayama, 1972; Editorial, 1975; Krisnamurthy, 1966; Mittal, 1972).

In the United States, Shuttleworth and Ross (1956), in a necropsy study, found that 10 out of 18 patients who had been treated at Carville (Louisiana) had amyloidosis (55%). Bernard (1956), in the Argentine Republic, found that 28 out of 40 patients who underwent autopsy at the Sommer Sanatorium had amyloidosis (70%). Williams *et al.* (1965) at Carville, found that 31% of patients had amyloidosis. Williams' work is a most important contribution in favour of the existence of intergroup differences non-attributable to the clinical forms. In work published in 1965 and conducted among reactional lepromatous patients studied with the Bennhold's test, we found a 15% incidence of amyloidosis.

These high indices are opposed by the data cited by certain authors from eastern countries and Mexico. Williams *et al.* found a percentage of amyloidosis of 3.3 among farmers in Mexico suffering from hanseniasis. In India, Satyanarayama *et al.* (1972) mention a percentage of 7.5%. Krishnamurthy, at Vellore, India, found 8% among 25 patients. Finally, Mittal *et al.* (1972) at New Delhi, did not find any case of amyloidosis in 30 kidney biopsies.

From the above data, apparently, there exists a marked difference in incidence of amyloidosis between orientals and occidentals.

Williams *et al.* (1965), interested in the reasons for the differences between the Carville patients and the Mexican farmers (31% and 3.3% respectively on the basis of gingival biopsies), studied the diets and work habits of both groups, to determine differences in their alimentary habits and way of life. They suspected that these might be the cause of the different behaviour of the 2 populations. We tried (1972) to test Williams' hypothesis in the patients at the Sommer Leprosarium in the Argentine Republic, and on that occasion we were not able to confirm their findings. In our group the amyloidosis incidence was similar to that registered at Carville, while the diet was more like the Mexican one.

Before discussing our position, we wish to point out some observations that we believe are important in order to attempt the elucidation of the problem in question.

(a) Amyloidosis incidence varies according to the clinical forms and complications of the disease. Comparing the incidence of amyloidosis among 3 groups of patients (1962) we found 15% in reactional lepromatous leprosy, 4% in non-reacting lepromatous leprosy, and 5% in the relatively benign forms (tuberculoid, borderline, uncharacteristic, etc.). In other work (1963) we found that patients suffering from reactional lepromatous leprosy and/or infections were the most affected by amyloidosis.

(b) In a study conducted with Jonquieres (1968) we found among 200 urine samples from hanseniasis outpatients at a preventorium specializing in leprosy only 3 urines with proteinuria, which at best would represent 1.5% or renal amyloidosis and, taking into account that the kidney is usually affected in about 80% of cases, nearly 2% or amyloidosis in general.

We believe that the above data are sufficient to affirm that the incidence of amyloidosis in leprosy varies remarkably according to the clinical forms, the complications (reactions, infections, etc.) and the place where the patients are seen (external consulting offices, sanatoria).

The importance of this last item does not lie in the kind of food patients receive or the environment where they live, but in the fact that to a great extent it determines the type of patients that are included in the studies. Inpatients from sanatoria are generally affected by the most severe and complicated forms of leprosy while outpatients include a very high percentage of milder forms without complications.

In the same country (Argentina) amyloidosis incidence varied according to the patients that were considered; 2.0% in patients seen in external consulting offices, 4% in non-complicated lepromatous, 5% in benign forms (tuberculoid, borderline, etc. (generally complicated with chronic infections), 15 to 22% in reactional lepromatous or lepromatous with chronic infections, and 70% in necropsied patients.

The data outlined above lead us to think that the comparisons which suggest a different behaviour in different countries are possibly erroneous; in fact we believe that groups that are not really comparable are being compared. In our opinion the group of inpatients cannot be compared with outpatients.

