Volume 48, Number 4
Printed in the U.S.A.

Microscopic Counts Carried Out on *Mycobacterium leprae* and *M. tuberculosis* Suspensions. A Comparison of Three Staining Procedures[1]

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Total counts for mycobacteria are usually carried out by direct microscopic enumeration of acid-fast bacilli in smears stained by the Ziehl-Neelsen method or its Fite-Faraco adaption ([4, 5]). The number of bacilli determined to be present per ml of suspension depends upon the ability of the heat-killed mycobacteria to retain the fuchsin dye during acid decolorization. Dead leprosy bacilli frequently take on a highly beaded appearance ([1]) and may not retain sufficient dye to be recognized in smears exposed to the standard acid-alcohol decolorization procedure ([3]). Several alternative decolorization procedures have been recommended for *M. leprae*-infected tissues which could result in higher estimates for these microscopic counting procedures ([4, 10, 12]).

Lepromin is prepared from autoclaved human biopsy material following appropriate enzymic digestion and differential centrifugation to remove the residual tissue components ([7, 8]). Many of the *M. leprae* cells present in the final preparation will have been damaged during this process. As a result, they may not retain their acid-fastness when stained by the Ziehl-Neelsen method. Other staining procedures may give higher counts because they do not depend upon the bacilli retaining the acid-fast stain. This difference could have important implications where the total bacillary count is used to standardize the bacterial suspension in terms of bacillary mass per ml ([7, 13]).

The present study compares the number of mycobacteria in standardized heat-killed suspensions of *M. leprae* and *M. tuberculosis* which were counted following treatment of the smears by three different staining procedures. Substantial variations in the number of bacilli were reported depending upon the staining method.

MATERIALS AND METHODS

Bacterial suspensions. *M. tuberculosis* $H_{37}Rv$ (TMC #102) was grown in synthetic modified Sauton's liquid medium (MSTA), incubated in a 1 liter roller bottle at 37°C for 10 days ([2]). Viable counts carried out at the time of harvest indicated that 18.5×10^8 viable units were present per ml. The cells were washed with sterile 0.05% Tween-saline and killed by boiling for 10 min. The suspension was checked for sterility by plating several aliquots onto Middlebrook 7H10 agar and incubating the plates at 37°C for 6 weeks in sealed plastic bags. All of the cultures were negative. The suspension was standardized turbidimetrically; approximately 10^8 AFB per ml preserved with 0.1% cresol were stored at 4°C until required.

M. leprae. Human skin biopsies were collected at the Leonard Wood Memorial Laboratories, Cebu, in the Philippines. The tissue was autoclaved, homogenized in phosphate buffered saline (pH 7.2), treated with 1% Triton X-100 in 0.3 M sucrose-EDTA at room temperature for 1 hr before centrifugation at $15{,}000 \times g$ for 15 min. The washed pellet was treated with 100 μg of collagenase (Worthington Biochemicals, Freehold, New Jersey, U.S.A.) per ml of buffer (pH 7.2) at 37°C for 24 hr in the presence of sodium azide ([7]). The bacilli were

[1] Received for publication on 13 May 1980; accepted for publication on 25 June 1980.

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TABLE 1. *Number of mycobacteria per field when stained by the Ziehl-Neelsen, auramine fluorescence, or silver-methenamine methods.*

Bacterial suspension	Count No.	Average number of bacilli per field		
		Ziehl-Neelsen	Auramine	Silver-methenamine
M. leprae #1	1	25.7 ± 1.26[a]	28.5 ± 1.60	58.3 ± 4.30
	2	15.9 ± 1.67	29.5 ± 2.03	35.9 ± 5.50
	3	20.9 ± 1.19	23.8 ± 1.98	47.2 ± 3.25
	4	20.0 ± 1.67	22.1 ± 1.68	51.1 ± 2.95
	Average	20.0 ± 1.45	26.0 ± 1.79	48.1 ± 4.68
	AFB per ml	4.7×10^7	6.1×10^7	12×10^7
	p	—	N.S.	<0.01
M. leprae #2	1	14.2 ± 1.09	23.8 ± 1.98	38.7 ± 3.15
	2	19.9 ± 1.67	22.1 ± 1.68	45.3 ± 3.10
	3	13.8 ± 1.26	20.8 ± 1.63	47.3 ± 4.95
	4	18.2 ± 1.15	28.9 ± 1.02	39.9 ± 4.00
	Average	16.5 ± 0.75	23.9 ± 1.78	42.8 ± 3.94
	AFB per ml	3.9×10^7	5.6×10^7	10×10^7
	p	—	N.S.	<0.01
M. tuberculosis	1	11.8 ± 1.06	26.1 ± 2.01	44.2 ± 8.90
	2	14.2 ± 1.10	26.6 ± 2.60	61.5 ± 8.72
	3	11.8 ± 1.30	23.1 ± 1.47	—
	4	18.7 ± 1.26	20.8 ± 1.63	—
	Average	14.1 ± 0.69	24.1 ± 1.35	52.9 ± 1.40
	AFB per ml	3.3×10^7	5.7×10^7	13×10^7
	p	—	N.S.	<0.01

