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Report

Size Characterization of *Mycobacterium bovis* BCG (*Bacillus* Calmette Guérin) Vaccine, Tice™ Substrain

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Reconstituted, lyophilized, attenuated *Mycobacterium bovis, Bacillus* Calmette Guérin (BCG) vaccine, Tice™ substrain, was characterized using a Coulter Multisizer and a HIAC/Royco counter. The primary organism has an equivalent spherical diameter approximating 1 μm but the BCG cell suspension is heavily aggregated. The cumulative size distribution of the suspension fits a log-probit plot and this information can be used to determine the total number of particles per ampoule. The instrumental count may be related to the viable count. The state of dispersion was unaffected by mild shear (syringe aspiration or ultrasound) and only slightly affected by the addition of cetylpyridinium chloride or sodium tauroglycolate.

KEY WORDS: *Bacillus* Calmette Guérin (BCG), Tice™; HIAC/Royco; Coulter; size analysis; aggregation number; colony-forming units (CFU); aggregation mechanism; surface charge; *Mycobacterium bovis*.

INTRODUCTION

The BCG (*Bacillus* Calmette Guérin) vaccine was first employed clinically as a treatment for tuberculosis in 1921 (1). The Tice substrain is generally recognized to be an effective and safe vaccine. It is currently on clinical trial as an immune stimulant and treatment for various cancers, in particular bladder cancer, where it appears to be unique (2).

The BCG vaccine consists of a suspension of a living culture of an attenuated *Mycobacterium bovis*. The suspension therefore contains viable cells, together with a proportion of damaged cells, cell fragments, and other debris that does not contribute to the clinical effectiveness of the vaccine but constitutes particulate matter in the dispersion (3).

The dose is measured in terms of the number of colony-forming units (CFU) per milliliter of volume, equivalent to the number of organisms per ampoule. For manufacturing purposes a faster method of estimating the CFU dose is needed.

Although Calmette and Guérin discovered in 1908 that the hydrophobic *M. bovis* could be dispersed in culture by the addition of bile salts (4), wetting agents are generally not added to cultures of the organism, the Glaxo (UK) strain being an exception (5). The hydrophobic cells constituting the vaccine are therefore substantially aggregated together. Kim (6) noted that the vaccine was aggregated to a degree that could not be adequately counted by direct microscopy.

The *M. bovis* organism is approximately cylindrical in shape, 2–4 μm long and 0.2–0.5 μm in diameter (7). Microscopy is a poor technique for accurate counting and discrimination of particles in the micrometer region (8,9). For quality-control purposes, as well as accuracy of dosage, there is clearly a need to reexamine the state of dispersion of this important vaccine. An appropriate method for rapidly determining the equivalent of the CFU and the state of dispersion of the microorganisms constituting the vaccine is required. The purpose of this present investigation was to provide preliminary data prior to developing improved methodology for vaccine characterization.

EXPERIMENTAL

Materials

Tice™ substrain BCG vaccine, various lots, was manufactured by the Institute for Tuberculosis Research (ITR), University of Illinois at Chicago. Other materials were as follows: water, glass distilled; Isoton II, Coulter Inc., Hialeah, Fla.; sodium tauroglycolate, Sigma Chemical Co., St. Louis, Mo.; cetylpyridinium chloride, Hexagon Chemical Co., Chicago; Monoject syringes; and 18-gauge 1-in. needles.

Instrumentation

An HIAC/Royco light blockage counter Model 4100, fitted with a Model 3000 automatic sampler and a Model 1-60 HR60HA sensor, was used. Samples of 1.0 ml were taken. The Coulter Multisizer counter was fitted with a 50-μm-diameter orifice tube. The sampling mode was 0.5 ml and up to 256 channels of discrimination were available. The Sonicor ultrasonic cleaning bath was 1 amp and 30 MH_z in frequency.

