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Enumeration of *Mycobacterium leprae* Stained with and without Prior Periodate Oxidation

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Slides prepared with suspensions of *Mycobacterium leprae* recovered from skin biopsy specimens obtained from treated lepromatous leprosy patients and oxidized with periodate contained on the average 36% more acid-fast bacteria (AFB) than did duplicate slides stained by the standard acid-fast stain. By contrast, slides prepared with suspensions of *M. leprae* recovered during logarithmic multiplication in mouse foot pads and treated with periodate contained on the average 16 or 33% fewer AFB than did duplicate slides stained by the standard technique. These results are inconsistent with a non-acid-fast stage in the growth of *M. leprae*.

Introduction

The claim has been made that oxidation of *Mycobacterium tuberculosis* and *M. leprae* renders more of these organisms stainable by an acid-fast staining technique than when acid-fast staining is carried out without prior oxidation (Nyka, 1963, 1967; Reich *et al.*, 1972; Harada, 1973; Mohysen and Alemayehu, 1973). This claim has been reexamined for *M. leprae*.

Materials and Methods

In this laboratory, which has been engaged in the inoculation of mice with *M. leprae* for the past 9 years, it is standard practice to make duplicate preparations of bacterial suspensions on Reich counting slides* for acid-fast staining and enumeration of acid-fast bacilli (AFB). Both slides are routinely fixed in formalin fumes; one slide is stained with a carefully standardized acid-fast stain at room temperature, and the AFB are enumerated (Shepard and McRae, 1968); both the stained and unstained slides are filed in a light-tight box. The exposure of the stained slide to light is minimized between the time it is stained and the time it is finally filed.

For the purpose of this study, 50 pairs of slides were selected from the files. These included 20 pairs prepared between September, 1971 and April, 1973 from

* Bellco Glass, Inc., Vineland, New Jersey, U.S.A.

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the biopsy specimens of patients who had been treated with acedapsone or rifampicin for 3 or 6 months (Group 1—"human"), 20 pairs prepared with logarithmic-phase *M. leprae* from mouse foot pad homogenates during the first six months of 1974 (Group 2—"new mouse"), and 10 pairs prepared from mouse foot pad homogenates between January and May, 1971 (Group 3—"old mouse"). Only those pairs were selected that had yielded a minimum of 100 AFB per 60 oil immersion fields when originally counted.

The 50 unstained slides were immersed in a freshly-prepared 10% (w/v) aqueous solution of periodic acid for 4 h, after which they were rinsed with water and stained by the standard acid-fast stain (oxidation for 24 h was found to yield the same results as periodate treatment for 4 h).

C.H. examined all 100 slides from Groups 1, 2 and 3. In addition, 20 slides from Groups 1, 2 and 3 were recoded and reexamined by C.H.—10 stained by the standard technique and 10 after periodate oxidation. L.P.M. examined 20 of the slides from Groups 1, 2 and 3—10 stained by the conventional acid-fast stain and 10 stained after treatment with periodate. In addition to these 100 slides, 10 "human" and 10 "new mouse" acid-fast stained slides previously found by L.P.M. to yield at least 100 AFB per 60 fields were selected for recounting by the same examiner. All 120 slides were coded with a three-digit random number.

The results have been analyzed by the linear regression technique (Goldstein, 1964).

Results

REPRODUCIBILITY OF COUNTS OF AFB

The results of the duplicate counts are plotted in Fig. 1. All 40 points representing duplicate counts made by the same observer were plotted with the first count as the "observer no. 1" value and the second count as the value for "observer no. 2". The duplicate counts by L.P.M. are represented by the open circles. The slope of line a, the regression line calculated for the 20 comparisons, is 0.74, a value significantly smaller than 1. The slope of the regression line for duplicate counts by C.H. (closed circles, line b) is 0.82, a value not significantly different from 1.

It may be more appropriate to consider the absolute differences (the differences without regard to sign) between duplicate counts as the measure of observer error. The mean absolute difference between duplicate counts, expressed as the percentage of the mean of the two counts, is 25% for L.P.M. and 24% for C.H. One may also calculate the regression of the smaller counts on the larger counts in each pair; the slopes of these regression lines are 0.62 for L.P.M. and 0.84 for C.H.; both values are significantly smaller than 1 but not different from each other.