[a] Average of 20 fields ± S.E.M.

concentrated by centrifugation at 15,000 × *g* for 20 min, suspended in 0.001% Tween-saline plus 0.1% cresol, and stored at 4°C until required.

Preparation of standard bacterial smears. Tenfold dilutions of the bacterial suspensions were prepared in 0.01% Tween-saline and homogenized briefly with a Teflon tissue homogenizer ([2]). The suspensions were mixed with an equal volume of formol-milk ([13]) and 20 μl of each dilution were spread in triplicate over 1 cm diameter circles on Reich slides (Bellco Glass, Vineland, New Jersey, U.S.A.). The suspension was spread evenly over the whole circle and allowed to air dry on a levelling table. The slides were fixed by immersion in absolute methanol for 5 min at room temperature, drained, and allowed to air dry.

Staining methods. Ziehl-Neelsen (Z-N) stain. The fixed slides were stained for 5 min with strong carbol fuchsin at 80°C on a hot plate ([3]). In most experiments, the slides were rinsed with water and decolorized with 3% aqueous hydrochloric acid for up to 1 min. In some studies, the stained slides were decolorized with 3% hydrochloric acid made up in 95% alcohol ([16]). The acid-alcohol mixture was applied until no more stain was observed to wash out of the smear, which was then immediately rinsed with water and counterstained with aqueous methylene blue for 5 min. The smears were washed thoroughly in distilled water, drained, and allowed to air dry.

Auramine stain. The slides were stained with 0.1% carbol auramine at room temperature for 15 min ([16]). After washing with water, they were decolorized with 0.5% hydrochloric acid-alcohol and counterstained with 0.01% acridine orange and allowed to air dry ([10]).

Gomori silver-methenamine stain. The fixed slides were oxidized with 5% chromic acid for 1 hr and then stained with silver nitrate-methenamine for 1 hr at room temperature ([6, 15]). The slides were treated with 0.1% gold chloride and the unreduced silver

TABLE 2. *Number of* M. leprae *per field when the lepromin suspension was stained by Ziehl-Neelsen using 3% acid-alcohol or by the auramine method (0.5% acid-alcohol) or by the silver-methenamine method (no acid decolorization step).*

Preparation No.	Ziehl-Neelsen	Auramine	Silver methenamine	p value
1	7.20 ± 1.09[a]	23.8 ± 1.98	58.2 ± 7.25	
2	9.50 ± 1.49	22.1 ± 1.68	69.8 ± 5.95	
3	5.80 ± 1.06	22.2 ± 1.11	46.4 ± 5.12	
4	7.70 ± 0.54	23.9 ± 1.36	—	
Average	7.55 ± 0.76	22.9 ± 0.5	58.1 ± 6.76	<0.001

[a] Mean of 10 counts ± S.E.M.

removed by washing the slides in 2% sodium thiosulfate for 1 min, followed by thorough washing with distilled water and allowing to air dry.

The auramine stained slides were coded and examined with a ×63 oil immersion lens using a Zeiss epifluorescence microscope fitted with a IV FL vertical illuminator and a FL 580 barrier filter. The Ziehl-Neelsen and silver-methenamine stained slides were counted, using the same magnification but with transmitted white light and a blue filter. The number of bacilli per field were counted in 40 fields using an eyepiece grid. The number of bacilli per ml of suspension was calculated using the formula provided by Shepard and McRae ([13]).