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Operational Principles and Limitations of the Coulter and HIAC/Royco Instruments When Applied to Dispersions

Operational principles and limitations of both instruments have been reviewed elsewhere (8,10). The Coulter instrument requires electrolyte and detects particles as equivalent spherical diameters. Within broad limits, particle shape is not therefore an issue with this instrument. As operated in the present investigation, with a 50-μm orifice tube, the instrument will detect particles down to approximately 0.9 μm in spherical diameter, with a maximum size of approximately 30-μm diameter being detected.

The HIAC/Royco instrument is a light blockage device, detecting a cross-sectional area of the profile, and is affected by the shape of the particle to some extent (10,11). Thus, a cylinder 4 μm long and 0.5 μm in diameter, the largest size of an individual *M. bovis* cell (7), could be detected as end-on or lengthwise profiles. In these extreme situations the end-on profile would be reported as a circle of 0.5-μm diameter and the lengthwise presentation as a circle of 1.6-μm diameter. The actual presentation to the sensing zone of the instrument will be an average of these two extremes, 1.05 μm, for a sufficiently large number of counts or samples (12). This maximum size of the organism is close to or just under the effective limit of detection using the HIAC/Royco, most particulate detected by the instrument being aggregates of the primary bacterial particles. The smallest reported size of the organism (2 × 0.5 μm) has an equivalent circle diameter of 0.71 μm, well below the instrument detection limit. The main advantage of the HIAC/Royco instrument is that it does not require electrolyte so that dilutions for counting can be made in water.

Similar calculations for the Coulter instrument show that the maximum size of the organism (4 × 0.5 μm) has an equivalent spherical diameter of 1.22 μm for the large form and 0.35 μm for the small (2 × 0.2 μm) organism, the former well inside the limit of instrument detection and the latter outside. The instrument must be operated in saline and this may suppress surface charges on the surface of cells.

A distribution between cumulative number and diameter conforming to a log-normal law will be linearized when plotted as the cumulative percentage (as a probit) and the corresponding size (13). In this case the HIAC/Royco detects the "size" of the particle as the diameter of a circle of equivalent area and determines the surface/number diameter, *dsn* (8). The advantage of *dsn* is that it can be converted to a specific number per unit volume (*N*), by using the Kapteyn transformation to derive a volume number distribution mean, *dvn* (13),

$$\ln dvn = \ln dsn + 0.5 \ln^2 \sigma_g$$

where ln is the natural logarithm and σ_g is the geometric standard deviation of the number distribution.

Specific number per unit volume =

$$N = \frac{6 \times 10^{12}}{d^3 vn} \text{ particles per cm}^3$$

The specific number per cubic centimeter is equivalent to the total number of organisms per ampoule.

In addition, if a particular instrument is able to count particles at or above its limiting threshold, a plot of the cumulative oversize count against the size threshold will be a sigmoidal, the count per unit volume being asymptotic or relatively linear at the smallest size thresholds. This estimated limiting count, corrected for dilution, should be equivalent to the CFU determined by culture methods, irrespective of any distribution law controlling the actual shape of the sigmoid. The display as part of the Coulter Multisizer data processing unit enables this linear portion of the sig-