Finally, we would like to make one more comment about the heterogeneous character of some of the groups that have been compared and the mistakes made when analysing them. Krishnamurthy *et al.* (1966) report only 8% of amyloidosis among 25 autopsies of leprosy patients. If the data are further analysed, one can see that only 17 of the cases were lepromatous and in this group the percentage would go up to 11%. Unfortunately, as the authors do not present any more information on the patients (existence or not of reactions and infections) one cannot go deeper into the data. Mittal *et al.* (1972) did not find any case of amyloidosis among 30 leprosy patients who underwent kidney biopsy. This affirmation, apparently lapidary, loses much of its force if the following is taken into account. Twelve out of the 30 patients were not lepromatous and only 8 out of the 18 lepromatous patients were reactional. The sample is very small and therefore it may happen that by chance no case of renal amyloidosis appears. After what has been said, and despite the interesting study of Williams *et al.*, our doubts have not vanished. Really, are there any regional differences based on race, life habits, etc., or in fact, have "eggs" been compared to "oranges"?

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Comparison of *o*-Diphenoloxidase of *Mycobacterium leprae* from Armadillo Tissues and from Human Sources: A Few General Observations

ENZYME LEVELS

Preparations of *Mycobacterium leprae* obtained from infected armadillo tissues show great variations in the level of phenoloxidase activity. Some preparations contain high enzyme levels while others have very low activities. In preparations with high activity, the enzyme has the same properties as those of *M. leprae* separated from infected human tissues. They oxidize D-dopa rapidly, giving rise to indole-5,6-quinone with a peak at 540 nm in the spectrum. (Mammalian tyrosinase does not oxidize D-dopa.) In preparations with low activity, the reaction is rather slow, and the spectrum of the supernatant fraction shows only general absorbance with no well-defined peak. However, these bacilli also oxidize D-dopa producing melanin pigment. *M. leprae* from human sources do not show such wide variations in the enzyme levels.

TISSUE INHIBITORS

Bacterial preparations from the liver tissue of certain armadillos (especially those infected with *M. leprae* intravenously) sometimes show a greenish tinge, indicating the presence of bile pigments. Such preparations contain little dopa oxidase activity. However, bacilli separated from the spleen of the same animals do oxidize D-dopa. Obviously, some inhibitor(s) interfere with the oxidation of the substrate by the liver organisms. *M. leprae* obtained from most liver tissues does not show this type of inhibition.

BINDING OF DOPA AS AN IDENTIFICATION TEST

It may be interesting to note that in the preparations described above, only the oxidative mechanism is inhibited, while the binding of dopa is not. This is readily

demonstrated by incubating the bacilli with [^{14}C]-dopa and measuring the radioactivity of the organisms due to the bound substrate. Since other mycobacteria have been found not to take up dopa, binding of the substrate could serve as a reliable indentification test for *M. leprae* (in laboratories where the necessary facilities exist).

A SIMPLER PROCEDURE FOR IDENTIFYING *M. LEPRAE*

We have demonstrated oxidation of dopa by *M. leprae* spectrophotometrically (by measuring the quinone formed), polarographically and manometrically (by measuring the amount of oxygen consumed), and radiographically (by determining the amount of labelled water formed when tritiated dopa is used as substrate). When quantitative readings are not needed, a simpler method may be adopted for identification of the bacilli. The purified bacterial preparations (10^9-10^{10} organisms or more) are incubated with dopa at 37° C (pH 6.8) for 30 min or 60 min, depending on the level of enzyme activity in the bacilli. (The colour development in the incubation mixture can be visually assessed.) When the reaction mixtures are centrifuged, the sediment of *M. leprae* incubated with dopa would be black. Other mycobacteria do not show any change in colour. Controls should be run with bacilli alone, and with heat-inactivated *M. leprae* to which dopa is added. The bacterial suspensions are heated at 100° C for 30 min and then cooled. Heated *M. leprae* with dopa might show a light brown colour or no colour change at all.

