Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1967, 37, 967-972

Lysed BCG Vaccines

1. Observations on Optimal Conditions for BCG Growth and Lysis *

H. SATO, B. B. DIENA & L. GREENBERG

As a first step towards the production of a lysed BCG vaccine that would have reduced toxicity and allergenicity and yet be immunogenic, the growth and lysis of BCG bacilli under strictly controlled conditions have been studied.

BCG grown in both Dubos and Aldridge liquid media showed an arithmetic linear growth, preceded by a very short logarithmic phase. This suggested that some of the cell population became metabolically inactive at a very early stage, possibly owing to suboptimal conditions of growth.

Glycine, lysozyme and lithium chloride initiated lysis of BCG growth in the aforementioned media 24–48 hours after inoculation. After 2 weeks' incubation approximately 50 % of the original BCG culture was lysed and the viable count had fallen 1000-fold. The autolytic pattern of BCG in a nitrogen-deficient, chemically defined Aldridge medium is also described.

Previously (Sato et al., 1966) we reported the lysis of BCG cells under certain conditions of metabolic disturbance: glycine, lysozyme and lithium chloride were found to be essential for such lysis. These findings suggested the possibility of producing a lysed BCG vaccine possessing reduced toxicity and allergenicity but retaining immunogenicity. In recent years, a number of workers have attempted to prepare such vaccines (Weiss et al., 1956; Crowle & Teranuma, 1964; Ribi et al., 1966; Youmans & Youmans, 1966) by the purification of BCG cells.

The purpose of the investigation reported here was to study the growth and lysis of BCG bacilli under strictly controlled conditions (this paper) and then to assess the immunogenicity of these experimental lysates (Sato et al., 1967; following paper).

MATERIAL AND METHODS

Strain

The Glaxo strain of BCG was employed throughout these experiments.

Media

The following media were used:

(1) DOA: Dubos oleic-acid–agar medium.

(2) DL: Dubos Tween-80–albumin liquid medium (Dubos & Middlebrook, 1947).

(3) DLGLL: Dubos Tween-80–albumin liquid medium (DL) with glycine (1.5%), lysozyme (100 μg/ml) and LiCl (0.1%).

(4) DLS: Dubos Tween-80–albumin liquid medium (DL) with 0.17 M sucrose (with either bovine serum albumin (BSA) or human serum albumin (HSA)).

(5) DLSGLL: Dubos Tween-80–albumin liquid medium (DL) with sucrose (0.17M), glycine (1.5%), lysozyme (100 μg/ml) and LiCl (0.1%).

(6) ACN: Aldridge chemically defined medium (Aldridge et al., 1959; Sato et al., 1966).

(7) ACNGLL: Aldridge medium (ACN) with glycine (1.5%), lysozyme (100 μg/ml) and LiCl (0.1%).

(8) AC–N: Nitrogen-deficient Aldridge medium (Sato et al., 1966).

Culture studies

A 7-day culture of BCG grown in DL was used as inoculum; 5 ml were used to inoculate Roux flasks containing DOA which were incubated for 1 week at 37°C. The resultant growth was then harvested and washed once with physiological saline. The washed cells were used for inoculation of all the experimental media used.

* From the Biologics Control Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

Changes in the turbidity of BCG cultures grown in Nepheloculture flasks were determined by reading in a Coleman Model 9 Nephelo-colorimeter at 450 nm. Viable counts were made in Löwenstein-Jensen plates.

RESULTS

Dubos liquid media (DLS and DL) and Aldridge chemically defined medium (ACN) were used in our efforts to determine the best physiological conditions for growth of BCG bacilli. Diverse patterns of growth were obtained when varying inocula were used; representative results are shown in Table 1 and Fig. 1. From these data, it is apparent that growth of BCG in the previously mentioned media follows a pattern somewhat different from that usually displayed by bacteria. After a very short exponential phase (1–2 days), an arithmetic linear growth ensues for 10–12 days. This type of linear growth was further studied by comparing the turbidity of BCG cultures with viable counts. Fig. 1 shows that increases in optical density values (6–7 times the original inoculum) were not propor-