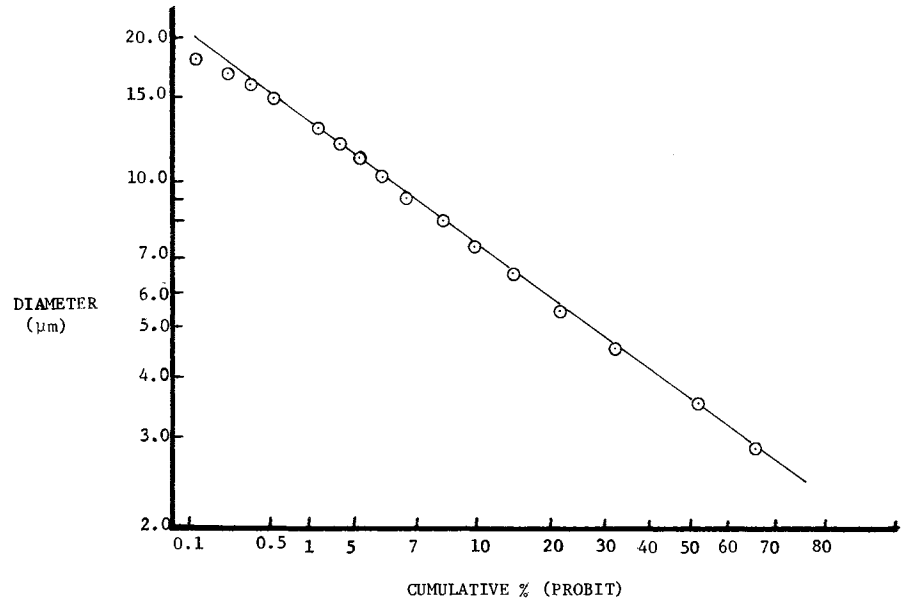

Fig. 1. Cumulative size distribution of BCG, Tice substrain, Lot No. 105171, after 1 min of mild ultrasonic irradiation (experiment similar to that described in text), obtained using the HIAC/Royco instrument and plotted as a log-probit graph. CFU = 6.60 × 10^8; *dsn* = 3.50 μm; σ = 1.75; *N* = 2.78 × 10^{10} organisms/ampoule (see text).

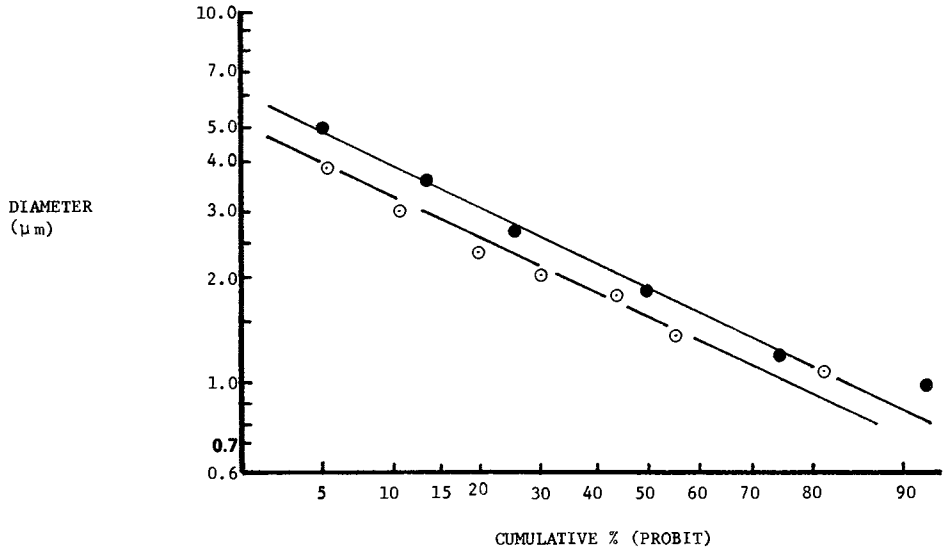

Fig. 2. Cumulative size distributions of BCG, Tice substrain, Lot No. 105171, dispersed in (a) Isoton II (●) and (b) Isoton II containing 5×10^{-4} *M* cetylpyridinium chloride (⊙), measured by a Coulter Multisizer counter fitted with a 50-μm orifice tube (see text).

moid to be determined visually, allowing the particle aggregate count per ampoule to be estimated by appropriate correction for dilution.

RESULTS

Initial investigations using the HIAC/Royco counter indicated that the aggregated organisms in the vaccine approximated to a log-normal (Poisson) size distribution (Fig. 1). An example of a size distribution obtained by Coulter is shown in Fig. (2), confirming the previous observation of a Poisson distribution for the cellular aggregates.