SPECIFICITY OF THE REACTION AND PRECAUTIONS

We tested mycobacteria separated from the skin and liver tissues of 3 different mammalian species, as well as several cultivable mycobacteria. Some of the mycobacterial cultures were claimed to be *M. leprae,* and 2 strains were claimed to oxidize D-dopa. These mycobacteria (both from infected tissues and from cultures) showed no reaction with dopa. Cultivable mycobacteria have to be thoroughly washed free of the growth medium; otherwise, false positive results may be obtained. Components of certain culture media (especially metal ions) might stimulate auto-oxidation of dopa; however, heated samples also would stimulate the auto-oxidation of the substrate, indicating that this is not an enzymatic reaction. The enzyme activity in *M. leprae* is abolished on heating. After separating *M. leprae* from infected organs, host-tissue materials are inactivated or removed by treating the bacilli with 0.1 N NaOH, trypsin, or acetone and ether. Since tissue enzymes do not act on D-dopa, these treatments may not always be necessary in routine tests. If the *M. leprae* preparations have little activity to start with (due to presence of inhibitors or inactivation of the enzyme as a result of prolonged storage), both the heated and the unheated samples would show no colour development or might give only a light brown colour (caused by any residual enzyme activity).

LABILITY OF *o*-DIPHENOLOXIDASE IN ARMADILLO BACTERIA

A significant feature of the phenoloxidase of the armadillo bacteria is that it is more labile than the enzyme in *M. leprae* obtained from human tissues. We have stored lepromatous human spleen and skin nodules for a year or more at −20° or −80° C. In the bacilli separated from the stored human tissues, the enzyme remains active, although at a slightly diminished rate. We have obtained *M. leprae* preparations from armadillo tissues which readily convert D-dopa to indole-

5,6-quinone. However after storage for about a year, the bacilli separated from these organs were found to have completely lost their ability either to oxidize or to bind dopa, indicating that the enzyme had been inactivated. These tissues had been thawed and refrozen previously to remove material for other experiments. Very little activity was lost by *M. leprae* from human tissues treated similarly. We have reported before that the *o*-diphenoloxidase of *M. leprae* is associated with a decarboxylase. The phenoloxidase oxidizes dopa to dopachrome (with a peak at 480 nm in the spectrum); it is the decarboxylase that converts dopachrome to indole-5,6-quinone (with a peak at 540 nm in the spectrum). During storage of the infected tissues or the separated bacteria, the decarboxylase activity is lost sooner than the phenoloxidase. In such instances, the immediate reaction product would be dopachrome and not indole-5,6-quinone.

HYPOTHESIS

At present we can only speculate on why the phenoloxidase of *M. leprae* from armadillo tissues is relatively more labile. In most armadillos, at the time they are killed, the bacilli apparently are continuing to multiply in the tissues; i.e. the bacilli are in the growth phase. In rapidly multiplying organisms, as many enzyme molecules may not accumulate, as in organisms in the stationary phase. Moreover, the structure of the cell membrane of the armadillo bacteria could be of a more "leaky" nature, as compared to the cell membrane of *M. leprae* from human tissues. These phenomena might explain the lower level of phenoloxidase activity (per unit number of bacilli) and the earlier inactivation of the enzyme in the armadillo bacteria than in *M. leprae* from human sources. Other explanations are possible. However, without experimental evidence, they remain as hypotheses.

CONCLUSION

The armadillo bacteria contain *o*-diphenoloxidase as *M. leprae* from human tissues. The activity has been demonstrated in organisms separated from the spleen, liver, lymph nodes and skin nodules of armadillos, from the spleen, testis and skin nodules of lepromatous patients, and from the mouse foot-pads. The enzyme has been solubilized from the bacterial particles by detergent-treatment and shown to be a copper-containing protein. *o*-Diphenoloxidase is the only specific metabolic activity detected in *M. leprae* so far.

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