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Viability Counts on BCG Vaccines for Tumour Immunotherapy: Divergent Effects on Different Growth Media*

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Abstract—*Viable unit determinations have been carried out on nine BCG preparations covering six different strains and using two recognised mycobacterial growth media with or without whole human blood supplement. Viable unit determinations were comparable on all four media for four of the BCG preparations, but the other five, including Connaught, Glaxo and Tice strains, were more fastidious in their growth requirements giving higher viable unit counts on Middlebrook 7H11 medium, or Dubos oleic agar supplemented with whole human blood. This was particularly important with both clinical and experimental preparations of Tice and Glaxo BCG, where viable unit counts were increased up to 30-fold with more enriched media. These findings indicate that BCG doses, such as used in tumour immunotherapy protocols, compared on viable unit bases may be erroneous unless optimum growth conditions are achieved for the individual vaccines.*

INTRODUCTION

BCG is widely used in immunotherapy of experimental animal tumours and is being incorporated into clinical immunotherapy trials [1, 2]. There are, however, many different strains and preparations of the vaccine currently in use and the dose of BCG is frequently expressed in viable units. This is not an ideal situation, since vaccines will vary widely in the proportion of viable to dead organisms and in the size of bacterial aggregates making up a viable unit [1]. Furthermore, since BCG strains are known to vary in biochemical growth requirements [3] the results of viable unit determinations will depend upon the composition of growth media employed. This will be particularly important if viability determinations are carried out on a range of BCG strains, since medium suitable for one strain may not be optimal for a more fastidious strain. Recent studies in this laboratory to compare the relative antitumour effects of a number of different BCG preparations [4] necessitated such determinations of viable unit concentrations and the present paper describes the divergent results obtained on different growth media.

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MATERIALS AND METHODS

BCG preparations

Glaxo freeze-dried percutaneous vaccine was a gift from Glaxo Laboratories, Greenford, Middlesex, England.

Frozen suspensions of Tice (TMC1032), Montreal (TMC1012), Pasteur (TMC1011), Phipps (TMC1029) and Glaxo (TMC1032) strains of BCG were purchased from the Trudeau Institute, Saranac Lake, New York, U.S.A., shipped in dry ice and stored at −80°C. These preparations are suspensions for experimental immunotherapy and are not intended for clinical use.

Freeze-dried Tice BCG was a gift from Dr. R. G. Crispin, University of Illinois, Chicago, Ill., U.S.A.

Freeze-dried Connaught BCG was a gift from Professor S. Landi, Connaught Medical Research Laboratories, Willowdale, Ontario, Canada.

Liquid suspension Pasteur BCG, as

Immuno BCG Pasteur F, was purchased from Institut Pasteur, Paris, France.

Growth media

Mycobacterial growth media were purchased from Difco (Detroit, Michigan, U.S.A.). Dubos oleic agar base was supplemented with Dubos oleic albumin complex and 50 U/ml penicillin. Mycobacteria 7H11 agar was supplemented with Middlebrook OADC enrichment. Both media were prepared to Difco's instructions and dispensed in 10–15 ml amounts in 9 cm Petri dishes. Blood enriched media were prepared by the addition of 5% (v/v) freshly drawn whole human blood to the complete media at 55°C immediately before dispensing.

Viable unit counts

BCG vaccines were thawed from the frozen state or reconstituted from freeze-dried form as directed by the supplier and serial dilutions made in glycerol:Triton solution (4% glycerol in 0.025% Triton WR1339 in pH 7.2 phosphate buffered saline, sterilised by autoclaving). Twenty microlitre amounts of three serial dilutions were plated simultaneously onto two or more different media in replicates of eight with a Finnpipette (Finnpipette, Helsinki, Finland), spread over an area of approximately 5 cm^2 by gentle rotation of the dishes and allowed to dry at room temperature. Inverted Petri dishes were incubated in sealed polythene bags at 37°C, in air, for 20 days. Macroscopically visible discrete surface colonies were counted for vaccine dilutions giving 5–100 colonies.

RESULTS

Table 1 shows the results of viable unit concentration determinations of the 14 batches of vaccine of the nine preparations examined. Essentially, four out of nine preparations gave virtually identical viable unit counts on both Dubos and Middlebrook media, with or without blood supplementation. Five preparations, those of Glaxo, Connaught and Tice BCG's, gave divergent results on the different media. Thus, three preparations, those of Phipps, Montreal and Pasteur BCG's, from the Trudeau mycobacterial collection showed no significant differences in viable counts on the four media, and Pasteur BCG from the Pasteur Institute (Pasteur F) similarly gave comparable results on Dubos medium with or without blood, although slightly reduced counts on Middlebrook 7H11. However, Tice BCG, both from the Trudeau Institute (TMC1032) and from Illinois was more fastidious in its growth requirements. The counts on Dubos medium alone were $1.3–2.0 \times 10^7$/ml and 1.0×10^7/ml for the two preparations, but blood supplementation in-

