13. GERWIG, G. J., KAMERLING, J. P. and VLIEGENTHART, J. F. G. Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary g.l.c. Carbohydr. Res. **62** (1978) 349–357.
14. GIGG, R., PAYNE, S. N. and CONANT, R. The allyl group for protection in carbohydrate chemistry. Part 14. Synthesis of 2,3-di-*O*-methyl-4-*O*-(3, 6-di-*O*-methyl-β-D-glucopyranosyl)-L-rhamnopyranose (and its α-propyl glycoside): a haptenic portion of the major glycolipid from *Mycobacterium leprae*. J. Carbohydr. Chem. **2** (1983) 207–223.
15. HUNTER, S. W. and BRENNAN, P. J. A novel pheolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147** (1981) 728–735.
16. HUNTER, S. W., FUJIWARA, T. and BRENNAN, P. J. Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J. Biol. Chem. **257** (1982) 15072–15078.

Effect of Shipment of Skin-Biopsy Specimens to a Distant Laboratory on Viability of *Mycobacterium leprae*

TO THE EDITOR:

In some clinical trials of antileprosy chemotherapy, measurement of the response to treatment requires shipment of biopsy specimens to a distant laboratory where *Mycobacterium leprae* are recovered from the specimens and inoculated into mice. Earlier work ([4]) had demonstrated that *M. leprae* survived periods of storage at 0–4°C, justifying shipment of specimens to distant laboratories for inoculation into mice, but the number of specimens studied and the method employed were insufficient to exclude a small but systematic loss of viable organisms in the course of shipment.

In a series of clinical trials among patients with lepromatous leprosy at the Leonard Wood Memorial Leprosy Research Laboratory, Cebu, The Philippines ([1, 2]), conducted between mid-1969 and early 1974, patients were subjected to skin biopsy at intervals, and 409 biopsy specimens were divided, one portion of each specimen being processed for inoculation of mice in Cebu and the second portion air-shipped on wet ice to the U.S. Public Health Service Hospital, San Francisco, California, where mice were also inoculated. Specimens obtained during the morning in Cebu were usually put aboard an afternoon flight to Manila, and transferred to an international flight that left Manila that evening, arriving in San Francisco on the same evening (because of the west-to-east crossing of the International Date Line). In San Francisco, the specimens were usually picked up during the evening of arrival and, when possible, processed for mouse inoculation the following day. Thus, the elapsed time between biopsy in Cebu and inoculation of mice in San Francisco was frequently no more than 48 hr. Occasionally, a longer period of storage intervened between biopsy and inoculation of mice in San Francisco.

In both Cebu and San Francisco, the technique of Shepard ([3, 5]) was employed for recovery of *M. leprae* from the biopsy specimens, counting the organisms, inoculating mice, and harvesting the organisms from mice. The generation time (G), defined as the number of days per doubling of *M. leprae,* was calculated according to the relationship:

$$G = \frac{\text{number of days between inoculation and harvest}}{\text{logarithm}_2 \left(\text{number AFB harvested} / \text{number of AFB inoculated}\right)}.$$

This calculation assumes that all of the inoculated organisms were capable of multiplication, began multiplying on the day of inoculation, and multiplied at a constant rate from the day of inoculation to the day of harvest. Although these assumptions are untenable, this measurement has provided a useful means of evaluating the effects of treatment in a number of clinical trials among patients with lepromatous leprosy. In addition to the values for G, the difference between the dates of inoculation in the two laboratories (D) and the year of biopsy (Y) were recorded for each specimen.