Twenty of the points plotted in Fig. 1 (Δ's, line c) represent counts made by L.P.M. (observer no. 1) and C.H. (observer no. 2). The slope of the regression of the counts by C.H. on those of L.P.M. was 0.76, a value significantly smaller than 1 but not different from 0.74 or 0.82.

Because L.P.M. reexamined 10 slides prepared from human material that had been first examined from 1 to 3 years earlier, it was possible to assess the effect of storage on the number of AFB stained by the conventional AFB stain. The regression of the recent counts on those made originally was 0.78, which is not significantly smaller than 1.

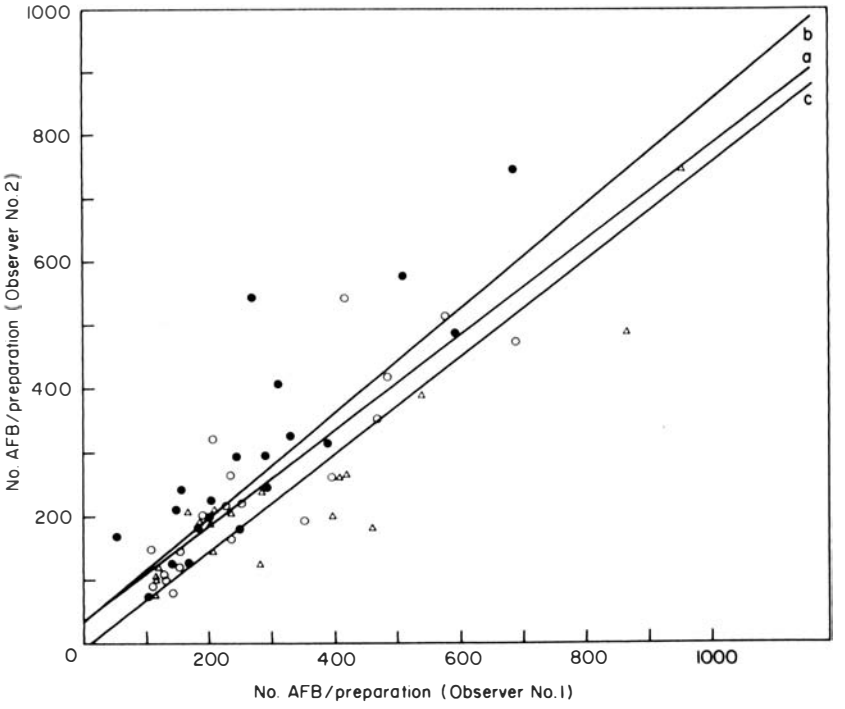

Fig. 1. The number of AFB counted by observer no. 2 as a function of the number counted by observer no. 1 in the same preparation. The lines represent the regression of the number counted by observer no. 2 on that counted by observer no. 1.

L.P.M. vs L.P.M. - (○), line a;
C.H. vs C.H. - (●), line b;
C.H. vs L.P.M. - △'s, line c.

Equations of the regression lines and their correlation coefficients (r) are as follows:

line a - Count no. 1 = 246.4 + (0.744 ± 0.208) (Count no. 2 - 282.3); $r = 0.871$;
line b - Count no. 1 = 275.6 + (0.820 ± 0.224) (Count no. 2 - 298.0); $r = 0.876$;
line c - Count no. 1 = 271.6 + (0.763 ± 0.152) (Count no. 2 - 367.4); $r = 0.926$.

EFFECT OF PERIODATE TREATMENT

In Fig. 2, the numbers of AFB in the acid-fast stained preparations are plotted as a function of the numbers of organisms counted in the periodate-treated preparations. The slope of the regression of the counts in the acid-fast stained preparations on those in the periodate-treated slides prepared from skin biopsy specimens from patients (open circles, line a) was 0.38, a value significantly smaller than 0.6. The slope of the regression of the numbers of organisms in acid-fast stained preparations on those in corresponding periodate-treated slides prepared with *M. leprae* harvested from infected mouse foot pads (closed circles, line b), is 1.07; this value is not significantly different from 1, but is different from 0.38.