The Effect of Mild Shear on the State of Dispersion

Initially the effect of repeated inspiration of the reconstituted vaccine into and out of a syringe was investigated using a HIAC/Royco instrument since this is the usual method of mixing the ampoule contents. Sampling after groups of four aspirations, for a total of 37, indicated no more than slight random fluctuation in the numbers (Table I).

An ampoule of the same lot was subjected to ultrasonic irradiation by immersion in an ultrasonic cleaning bath filled to a depth of 10 cm with water, sampling every minute. Data are shown in Table II, indicating little, if any, deviation with time of agitation after the first minute. Slight differences between ampoule analysis of the same batch (Tables I and II) may be attributed to ampoule-to-ampoule variation.

The Effect of Surfactants on the State of Dispersion

By using the facility of the Coulter to provide a total

Table I. The Surface Number Mean (μm) Measured by HIAC/Royco and Total Number of Particles per Ampoule Following Repeated Aspiration into and out of a Reconstituted Vaccine Ampoule with an 18-Gauge Needle Attached to a 10-ml Syringe[a]

Aspiration No.	Surface number mean (μm)	σ_g	Total number per ampoule ($\times 10^{10}$)
1	3.75	1.74	2.29
5	3.75	1.69	2.40
9	3.73	1.75	2.30
13	3.65	1.75	2.46
17	3.63	1.73	2.54
21	3.73	1.73	2.34
25	3.75	1.72	2.33
29	3.70	1.76	2.33
33	3.65	1.74	2.48
37	3.73	1.76	2.78
X	3.71 (SE = 1.26%)		2.43 (SE = 6.20%)

[a] BCG, Tice® substrain, Lot No. 105171; CFU (averaged over 12 months, determination every 3 months) = 6.60×10^8.

Table II. The Effect of Mild Ultrasonic Irradiation Obtained by Immersing an Ampoule of Reconstituted BCG Vaccine, Tice® Substrain, Lot No. 105171 [CFU (Table I), 6.60×10^8], in 10 cm Water in an Ultrasonic Cleaning Bath for Various Lengths of Time (HIAC/Royco)

Time (min)	Surface number mean (μm)	σ_g	Total number per ampoule ($\times 10^{10}$)
1	3.80	1.73	2.22
2	3.55	1.78	2.59
3	3.60	1.76	2.53
4	3.60	1.71	2.66
5	3.45	1.82	2.72
6	3.60	1.71	2.66
7	3.50	1.77	2.73
8	3.55	1.78	2.59
9	3.55	1.73	2.72
10	3.60	1.71	2.66
X	3.58 (SE = 2.57%)		2.61 (SE = 5.79%)

Table III. Effect of Sodium Tauroglycolate on the Total Number of Particles per Ampoule of BCG Tice™, Lot No. 105178 (CFU = 2.5 × 10^8), as Measured by Coulter Counter (Dilution in Isoton II, 1:4000)

Concentration of sodium tauroglycolate (mg/ml)	*M*	Estimated number of particles per ampoule (× 10^8)
0	0	6.41
0.01	1.86×10^{-5}	6.48
0.10	1.86×10^{-4}	9.37
0.20	3.72×10^{-4}	11.67
0.50	9.31×10^{-4}	11.91
1.00	1.86×10^{-3}	11.98
2.00	3.72×10^{-3}	12.00

Table IV. Effect of Cetylpyridinium Chloride on the Total Number of Particles per Ampoule of BCG Tice™, Lot No. 105178 (CFU = 2.5 × 10^8), as Measured by Coulter Counter (Dilution in Isoton II, 1:4000)

Concentration of cetylpyridinium chloride (*M*)	Estimated number of particles per ampoule (× 10^8)
0	7.00
2×10^{-4}	10.09
5×10^{-3}	10.43
1.5×10^{-3}	11.53
1.0×10^{-2}	10.73
1.5×10^{-2}	10.00

number in the dispersion, the effect of various surfactants on the total count per ampoule was evaluated (Tables III and IV). In all cases the addition of surfactant resulted in a modest increase in the numbers per ampoule.