TABLE 1. *Distribution of D and Y for 409 specimens studied in Cebu and San Francisco.*

Y	D (days)						
	0	1	2	3	4	6	Total
	(Numbers of specimens)						
1969	1	1	4	3	2	0	11
1970	0	19	61	29	7	1	117
1971	6	20	113	13	1	1	154
1972	3	42	35	4	4	0	88
1973	4	25	7	0	0	0	36
1974	0	3	0	0	0	0	3
Total	14	110	220	49	14	2	409

The distribution of the values for D and Y is presented in Table 1, in which a value for D of 1 day is equivalent to an elapsed time of 48 hr, a value of D of 2 days to 72 hr, etc. In the course of the 5-year period, organisms from 30% of the specimens were inoculated with an elapsed time no longer than 48 hr, and 84% with an elapsed time no longer than 72 hr.

Of the 409 specimens, the *M. leprae* recovered from 170 failed to multiply in mice in either laboratory. Virtually all of these specimens had been obtained from patients in the course of treatment; one may conclude only that the proportions of viable *M. leprae* in these specimens were insufficient to infect mice. These 170 specimens are not useful in comparing results in the two laboratories for the purpose of investigating the effects of storage in the cold on viability of the organisms, and are not further considered.

The organisms recovered from 39 additional specimens multiplied in the mice of one laboratory, but failed to multiply in the mice of the other (Table 2). Were death of *M. leprae* during storage quantitatively important, one would expect a disproportionately large fraction of these specimens to be associated with larger values for D; yet, this was not the case. Considering the 239 specimens yielding multiplication in at least one laboratory, 8 of 86 specimens (9.3%) with $D \leq 1$ day failed to yield multiplication in San Francisco, compared to 13 of 153 specimens (8.5%) with $D \geq 2$.

The results of the study of the 200 specimens yielding multiplication in both laboratories demonstrate that the mean values for G—32.7 days [standard deviation (S.D.) 14.3] in Cebu and 34.0 days (S.D. 12.3) in San Francisco—are remarkably similar, suggesting that the values estimated for G in the two laboratories do not differ systematically (to conserve space, only the results of the 113 specimens studied during the years 1971 and 1972 are presented in Table 3).

The formula defining G suggests that the reciprocal, 1/G, may be more directly re-

TABLE 2. *Comparison of results from the study in Cebu and San Francisco of 39 specimens not yielding multiplication in mice in one of the two laboratories.*

D	G_{CEBU}	G_{SF}
	1970	
1	>100	22.9
1	>100	35.6
1	>100	39.8
1	>100	43.6
1	>100	68.3
2	36.4	>100
2	48.0	>100
2	65.5	>100
2	>100	40.4
2	>100	46.5
2	>100	62.8
2	>100	66.2
2	>100	86.1
3	71.3	>100
3	78.7	>100
3	>100	36.8
3	>100	89.8
4	51.7	>100
	1971	
1	62.0	>100
1	>100	79.0
2	>100	30.3
2	68.8	>100
2	85.8	>100
2	88.7	>100
2	91.0	>100
2	>100	63.5
2	>100	86.3
2	>100	91.5
3	31.0	>100
3	45.3	>100
6	71.6	>100
	1972	
1	26.1	>100
1	57.2	>100
1	68.8	>100
1	>100	31.4
	1973	
0	45.7	>100
1	47.0	>100
1	65.1	>100
1	74.2	>100

TABLE 3. *Results of inoculation of mice in Cebu and San Francisco with* M. leprae *from 200 specimens.*