TABLE 1. GROWTH OF BCG, GLAXO STRAIN, IN VARIOUS LIQUID MEDIA AS MEASURED BY OPTICAL DENSITY

Day	Medium			
	DL[a] (optical density)	ACN[b] (optical density)	DLS (HSA)[c] (optical density)	DLS (BSA)[d] (optical density)
0	0.045	0.040	0.060	0.060
1	0.065	0.080	0.090	0.075
2	0.100	0.090	0.100	0.085
3	0.140	0.110	0.135	0.110
5	0.190	0.185	0.180	0.155
6	0.205	0.195	0.190	0.165
7	0.220	0.225	0.210	0.185
8	0.250	0.235	0.220	0.200
10	0.260	0.280	0.230	0.210
14	0.300	0.320	0.285	0.240

[a] Dubos liquid medium (bovine serum albumin).
[b] Complete Aldridge medium.
[c] Dubos liquid medium with sucrose (0.17 M) and human serum albumin.
[d] Dubos liquid medium with sucrose (0.17 M) and bovine serum albumin.

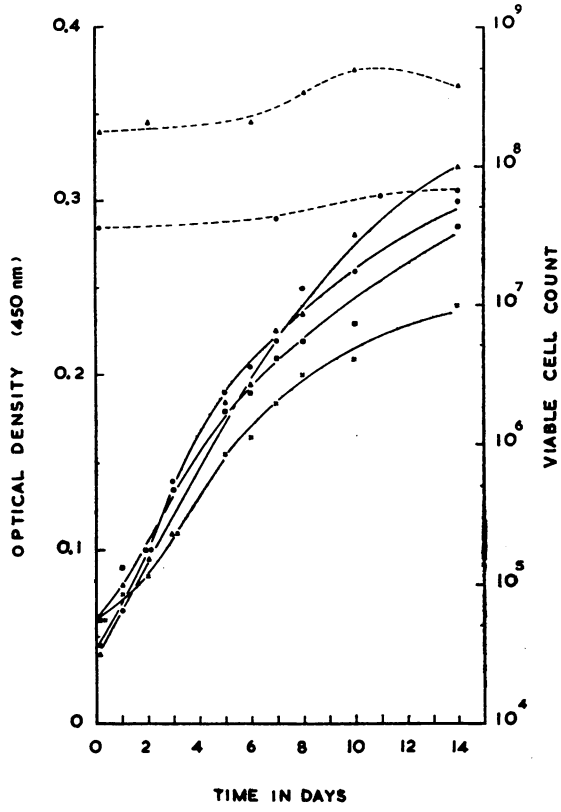


FIG. 1
PATTERN OF BCG GROWTH IN DIFFERENT MEDIA

TABLE 2. OPTICAL DENSITY AND VIABLE CELL COUNTS OF BCG, GLAXO STRAIN, IN MEDIA CONTAINING GLYCINE, LYSOZYME AND LITHIUM CHLORIDE

Day	Medium					
	DLSGLL[a]		DLGLL[b]		ACNGLL[c]	
	Optical density	Viable cell count	Optical density	Viable cell count	Optical density	Viable cell count
0	0.280	9×10^8	0.330	9×10^8	0.330	9×10^8
1	0.330		0.385		0.350	
2	0.290	7.5×10^7	0.390	1.1×10^8	0.330	3.8×10^8
3	0.260		0.360		0.290	
4	0.205		0.310		0.270	
5	0.180	1.1×10^7	0.290	2.8×10^7	0.250	1×10^8
6	0.150		0.250		0.240	
7	0.135	3.5×10^6	0.220	5×10^6	0.200	4.5×10^6
10	0.120		0.210		0.195	
14	0.100	$<10^3$	0.190	2.8×10^5	0.195	5×10^5

[a] Dubos Tween-80–albumin liquid medium with sucrose (0.17 M), glycine (1.5 %), lysozyme (100 μg/ml) and lithium chloride (0.1 %).
[b] Dubos Tween-80–albumin liquid medium with glycine (1.5 %), lysozyme (100 μg/ml) and lithium chloride (0.1 %).
[c] Aldridge chemically defined medium with glycine (1.5 %), lysozyme (100 μg/ml) and lithium chloride (0.1 %).

tional to the increase in the living cell count, especially in Dubos media. After 2 weeks, turbidity continued to rise in all media while the viable count steadily declined, suggesting that at least a portion of the living cells continued to multiply while an increasing number of the cell population was either dead or metabolically inactive.