*Table 1. Viable unit determinations on BCG vaccines**

Vaccine	Suppliers lot No.	Dubos oleic agar +Blood	Dubos oleic agar −Blood	Middlebrook 7H11 +Blood	Middlebrook 7H11 −Blood
Glaxo	P625	1.3×10^8	3.3×10^7	1.9×10^8	1.7×10^8
	P706	8.6×10^7	6.0×10^7	1.3×10^8	8.7×10^7
	P686	8.2×10^7	5.4×10^7	1.2×10^8	9.0×10^7
TMC1011 (Pasteur)	A4	1.5×10^8	1.4×10^8	1.4×10^8	1.7×10^8
TMC1012 (Montreal)	A2	2.0×10^8	1.6×10^8	2.0×10^8	1.8×10^8
	S1	1.0×10^8	7.0×10^7	1.0×10^8	8.0×10^7
TMC1029 (Phipps)	11	2.2×10^8	2.0×10^8	3.5×10^8	2.0×10^8
	12	1.7×10^8		1.6×10^8	1.3×10^8
TMC1032 (Tice)	4	1.0×10^8	1.3×10^7	1.9×10^7	1.1×10^8
		1.2×10^8	2.0×10^7	—	—
TMC1024 (Glaxo)	A3	2.3×10^6	9.0×10^5		5.6×10^6
Tice	Ill105 (S) 19	2.4×10^8	1.0×10^7	4.1×10^8	3.8×10^8
		3.8×10^8	1.0×10^7	3.5×10^8	2.9×10^8
Connaught	291–1	2.5×10^7	1.7×10^7	5.5×10^7	5.0×10^7
		2.5×10^7	3.0×10^7	5.0×10^7	5.0×10^7
Pasteur-F	30	5.0×10^8	4.5×10^8	3.5×10^8	2.6×10^8
	12	2.7×10^8	2.6×10^8	—	1.3×10^8

*Expressed as viable units/ml of vaccine thawed or reconstituted to manufacturers' instructions.

creased this up to 30-fold to 1.0×10^8/ml and 3×10^8/ml. Counts on Middlebrook medium alone were comparable to those on Dubos medium with blood, but addition of blood to Middlebrook medium did not markedly increase the count. Similar increased counts on more enriched media were seen with both the clinical vaccine and the experimental preparation of Glaxo BCG. For example, the Trudeau preparation (TMC1024) showed a 6-fold increase in viable unit count on Middlebrook 7H11 medium, without blood, compared with Dubos oleic agar. With Connaught BCG, viable counts on Middlebrook medium with or without blood were twice those on Dubos medium, but here blood supplementation of the Dubos medium did not increase colony formation.

DISCUSSION

The pathogenic mycobacteria are well known to be fastidious in their growth requirements generally requiring media with blood, serum or egg supplementation. Many media are available for isolation and propagation of these organisms often based on those of Dubos and Middlebrook and Löwenstein and Jensen and these media have also been used for culture and viability determination of BCG. The present work arose out of a programme to compare the antitumour properties of BCG strains and preparations which necessitated viable unit determinations and clearly demonstrates that such viable unit determination may give erroneous results if a suitably enriched medium for the strains under test is not used.

The dose of BCG administered in both experimental and clinical trials is often inadequately documented, it is often given as either numbers of units, numbers of viable units, number of organisms, number of viable organisms or weight of organisms and it is generally impossible to compare precisely one investigator's dose with another, bearing in mind the wide range of viabilities of vaccines, and the size of bacterial aggregates making up 'a unit'. When the dose is given in viable units it is frequently based on the manufacturer's estimate and it must be assumed that the viability count has been carried out on medium suitable for that particular strain. When individual workers carry out viability counts the media used cover a number of types (e.g. Middlebrook 7H10 [5]; Youman's medium [6]; Dubos oleic agar alone [7, 8] or supplemented with horse blood [9]; Löwenstein–Jensens medium [10, 11]), and it is not possible to tell whether these are optimum conditions for the particular BCG strain under test. Comparative viability counts of BCG vaccine on different media, as reported here, seem poorly documented, although Lugosi [7] examined ten vaccines using Löwenstein–Jensens medium and oleic acid agar supplemented with blood and reported wide discrepancies in viable unit determinations depending upon the medium, the dilution technique and the individual operator. In addition, Gallagher and Horwill [12] found that enrichment of Middlebrook 7H11 medium with 10% fresh bovine serum increased colony counts of "field isolates" of *Mycobacterium bovis* by about 7-fold compared with 7H11 alone, and this enriched 7H11 medium gave two to three times the colony formation of Tarshis, Löwenstein–Jensen or Stonebrink's medium. While the present work has not attempted a comprehensive analysis of conditions for BCG viability counting it does indicate that divergent results may be obtained on different media and that unless optimum culture conditions are achieved the stated dose of BCG used in immunotherapy regimes, already only poorly documented, may be inaccurate. With the vaccines examined in the present work, the indication is that Middlebrook 7H11 medium with further blood enrichment is the most reliable single medium for comparative viability counting.

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