D	G_{CEBU}	G_{SF}	D	G_{CEBU}	G_{SF}	D	G_{CEBU}	G_{SF}	D	G_{CEBU}	G_{SF}	D	G_{CEBU}	G_{SF}
	1971		2	23.4	26.1	2	42.5	41.5	1	22.5	26.3	2	20.4	24.2
0	20.5	23.6	2	23.5	22.1	2	54.9	32.8	1	22.7	25.3	2	22.2	22.1
0	21.0	19.0	2	23.5	26.2	2	63.6	96.2	1	22.8	29.1	2	22.6	44.4
0	24.5	24.3	2	24.5	22.6	2	77.9	29.0	1	23.9	26.0	2	22.7	40.7
0	28.3	32.3	2	24.9	41.9	2	83.2	45.6	1	24.2	28.3	2	22.8	25.5
1	18.9	29.5	2	25.5	27.0	2	98.4	38.0	1	26.0	23.2	2	23.4	24.7
1	19.9	27.0	2	26.5	28.4	3	30.3	41.5	1	26.5	29.3	2	23.7	25.4
1	22.2	26.5	2	27.4	34.0	3	44.1	78.3	1	27.2	24.9	2	24.6	32.3
1	27.2	37.6	2	27.5	28.0	3	45.0	29.4	1	29.2	28.8	2	25.1	27.7
1	27.4	31.9	2	27.6	37.2	3	46.0	31.1	1	29.5	30.6	2	26.5	28.9
1	46.3	38.0	2	27.8	30.3		1972		1	29.9	33.5	2	28.2	24.7
1	47.0	52.2	2	27.8	33.6	0	19.3	31.6	1	30.1	39.6	2	28.6	27.1
2	17.1	22.1	2	27.8	39.8	0	29.4	92.1	1	31.0	38.5	2	30.6	48.4
2	17.7	27.5	2	29.2	23.0	0	40.7	73.9	1	33.2	36.6	2	37.8	49.3
2	17.8	20.8	2	29.2	32.6	1	16.2	24.1	1	35.0	41.5	2	38.2	30.0
2	18.1	35.5	2	29.8	31.1	1	17.8	23.5	1	35.1	39.1	3	22.6	26.3
2	20.2	23.4	2	31.7	49.5	1	19.9	26.7	1	41.2	44.5	3	23.1	24.2
2	20.5	23.8	2	33.2	26.7	1	20.6	27.1	1	44.7	35.1	3	26.3	40.7
2	20.7	23.8	2	33.8	25.0	1	21.0	25.7	2	17.0	20.9	3	68.4	73.2
2	22.3	44.2	2	35.6	26.1	1	21.3	34.7	2	17.4	30.1	4	25.3	30.9
2	22.4	23.1	2	37.1	25.0	1	21.9	23.2	2	17.8	41.1	4	26.5	33.9
2	22.4	23.1	2	37.7	41.6	1	22.3	27.3	2	19.0	25.1	4	28.8	24.7
2	22.5	24.1	2	40.8	32.4	1	22.3	36.1	2	19.7	29.0	4	36.2	23.8

lated to the number of viable *M. leprae* inoculated than is the value G itself. In addition, as measured by the skewness and kurtosis parameters,[1] the distribution of 1/G was much more similar to a normal distribution than was that of G itself. For these reasons, 1/G in San Francisco ($1/G_{SF}$) and 1/G in Cebu ($1/G_{CEBU}$) were employed in the following analyses.

It was postulated that $1/G_{SF}$ and $1/G_{CEBU}$ are linearly related. The degree to which this relationship may have been perturbed by shipment of the specimens to a distant laboratory, or by some factor related to the passage of time in the course of the 5 years of the study, was examined by the techniques of multivariate and stratification analyses. Considering all 200 pairs of values, the technique of linear step-wise regression, employed to examine the regression of $1/G_{SF}$ on $1/G_{CEBU}$, D and Y, yields the relationship: $1/G_{SF} = 0.15 + 0.42 \times 1/G_{CEBU} - 0.0018 \times Y$; $p < 0.0001$, and $R^2 = 0.29$, indicating that only 29% of the variance between $1/G_{SF}$ and $1/G_{CEBU}$ is explained by this regression. D failed to enter the equation, implying that this variable exerted no measurable influence on the regression. The coefficient of Y, −0.0018, is very small, and the entry of Y into the step-wise regression was found to explain only 4% of the variance.