Lysis of BCG cultures in the same media, with the addition of glycine, lysozyme and lithium chloride, was studied, again comparing optical density curves with viable cell counts. The same pattern of lysis occurred in all media. After an initial period of metabolic activity (1–2 days), the turbidity began to diminish and continued for 10–11 days. Table 2 and Fig. 2 show these results. Although there was an increase in the initial turbidity, viable counts immediately diminished logarithmically. In ACNGLL, from a plateau of 9×10^8 cells/ml, the count by the fifth day was 1×10^8 cells/ml, and after 14 days fell to 5×10^5 cells/ml. In DLGLL from a maximum of 9×10^8 cells/ml, the count dropped to 2.8×10^5 cells/ml at 14 days; in DLSGLL it was less than 1000 cells/ml in the same time interval. Viable cells left in ACNGLL after 2 weeks were 1/1800th of the original inoculum, in DLGLL 1/3200th and in DLSGLL 1/90 000th, while turbidity values decreased by only about 50%.

Since a BCG vaccine prepared in a chemically defined medium would obviously be advantageous, the nitrogen-deficient Aldridge medium (AC–N) was investigated. Owing to the fact that lysis of BCG cultures in AC–N medium invariably occurs after short incubation, glycine, lysozyme and lithium chloride were not added. Fig. 3 and Table 3 show these results. Turbidity increased for 6 days, followed by lysis, which attained its maximum (50% of maximum turbidity) in 14 days. Contrary to expectations, the viable cell count began to decrease from the 2nd day of incubation, falling from an initial level of 1.75×10^8 cells/ml to 2×10^4 cells/ml in 14 days.

DISCUSSION

These studies were designed to develop a medium which would permit the best lysis of BCG suspensions intended for use as BCG vaccine. These investigations, on the other hand, raised some problems regarding the physiological properties of BCG bacilli. Basic media, such as DL, DLS and ACN, have all the ingredients necessary for good growth and yet the arithmetic linear growth of BCG bacilli after a very short logarithmic phase seems to indicate suboptimal growth conditions. Fisher et al. (1951) have reported a similar growth pattern for

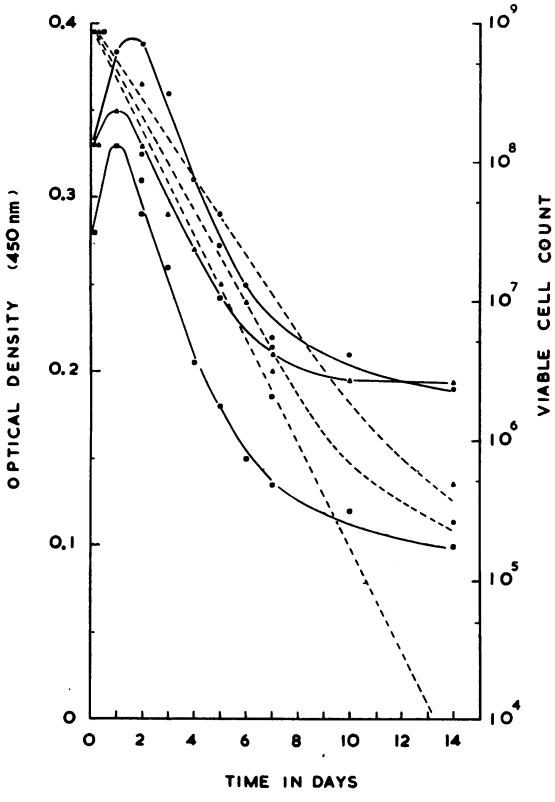


FIG. 2
PATTERN OF LYSIS OF BCG IN MEDIA CONTAINING GLYCINE, LYSOZYME AND LITHIUM CHLORIDE

o——o DLGLL (BSA), optical density.
□——□ DLSGLL (HSA), optical density.
△——△ ACNGLL, optical density.
●----● DLGLL (BSA), viable cell count.
■----■ DLSGLL (HSA), viable cell count.
▲----▲ ACNGLL, viable cell count.