DISCUSSION

There may be many orders of magnitude of difference between the CFU counts per ampoule and the actual number of primary particles with sizes approximating 1 μm (Tables I–IV). Both HIAC/Royco and Coulter data suggest that the diluted vaccines contain aggregates of 10-μm equivalent spherical diameter and larger. It is hardly surprising that there is at least a hundredfold difference between theoretical and actual, viable, counts. A single viable organism would grow up to a visible colony during a CFU measurement and this would provide the same result if the single viable cells were attached to an aggregate of nonviable cells.

The surfactant addition produced only a relatively modest increase in the total count. This would suggest that the mechanism for disaggregation may not be entirely electrostatic, resulting from charged groups at the surface of the cell membrane. George *et al.* (14) demonstrated that common species of *Mycobacterium* had amino groups, carboxylic acid, and phosphate groups at the membrane surface. From our own investigation (15) we believe the BCG Tice™ organisms to be similar, with a possibility of some liquid hydrophobic interaction occurring at a greater depth within the surface. It is evident that the magnitude of charge, irrespective of its direction, is insufficient to result in complete dispersion of aggregates to their primary cellular particles. This suggests that the actual particle interaction involves a mechanism that is only partially affected by charge. Hardman and James (16) observed a "slime-like" material at the surface of *Mycobacterium bovis* cells and suggested that this was probably bonding them together. Clearly this cannot be the entire picture since the 1908 observation by Calmette and Guérin (4) that bile caused a considerble improvement in dispersion appears to involve disruption of lipophilic interactions by a wetting mechanism. The mechanism of interaction is evidently complex, with a number of facets operating simultaneously.

Instrumental counting of the *Mycobacterium* suspensions offers the prospect of an improved accuracy of dosage of this vaccine from the point of view of both the producer and the clinical user.

REFERENCES

1. A. Calmette, C. Guérin, and B. Weill-Halle. *Bull. Acad. Med.* 91:787–796 (1924).
2. S. R. Rosenthal. *BCG Vaccine: Tuberculosis-Cancer.* P.S.G., Littleton, Mass., 1980.
3. G. B. Mackaness, D. J. Andair, and P. H. Lagrange. *J. Natl. Cancer Inst.* 51(5):1655–1667 (1973).
4. A. Calmette and G. Guérin. *Compt. Rend. Acad. Sci.* 149:716–718 (1909).
5. J. Ungar, P. W. Muggleton, J. A. R. Dudley, and M. I. Griffiths. *Br. Med. J.* ii:1086–1089 (1962).
6. T. H. Kim. *Appl. Environ. Microbiol.* 34(5):495–499 (1977).
7. B. D. Davis, R. Dalbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood (eds.). *Microbiology,* 2nd ed., Harper and Row, Evanston, Il., 1973, p. 848.
8. T. Allen. *Particle Size Measurement,* 3rd ed., Chapman and Hall, New York, 1981.
9. W. C. McCrone, R. G. Draftz, and J. G. Delly. *The Particle Atlas, 1,* Ann Arbor Science, Ann Arbor, Mich., 1967.
10. M. J. Groves. Proc. P.D.A. Int. Conf., *Liquid-Borne Particulate Inspection and Metrology,* Washington, D.C., 1987, pp. 267–300.
11. T. Barber. Proc. P.D.A. Int. Conf., *Liquid-Borne Particulate Inspection and Metrology,* Washington, D.C., 1987, pp. 317–375.
12. J. Wong and M. J. Groves. Presentation, Fine Particle Society Annual Meeting, San Francisco, July 29, 1986.
13. G. Herdan. *Small Particle Statistics,* 2nd ed., Butterworths, London, 1960.
14. K. L. George, A. T. Pringle, and J. O. Falkinham. *Microbios* 45:199–207 (1986).
15. A. Zhang, M. J. Groves, and M. E. Klegerman. *Microbios* 53:191–195 (1988).
16. L. E. Hardman, and A. M. James. *Microbios Lett.* 13:33–42 (1980).