RESULTS

Both *M. tuberculosis* $H_{37}Rv$ and *M. leprae* suspensions contained predominantly single and paired cells with a few clumps of three to six bacilli. This effect could be overcome by the chloroform declumping procedure during the preparation of the smears ([8]). While this treatment improved the accuracy of some counts involving severely clumped suspensions, the process had to be carefully controlled so that there was no substantial loss of acid-fastness by the treated cells. Clumped bacilli were often difficult to enumerate definitively, especially in the Ziehl-Neelsen stained slides, due to the uneven, beaded staining exhibited by the bacilli. As a consequence, the number of acid-fast bacilli counted in these smears was consistently lower than that recorded using the other staining methods (Table 1). In the auramine stained smears, highly fluorescent cell walls were clearly outlined, and the individual bacilli were readily enumerated using the ×63 oil immersion lens. Individual cell walls were seen more clearly outlined in the silver-methenamine stained preparations, and this was probably responsible for the still higher counts obtained using this staining method to visualize the leprosy bacilli (Table 1). Depending upon the staining procedure used, the number of *M. leprae* present in the lepromin preparation varied from 4 to 12×10^7 bacilli per ml. Similar counts

TABLE 3. *Replicate total counts carried out on heat-killed* M. leprae *stained with auramine and examined by fluorescence microscopy on 3 consecutive days.*

Experiment No.		Number of bacilli per field										Average ± S.E.M.
1	A	38	22	17	28	22	23	24	21	22	15	25.3 ± 2.27
	B	29	30	39	31	32	34	27	15	26	24	28.7 ± 2.03
2	A	25	22	30	36	38	30	22	14	31	15	26.2 ± 2.56
	B	31	23	34	26	21	30	21	27	29	21	27.3 ± 1.42
3	A	30	24	23	22	26	21	32	18	15	22	22.4 ± 1.29
	B	25	25	25	29	22	20	27	25	15	20	23.1 ± 1.27
											Average	25.5 ± 0.99

carried out on the heat-killed *M. tuberculosis* $H_{37}Rv$ suspensions showed a somewhat wider variation (3 to 13 $\times$ 10^7 per ml), but the overall trend seen in the three groups of stained smears was essentially the same for both test organisms (Table 1).

Two separate samples of lepromin were diluted to approximately the same optical density, and the standardized smears were counted using the three staining procedures. The bacillary counts for both lepromin preparations were significantly higher ($p < 0.01$) when the silver-methenamine stain was used (Table 1) than the four replicate auramine counts. However, the difference between the auramine and the Ziehl-Neelsen counts was much smaller and was not significant. In earlier studies, much greater differences had been noted between Ziehl-Neelsen and auramine counts. In an attempt to explain the modest differences shown in Table 1, the *M. leprae* smears were stained by the Ziehl-Neelsen method but decolorized with 3% acid-alcohol rather than with the 3% aqueous acid normally employed [3]. Under these conditions, the number of acid-fast bacilli was consistently lower ($p < 0.001$) than either the auramine or the silver-methenamine counts (Table 2). Total counts carried out on auramine stained lepromin suspensions on three sequential days by two separate investigators resulted in essentially identical bacillary counts (Table 3). Those variations which did occur appeared to be within normal experimental limits.

DISCUSSION

Human antileprosy vaccines being prepared for the IMMLEP trials must be standardized in terms of the total number of AFB per ml using the Ziehl-Neelsen staining procedures [7]. However, numerous studies carried out with *M. leprae*-infected tissues suggest that the auramine staining method may be preferable to the Ziehl-Neelsen stain, which results in visualization of noticeably lower numbers of bacilli within the tissue, especially during chemotherapy [10, 12]. This difference may result in as much as ten fold lower bacillary counts, especially when the preparation is badly clumped [8]. This lower count in the Ziehl-Neelsen stained *M. leprae* smears could be explained in terms of the lower acid-fastness of the leprosy bacillus compared to *M. tuberculosis*, and this seems to be especially true for smears decolorized with the 3% acid-alcohol mixture [3]. However, a similar difference between the Ziehl-Neelsen and auramine stains occurred with the *M. tuberculosis* smears (Table 1). This organism is strongly acid-fast and can easily resist prolonged decolorization with 25% aqueous sulfuric acid or even with 25% acid-alcohol [3]. Thus the lower counts cannot be explained simply as over-decolorization of the lepromin smears by the dilute acid. Significantly, the silver-methenamine stained counts for both organisms were very similar to each other and were always significantly higher than the acid-fast stained preparations. Earlier workers have reported that Gomori stained preparations contain more bacilli than conventionally stained smears [10]. Such findings were consistent with reports that periodic acid pretreatment of *M. leprae* suspensions resulted in significantly higher bacillary counts for Ziehl-Neelsen stained smears [9]. This may be equivalent to the chromic acid oxidation step of the Gomori stain [15], which allows the cells to take up the silver stain even when partially digested mycobacterial carcasses were present. The latter do not normally stain by the conventional acid-fast procedure.