Mycobacterium tuberculosis strain H37Rv. Our results, using optical density values as well as living counts (taking into consideration the limitations of plate-counting methods), underline the need for basic studies of the physiology of mycobacteria, as well as of growth requirements *in vitro* and *in vivo*. The pattern of arithmetic linear growth, which is typical for BCG in our media, suggests that a number of BCG cells become metabolically inactive at a very early stage and consequently do not multiply.

As a general observation, because of lack of parallelism between turbidity and viable count, we feel that cautious interpretation must be given to reports in which turbidity values alone are considered. This is shown very clearly by our results in the Aldridge nitrogen-deficient medium (AC–N), where turbidity increased for the first 6 days of incubation, while the viable count decreased from the very beginning.

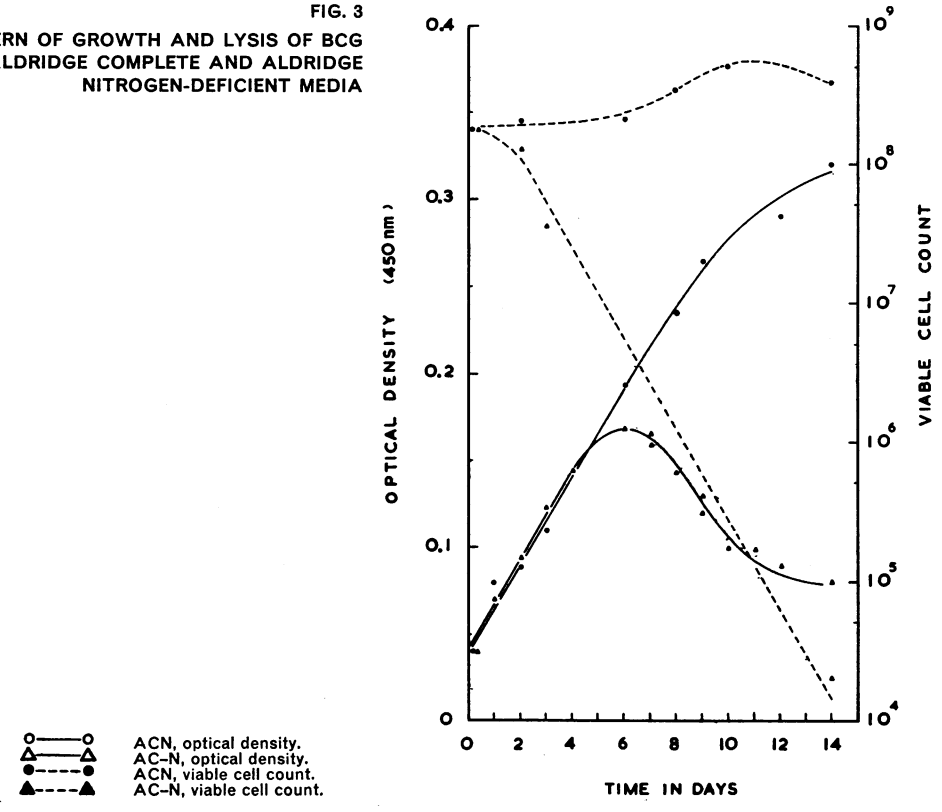
We have found previously that glycine, lysozyme and lithium chloride induce lysis of BCG suspensions (Sato et al., 1966). Lysis occurs after an initial

TABLE 3
OPTICAL DENSITY AND VIABLE CELL COUNTS OF BCG, GLAXO STRAIN, GROWN IN ALDRIDGE COMPLETE AND ALDRIDGE NITROGEN-DEFICIENT MEDIA

Day	ACN [a]		AC–N [b]	
	Optical density	Viable count	Optical density	Viable count
0	0.040	1.75×10^8	0.040	1.75×10^8
1	0.080		0.070	
2	0.090	2×10^8	0.095	1.3×10^8
3	0.110		0.125	3.5×10^7
4			0.145	
6	0.195	2×10^8	0.170	
7	0.225		0.160	1.1×10^8
8	0.235	3.3×10^8	0.145	
9	0.270		0.130	3.1×10^5
10		5×10^8	0.100	
11				1.75×10^5
12	0.285		0.090	
14	0.320	3.7×10^8	0.080	2×10^4

[a] Aldridge chemically defined medium.
[b] Nitrogen-deficient Aldridge medium.