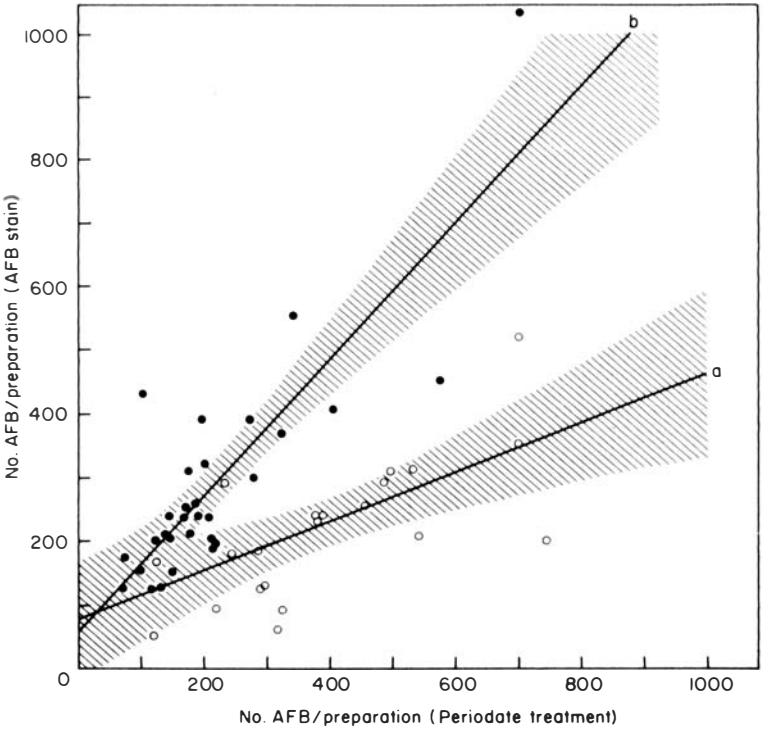

Fig. 2. The number of AFB stained by the standard acid-fast stain as a function on the number stained after periodate oxidation. The lines represent the regression of the number stained by the acid-fast stain on that stained after periodate treatment. Shaded areas represent the 95% confidence bands.

"Human" slides–(○), line a;
"Old mouse" and "new mouse" slides – (●), line b.

Equations of the regression lines and their correlation coefficients are as follows:

line a - Count no. 1 = 227.1 + (0.381 ± 0.214) (Count no. 2 - 393.8); r = 0.662;
line b - Count no. 1 = 289.7 + (1.067 ± 0.259) (Count no. 2 - 219.2); r = 0.847.

Discussion

The purpose of this study was to reexamine claims that periodate treatment rendered stainable *M. leprae* that could not otherwise be stained by the acid-fast technique. The results demonstrate that this is indeed the case for *M. leprae* recovered from patients after treatment sufficient to render the organisms non-infective for mice (Shepard *et al.*, 1972*a,b*). The number of these organisms stained after periodate treatment was, on the average, 36% larger than that directly stained by the acid-fast technique. These data appear consistent with those reported by others.

That we are not measuring the effects of storage has already been shown. The question of an artefact imposed by a systematic difference between observers is

TABLE 1

Comparison of counting error with effects of periodate treatment

Observer		Source	Stain	n	$\bar{D}$* (%)
No. 1	No. 2				
L.P.M.	L.P.M.	New mouse	AFB	10	6.93
L.P.M.	L.P.M.	Human†	AFB	10	20.3
C.H.	C.H.	Human	AFB	10	14.4
C.H.	C.H.	Human	Periodate	10	1.54
L.P.M.	C.H.	Old mouse	AFB	10	16.1
L.P.M.	C.H.	Human	Periodate	10	40.5‡
C.H.	C.H.	Human	Periodate vs AFB§	20	36.4‡
C.H.	C.H.	New mouse	Periodate vs AFB§	20	−32.9
C.H.	C.H.	Old mouse	Periodate vs AFB§	10	−16.4‡

* $\bar{D} = \Sigma$ |200 (Observation no. 1 - Observation no. 2)/(Observation no. 1 + Observation no. 2)|/n, where n = the number of pairs of observations.