In addition to providing higher total counts than the Ziehl-Neelsen preparation, the auramine stained smears had the advantage of providing greater visual contrast between the individual cell walls and the black background. This greatly increased the speed and reproducibility of counting in both bacterial preparations. The auramine and Gomori stains both offer the technical advantage of greater visual contrast compared with the conventional Ziehl-Neelsen method for staining killed mycobacterial suspensions. This has significance with respect to determinations of total bacterial mass within a heat-killed mycobacterial suspension. Studies carried out in intradermally vaccinated mice and guinea pigs suggest that the overall immunogenicity of killed *M. leprae* has a definite dose responsiveness [11, 14]. Human antileprosy vaccines must be standardized in terms of the total number of AFB per ml, and the present results suggest that the use of the con-

ventional Ziehl-Neelsen staining procedure may result in significantly lower bacillary counts compared to those obtained by other methods. It would therefore seem prudent to standardize antileprosy vaccines by both the acid-fast and Gomori silver-methenamine methods. This could indicate whether significant numbers of non-acid-fast bacillary carcasses (which could still represent substantial amounts of immunologically active antigen) were present in the vaccine. The presence of such non-acid-fast *M. leprae* or its antigen(s) within the standardized vaccine could constitute an important variable in the assessment of its protective value for human populations.

SUMMARY

Standard smears of heat-killed *Mycobacterium leprae* and *M. tuberculosis* $H_{37}Rv$ were counted microscopically following staining by the Ziehl-Neelsen, auramine, and silver-methenamine methods. The numbers of stained bacillary bodies were consistently higher in the silver-methenamine stained smears compared to the Ziehl-Neelsen and auramine stained smears. The auramine smears were examined under ultraviolet illumination and permitted the enumeration of the brightly fluorescent bacilli against a black background. The auramine counts were not as high as those obtained using the silver-methenamine stained preparations but were consistently higher than those obtained with the Ziehl-Neelsen preparations. Both the auramine and silver-methenamine stained preparations clearly outlined the cell walls of the bacilli in the smears and this greatly facilitated the counting process, especially if the cell suspension was badly clumped.

RESUMEN

Se prepararon extendidos sobre laminillas de vidrio de suspensiones de *M. leprae* y *M. tuberculosis* $H_{37}Rv$, muertos por calor. Los extendidos se tiñeron por los métodos de Ziehl-Neelsen, de la auramina y de la plata-metenamida, antes de hacer cuentas de bacilos. El número de bacilos teñidos fue consistentemente mayor en los extendidos teñidos por el método de la plata-metenamida que en aquellos teñidos por los métodos de Ziehl-Neelsen o de la auramina. Bajo iluminación ultravioleta, los bacilos teñidos con auramina aparecieron con una fluorescencia brillante resaltando sobre un fondo negro. Las cuentas de bacilos teñidos con auramina no fueron tan altas como las de los bacilos teñidos con la plata-metenamida pero fueron siempre mayores que las cuentas de bacilos teñidos por Ziehl-Neelsen. Tanto el método de la auramina como el de la plata-metenamida permitieron delinear claramente la pared de los bacilos en los extendidos y ésto facilitó mucho las cuentas, especialmente cuando las suspensiones bacterianas contenían muchos grumos.

RÉSUMÉ

On a compté au microscope le nombre de *Mycobacterium leprae* et de *Mycobacterium tuberculosis* $H_{37}Rv$, dans des frottis standards de bacilles tués par la chaleur, et colorés par la méthode de Ziehl-Neelsen, par l'auramine, ou par des techniques à base de methenamine-argent. Le nombre de corps bacillaires colorés était régulièrement plus élevé dans les frottis colorés par la methenamine-argent, que dans les frottis colorés par les deux autres méthodes. Les frottis colorés par l'auramine ont été examinés sous lumière ultra-violette, ce qui a permis de compter les bacilles brillamment fluorescents sur fond noir. Les nombres obtenus à la suite de la coloration par l'auramine n'étaient pas aussi élévés que ceux obtenus après coloration par la methenamine-argent; ils étaient cependant plus élevés que ceux obtenus dans les préparations colorées par la méthode de Ziehl-Neelsen. Les méthodes à base d'auramine et de methenamine-argent permettent toutes deux de définir clairement les parois cellulaires des bacilles dans les frottis. Il est ainsi beaucoup plus facile de compter les bacilles, surtout lorsque les suspensions cellulaires présentent des amas diffiles à délimiter.

Acknowledgements. We thank Roberta Morgan for preparing the auramine stained smears. The work was supported by Grant AI-14065 administered by the U.S.–Japan Cooperative Medical Science Program for the NIH, by Grant HL-19774 from the National Heart, Lung and Blood Institute, and by the Biomedical Research Support Grant RR05705 from the Division of Research Resources, NIH.

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