FIG. 3
PATTERN OF GROWTH AND LYSIS OF BCG IN ALDRIDGE COMPLETE AND ALDRIDGE NITROGEN-DEFICIENT MEDIA



turbidity increase which may perhaps be due to increased size of bacilli, since at this time the viable count is not altered. After 2 weeks, approximately 50% of the original BCG suspension is lysed: the number of living or metabolically active bacilli at this time is indeed very low. Perhaps the formation of osmotically sensitive spheroplasts, known to occur in these media (Sato et al., 1966), explains the logarithmic loss in viable count accompanied by a moderate loss in turbidity.

Glycine, lysozyme and lithium chloride seem to induce a basic change in the metabolic behaviour of BCG cells, causing a marked degeneration in their reproductive capabilities. Since these vaccines do not concurrently lose their ability to protect animals against experimental tuberculosis (Sato et al., 1967), it is possible that they contain degenerate or dormant cells which have lost only the ability to multiply in artificial media but not *in vivo*. This is especially important since the protective capacity of BCG vaccines is generally considered in terms of viable cells. Our criteria, therefore, for assessing these vaccines must be revised.

Finally, there is a similarity in the pattern of lysis on the one hand by the inducers glycine, lysozyme and lithium chloride (DLGLL, DLSGLL and ACNGLL media) and on the other by nitrogen starvation (AC–N) following the early period of incubation, during which nitrogen depletion occurs. While metabolic disturbance could be implicated in either of the resultant lysates, results to be reported in the subsequent paper (Sato et al., 1967) regarding loss of allergenicity ensuing from the inducers indicate that the basic mechanism is different.

ACKNOWLEDGEMENTS

Thanks are due to Dr E. T. Bynoe, Director, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada, and to Mr J. L. Byrne, Assistant Chief, Biologics Control Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada, for their suggestions in the preparation of this manuscript.

RÉSUMÉ

On s'est efforcé, en étudiant les modalités de la croissance et de la lyse d'une souche de BCG dans des conditions strictement contrôlées, de découvrir un milieu de culture favorable à la production de vaccin lysé.

L'expérimentation a porté sur le milieu liquide de Dubos, avec ou sans saccharose (milieux DLS et DL) et sur le milieu chimiquement défini d'Aldridge (milieu ACN). Dans ces milieux de culture, le développement du BCG présente des aspects assez différents de celui des bactéries ordinaires. Après une phase logarithmique très courte (1-2 jours), la croissance s'effectue selon une fonction arithmétique linéaire pendant 10 à 12 jours, ce qui semble indiquer que nombre de germes perdent très précocement toute activité métabolique et cessent de se multiplier.

Si l'on ajoute aux milieux liquides de Dubos et d'Aldridge de la glycine, du lysozyme et du chlorure de lithium (milieux DLGLL, DLSGLL et ACNGLL), on observe, après une période initiale d'activité métabolique de 24-48 heures, une lyse avec diminution de la turbidité qui se poursuit pendant 10-11 jours. A ce moment, 50% environ de la culture d'origine est lysée et la numération des particules viables donne des chiffres mille fois inférieurs aux valeurs initiales.

On a également étudié la possibilité d'obtenir un vaccin BCG lysé sans recourir à la glycine, au lysozyme et au chlorure de lithium. Dans le milieu d'Aldridge pauvre en azote (milieu AC-N), la croissance du BCG se poursuit pendant 6 jours, puis survient une lyse qui est maximale après 14 jours.

On a noté une similitude entre les caractéristiques de la lyse induite par la glycine, le lysozyme et le chlorure de lithium et la lyse provoquée par le manque d'azote. Si dans les deux cas, le phénomène résulte de perturbations métaboliques, le mécanisme de base apparaît cependant différent: la glycine, le lysozyme et le chlorure de lithium entraînent en effet une diminution du pouvoir allergisant et immunogène du vaccin, ainsi que l'ont montré des recherches décrites ailleurs.

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