† Observation no. 1 - those counts made in the period 1971 to 1973; observation no. 2 - recent counts.

‡ None of the values of $\bar{D}$ was significantly different from 0; the slopes of the regression lines calculated for these sets of data were significantly different from 1, however.

§ Observation no. 1 - those counts made on periodate-treated slides; observation no. 2 - counts made on slides stained by the standard acid-fast stain.

best answered by the data listed in Table 1. Here, the mean difference between duplicate counts or counts on duplicate preparations is expressed as the percent of the mean of the 2 counts. The absolute difference between duplicate counts on the same preparations is the same on the average for the 2 observers. C.H. appears consistently to count fewer organisms than does L.P.M.; this is particularly true for the periodate-treated "human" slides. It may be, then, that C.H. underestimated the effect of periodate treatment, perhaps by regularly dismissing as staining artefacts objects that the more experienced L.P.M. considered *M. leprae*. Thus, the effect of periodate treatment of *M. leprae* recovered from treated patients appears to be real, and may in fact be greater than that we have measured.

The situation with respect to *M. leprae* harvested from mouse foot pad tissues during logarithmic multiplication, however, appears different. In this case, observer C.H. found consistently more AFB stained by the conventional technique than after periodate treatment. And even if C.H. underestimated the number of these organisms stained after oxidation, there is no evidence of an excess of AFB stained after periodate treatment, as was the case with those recovered from treated patients. Thus, the phenomenon is not the result of fading of the acid-fast stained organisms on storage, nor is it the result of observer error.

Reich has reported (1971) that the number of viable organisms in a logarithmic phase culture of a cultivable mycobacterial strain—the "NQ bacillus"—was as much as 100-fold greater than the number enumerated as AFB. He suggested that the failure to be stained by the acid-fast stain was a characteristic associated with "physiologic youth" of this mycobacterial strain. In a later presentation, published only in abstract (Reich *et al.*, 1972), he reported that in approximately half of a group of smears of skin scrapings and bacterial suspensions recovered from the biopsy specimens of skin lesions of patients, periodate treatment

increased the number of AFB by at least 30% above the number counted in the corresponding conventionally-stained preparations. He concluded that, as was the case with cultures of the NQ bacillus, an important fraction of *M. leprae* goes unstained by the standard acid-fast stain, and that, because we have no independent measure of the number of *M. leprae*, "mycobacterial counting that included only the red stained bacilli would provide data that could be invalid for experimental conclusions that were based on the assumption that either the entire population or constant proportions of the population were being investigated." The data presented in this report suggest, on the other hand, that the failure of staining by the standard acid-fast technique is a characteristic of dead or decadent *M. leprae* that are unable to infect the mouse foot pad, whereas "youthful" organisms—those recovered from the mouse foot pad during logarithmic multiplication—stain at least as well by the standard technique as after periodate treatment.

The data presented here suggest, further, that the excess of "human" *M. leprae* stained after periodate oxidation is inconsequential. In an earlier, as yet unpublished study of the counting technique, we found that the *M. leprae* were often randomly distributed in the "circles" of the counting slide; that is, the numbers of organisms encountered in field-by-field counts were consistent with the Poisson distribution (Goldstein, 1964). Moreover, the numbers of organisms counted during the examination of 20 fields along the equator of each of the three circles on the slide fell in the range −50% to +100% of the mean for the 3 circles 90% of the time. Thus, some variation of the numbers of organisms counted in duplicate preparations is to be expected, even when both have been stained simultaneously by the same technique and were examined by the same observer. In order to demonstrate an effect of periodate treatment, the difference between numbers of AFB counted on duplicate slides must be shown to be greater than that attributable to counting error. Our data suggest that the increase of the number of stainable AFB from human material produced by periodate treatment is only a little larger than the apparent increase that might be encountered by chance when the same slide is reexamined by the same or another examiner, and may be no greater than the apparent increase encountered when duplicate but not identical preparations are examined. Certainly, there is no evidence to suggest that staining by the standard acid-fast technique results in a gross underestimate of the number of *M. leprae* in a preparation.

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