The influence of Y and D on the regression of $1/G_{SF}$ on $1/G_{CEBU}$ was examined further by first calculating the regression of $1/G_{SF}$ on $1/G_{CEBU}$, and subsequently calculating the regression of the residuals (RES)[2]—i.e., the departures of $1/G_{SF}$ from the value predicted by $1/G_{CEBU}$—on both D and Y. The regression obtained was: $RES = 0.092 - 0.0013 \times Y + 0.00064 \times D$. The coefficients of both Y and D are very small. The coefficient of D was not significantly different from 0 ($p = 0.29$); whereas that for Y was ($p = 0.014$). However, the value for R^2, 0.052, indicates that Y accounts for only 5% of the observed variance of RES. This analysis confirms that Y exerts only a small influence on the regression; whereas D exerts no measureable influence.

Finally, the influence of D on the regression of $1/G_{SF}$ on $1/G_{CEBU}$ was examined by

[1] Skewness is a measure of deviation from symmetry. Kurtosis is a measure of the peakedness or flatness of a frequency-distribution curve, compared to the bell-shaped normal curve.

[2] For each specimen, RES is defined by the relationship: $RES = [1/G_{SF} - (0.020 + 0.36 \times 1/G_{CEBU})]$, in which 0.020 and 0.36 are the intercept and slope, respectively, of the regression of $1/G_{SF}$ on $1/G_{CEBU}$.

omitting the data for the years 1969 and 1970, and by considering separately those specimens for which D = 1 and 2 (the numbers of specimens with D = 0, 3, 4, or 6 were too small to permit such an analysis). The regression of $1/G_{SF}$ on $1/G_{CEBU}$ for the 54 specimens with D = 1 obtained after 1970 is: $1/G_{SF} = 0.017 + 0.38 \times 1/G_{CEBU}$; $p < 0.0001$, $R^2 = 0.39$; and the corresponding regression for the 66 specimens with D = 2 is: $1/G_{SF} = 0.020 + 0.35 \times 1/G_{CEBU}$; $p < 0.0001$, $R^2 = 0.25$. These regression equations are virtually identical, again confirming the lack of influence of D on the regression, and suggesting that the influence of the year of biopsy on the regression of $1/G_{SF}$ on $1/G_{CEBU}$ is exerted primarily on those specimens obtained during the first 1½ years of the study.

A deleterious effect of prolonged storage in the cold on the viability of *M. leprae* might be expected to result in larger values of G in more distant laboratories than in laboratories in which organisms from the same biopsy specimens are inoculated without interim storage. However, the data that resulted from this study do not demonstrate a systematic difference between the results in the two laboratories consistent with a deleterious effect of storage.

To be sure, differences were observed between results in the two collaborating laboratories. However, the only identifiable factor influencing the results was Y, the year the biopsy was taken. This source of variation, which in fact exerted only a small influence on the results of inoculation of both laboratories, appeared to be present only during the first years of the collaboration; during this period, the mouse footpad technique was being established for the first time in Cebu, and all of the problems of trans-Pacific air shipment were being encountered. In fact, the major source of variation of the results of inoculation of mice in the two laboratories remains unrecognized.

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REFERENCES

1. Collaborative Effort of the U.S. Leprosy Panel (U.S.–Japan Cooperative Medical Science Program) and the Leonard Wood Memorial. Rifampin therapy of lepromatous leprosy. Am. J. Trop. Med. Hyg. **24** (1975) 475–484.
2. Collaborative Effort of the U.S. Leprosy Panel (U.S.–Japan Cooperative Medical Science Program) and the Leonard Wood Memorial. Spaced clofazimine therapy of lepromatous leprosy. Am. J. Trop. Med. Hyg. **25** (1976) 437–444.
3. Shepard, C. C. The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. J. Exp. Med. **112** (1960) 445–454.
4. Shepard, C. C. and McRae, D. H. *Mycobacterium leprae*: viability at 0°C, 31°C, and during freezing. Int. J. Lepr. **33** (1965) 316–323.
5. Shepard, C. C. and McRae, D. H. A method for counting acid-fast bacteria. Int. J. Lepr. **36** (1968) 78–82.