Distribution of *Mycobacterium leprae* in the Circles of Counting Slides

TO THE EDITOR:

In his recent article ([1]) entitled "Enumeration of purified suspensions of *Mycobacterium leprae*," Humber described a peculiar distribution of the organisms in his preparations, such that the *M. leprae* appear to be more concentrated in the periphery and center of the 4-mm-diameter circles he employed, and suggested that the problems he encountered also characterize those preparations of *M. leprae* made by the technic employed for many years in our laboratories ([2, 3]). Of course, the distribution of *M. leprae* in the preparations made according to this technic had been studied in the course of developing the technic; at that time, we were satisfied that, although the organisms were not randomly distributed, they were not concentrated in any portion of the circle of the counting slide we employ. In particular, there was no accumulation in either the periphery or the center of the circle. It is the purpose of this letter to present the early data from Atlanta and some data recently collected in Jerusalem, in order to demonstrate that Humber's concern was unwarranted.

In the early study, suspensions of *M. leprae* were prepared, as described ([2, 3]), from homogenates of 10 consecutive human biopsy specimens or mouse foot pad tissues by the addition of bovine serum albumin to a final concentration of 0.1%, "working up" the suspension by repeated aspiration into and expulsion from a pipette equipped with a rubber bulb, to promote coagulation of the particulate material in the homogenate, and then permitting the suspension to stand undisturbed for 2 minutes, after which the "2-minute supernate" was carefully aspirated. Without further purification, a measured volume of the 2-minute supernate was pipetted directly onto the circle, on which was first placed a measured volume of formol-milk. After drying in air, the circles were covered with gelatin-phenol, fixed with Formalin fumes, and stained by the standard room-temperature, acid-fast staining technic. *M. leprae* were then enumerated, employing an apochromatic 100 × oil-immersion objective and critical illumination, for this purpose in 11 microscope fields 1 mm apart, in each of the three circles of the counting slide. In the approximately 1-cm-diameter circles, the first field was located at one end of a diameter, the 6th field in the middle of the circle, and the 11th field at the opposite end of the diameter. The results of the counts of *M. leprae,* arranged in descending order of the total number of *M. leprae* enumerated, are presented in Table 1, in terms of the percent of the total number of organisms counted in 33 fields—

TABLE 1. *Distribution of* M. leprae *in the circles of counting slides—early study.*

Specimen no.	Percent of organisms in field no.[a]					Total no. organisms counted
	1	3	6	8	11	
1	1.4	2.7	8.8	17	2.0	147
2	16	7.8	3.9	4.9	22	102
3	7.7	5.2	21	14	1.3	77
4	19	12	1.3	12	16	75
5	0.0	0.0	21	1.6	13	62
6	2.5	7.5	28	10	0.0	40
7	2.6	10	2.6	5.1	5.1	39
8	5.6	0.0	0.0	31	33	36
9	0.0	32	0.0	7.0	0.0	28
10	3.8	0.0	7.7	15	3.8	26
Median	3.2	6.4	5.8	11	4.4	

[a] The pooled values for the 3rd and 8th fields are not different from those for the 1st and 11th fields, or from the values for the 6th field ($p > 0.10$ by the Mann-Whitney U test[3]).

TABLE 2. *Distribution of* M. leprae *in the circles of counting slides—new study.*

Specimen no.	Percent of organisms in field no.[a]					Total no. organisms counted
	1	3	6	8	11	
1	6.14	6.14	9.06	9.65	11.1	342
2	12.6	5.96	7.62	12.6	7.62	302
3	6.75	8.44	6.33	8.86	5.91	237
4	8.07	8.97	9.87	7.17	11.2	223
5	5.91	9.55	13.6	7.73	10.9	219
6	9.95	8.90	8.90	7.33	7.85	192
7	7.63	9.47	12.6	8.42	4.21	190
8	4.14	11.8	8.88	8.88	3.55	169
9	7.84	12.4	7.19	8.50	8.50	153
10	6.12	11.6	25.2	6.12	8.84	147
11	7.19	10.1	8.63	10.8	12.2	139
12	8.76	11.7	9.49	10.2	9.49	137
13	7.58	6.82	6.06	6.06	13.6	132
14	10.8	13.7	7.84	6.86	9.80	102
15	4.08	3.06	7.14	16.3	6.12	98
16	11.7	7.45	7.45	9.57	8.51	94
17	2.50	6.25	3.75	10.0	18.8	80
18	3.80	12.7	5.06	11.4	8.86	79
19	11.4	8.57	10.0	7.14	1.43	70
20	4.69	9.38	4.69	6.25	7.81	64
Median	7.38	9.18	8.24	8.68	8.68	

[a] The pooled values for the 3rd and 8th fields are not different from those for the 1st and 11th fields ($p > 0.06$), or from the values for the 6th field ($p > 0.19$ by the Mann-Whitney U test[3]).

the 1st and 11th fields, representing the periphery of the circle; the 6th field, representing the center; and the 3rd and 8th fields, representing indifferent areas of the circles, in each of three circles. As shown in Table 1, there is no suggestion of the distribution reported by Humber.

To assemble additional data from a second laboratory employing the same counting technic, a similar study was recently carried out. For this purpose, the last 20 slides, all representing harvests of *M. leprae* from mice from which at least 2×10^5 organisms per foot pad had been harvested, were reexamined, together with their duplicates, the numbers of organisms being summed for six circles (two slides) for each of the 11 fields selected. The results of this study are presented in Table 2, in which the specimens have been arranged in descending order of the total number of organisms counted in 66 fields. There appears to be less field-to-field variation in this study than was encountered in the early study, presumably because larger numbers of *M. leprae* were counted in each preparation. Again, there is no suggestion of the distribution described by Humber. In addition, analysis of the results of both studies, comparing the pooled results of the 3rd and 8th fields with those of the 1st and 11th fields on the one hand, and with the results of the 6th field on the other, by means of the Mann-Whitney U test ([4]) failed to reveal a significant difference. Thus, in neither study is there evidence of concentration of organisms at either the periphery or the center of the circle.

At least two explanations for the difference between these observations and those of Humber ([1]) are apparent. First, Humber employed *M. leprae* purified according to IMMLEP Protocol 1/79; whereas we routinely employ organisms that have been separated only by gravity from the larger particles present in tissue-homogenates. In the experience of one of us (CCS), the distribution of purified organisms on the circle displays partial concentration at the rim of the drop; whereas organisms that have not been purified do not show this "rim effect."

A second, not necessarily alternative, explanation lies in the differing geometry. Humber applied a volume of 5 μl to a circle with a diameter of 4 mm; whereas we apply a volume of 20 μl (10 μl formol-milk fol-

lowed by 10 μl bacterial suspension) to a circle with a diameter of 10 mm. Thus, Humber's drops are deeper (0.4 vs 0.25 μl per mm^2), and the circumferences of his circles are relatively greater (2.52 vs 1.57 mm per μl). We suggest that the rim effect is caused by greater evaporation from the rim, as compared with the central portion of the drop, and greater transportation of organisms to the rim, while the liquid is still deep enough to permit horizontal transport. Moreover, we make a point of quickly spreading the volume over the entire area after it has been placed on the circle, in order to avoid central deposition such as that described by Humber.

The microscopic counting of acid-fast bacteria has come to be an important technic in leprosy research. Because the procedure demands so much time of a highly skilled technician, we wish very much that it could be improved. Nevertheless, we do not think that the corrective calculations advocated by Humber are justified.

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