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Host-Parasite Relationships in Experimental Airborne Tuberculosis II. Reproducible Infection by Means of an Inoculum Preserved at −70 C

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The use of an inoculum preserved at low temperature for the infection of guinea pigs by the respiratory route was evaluated. In a preliminary study with *Mycobacterium bovis* (BCG), some of the conditions required for maximal recovery of viable cells stored at low temperature were examined. Survival of BCG was decreased by rapid freezing to −70 C and by storage at −20 C, but there was no decrease when BCG was frozen slowly and stored at −70 C or −196 C. In a subsequent study, the effect of storage at −70 C on viability and infectivity of *M. tuberculosis* (H37Rv) was considered. There was no loss of viability of H37Rv cells suspended in Dubos broth and stored 1 year at −70 C. This suspension showed no loss of infectivity as assessed by the number of primary pulmonary lesions initiated in guinea pigs. Constant viability and infectivity of a suspension stored at low temperature assures the reproducibility of the amount of infection and facilitates comparisons between experiments. This advantage, as well as others, of storage at low temperature are discussed.

In experiments in which host response is critically dependent upon the level of infection of animals, a technique that will produce a precise level of infection is essential. Photometric, nephelometric, and gravimetric methods are not satisfactory for this purpose, because they estimate only the number of particles in a suspension without providing information on their viability. Glover (3) suggested preservation of mycobacterial inocula at low temperature when animals are to be inoculated with a small number of bacilli with maximal reproducibility. Storage at low temperature is frequently employed for preserving microorganisms used in microbiological assay (8, 12-14).

This paper describes an investigation designed to evaluate the use of an inoculum preserved at low temperature for the infection of guinea pigs by the respiratory route. A preliminary study with *Mycobacterium bovis* (BCG) was directed toward an examination of some of the conditions required for maintaining maximal viability of cells stored at low temperatures. A later study considered the effect of storage at −70 C on viability and infectivity of *M. tuberculosis* (H37Rv)

MATERIALS AND METHODS

M. tuberculosis (H37Rv; obtained from the Trudeau Institute, Saranac Lake, N.Y.) and *M. bovis* (BCG; obtained from the Tice Laboratories, Chicago, Ill.) were maintained as surface pellicles on Sauton medium. Cells were prepared for storage as follows: a loopful of a 7- to 10-day-old pellicle was transferred to 5 ml of Dubos Broth (Difco) and was homogenized with a Teflon-glass homogenizer; 0.2 ml of the resulting suspension was then inoculated into 10 ml of Dubos Broth in 3-oz (ca. 90-ml) prescription bottles. After incubation at 37 C, four cultures were combined and centrifuged at 700 × *g* for 20 min. The cells were washed twice with Dubos Broth and resuspended in a volume of broth equal to that of the combined original cultures. These cells were dispersed with a Teflon-glass homogenizer and were filtered through a membrane filter (Millipore Corp., Bedford, Mass.) of 5-μ pore size to provide a suspension of single bacilli. With some preparations, difficulty was experienced in passing a sufficient quantity of bacilli through the 5-μ pore size membrane filter. Recently, suspensions of predominantly single cells have been obtained after filtration through 8-μ pore size membranes.

Methods for bacterial enumeration. The number of viable mycobacteria was determined by counting the colonies on the surface of Oleic Acid Albumin Agar (Difco) to which 0.01% cycloheximide (The Upjohn Co., Kalamazoo, Mich.) was added to inhibit molds. Two methods of inoculation were used. The first consisted of spreading 0.1-ml samples of selected dilutions over the surface of 3 to 10 replicate plates; the second, recommended by Fenner (2), consisted of inoculating plates with 25 replicate drops (0.025 to 0.03 ml each)

of appropriate dilutions. All plates were sealed in plastic bags during incubation, and colonies were counted after 14 to 21 days at 37 C.

Preparation of BCG cells for storage. Dubos Broth cultures were incubated for 5 days prior to the steps of preparation described above. The single cell suspension was divided into three parts, each diluted 10-fold in: Dubos Broth, Dubos Broth with 5% glycerol, or Dubos Broth with 5% dimethyl sulfoxide (DMSO). Samples of 1 ml of each suspension were sealed in sterile ampoules.

Preparation of H37Rv cells for storage. Dubos Broth cultures were incubated for 7 days prior to the preparation of a suspension of single cells as described above. Samples (1.5 ml) were sealed in sterile ampoules.

RESULTS

Viability of BCG cells after storage. An early experiment evaluated conditions required to maintain mycobacteria at a constant viability during long periods of storage. The requirement for a protective additive, the rate of freezing, storage at different subzero temperatures, and the duration of storage were studied for their effect on viability. Washed cells of BCG were suspended in Dubos Broth alone or with either 5% glycerol or 5% DMSO as protective additives. Ampoules of each of the three suspensions, frozen rapidly by immersing in a mixture of alcohol and solid carbon dioxide, were stored at −20, −70, and −196 C. Other ampoules of the three suspensions were frozen slowly by placing them directly into −20 and −70 C deepfreezes, and those to be stored at −196 C were frozen first in a Nitro Freeze (Canalco Corp., Bethesda, Md.) at a controlled rate of 1 degree per min to −60 C.

Ampoules of each of the three suspensions, frozen quickly or slowly and kept at the three temperatures, were removed after various intervals of storage. The contents of the ampoules were thawed quickly by immersion in water at 37 to 40 C, and the number of surviving mycobacteria was determined. Rapid freezing in alcohol-solid carbon dioxide reduced the number of viable BCG cells (Table 1). Glycerol and DMSO in the concentrations used did not protect BCG cells against damage from rapid freezing. After slow freezing, storage at −20 C resulted in a 50% loss of viability in the preparation without glycerol or DMSO. When BCG cells were frozen slowly and stored at −70 or −196 C, the use of additives was unnecessary to maintain viability.

When BCG was frozen rapidly and stored at −20 or −70 C, early samples of all three suspending fluids contained not only fewer cells, but the survivors produced colonies which measured 3 to 10 times their normal size. This altered growth characteristic did not occur in samples of the cells frozen rapidly and stored at −196 C, nor in any of the samples of cells frozen slowly. In addition, this change in colony form became progressively less marked with time of storage.

Conditions for storage of H37Rv cells. From the preliminary experiment with BCG cells, conditions for storage were selected for a more extensive study with H37Rv cells. The suspension in Dubos Broth was frozen slowly by placing ampoules in a −70 C deepfreeze. This temperature was selected because of the preliminary experiment with BCG cells showed no advantage for storage at −196 C.

TABLE 1. *Viability of BCG cells suspended in Dubos Broth and stored at low temperature*

Storage		No. of viable units per ml with additive of					
		None		5% Glycerol		5% Dimethyl sulfoxide	
Temp (C)	Duration	Slow[b]	Quick[b]	Slow	Quick	Slow	Quick
−20 C	1 day	65×10^4	25×10^4	74×10^4	11×10^4	60×10^4	33×10^4
	1 week	39×10^4	34×10^4	58×10^4	29×10^4	58×10^4	25×10^4
	16 weeks	30×10^4	13×10^4	62×10^4	15×10^4	84×10^4	36×10^4
−70 C	1 day	70×10^4	42×10^4	54×10^4	42×10^4	61×10^4	42×10^4
	1 week	56×10^4	44×10^4	81×10^4	45×10^4	58×10^4	36×10^4
	16 weeks	88×10^4	70×10^4	104×10^4	41×10^4	62×10^4	30×10^4
−196 C	1 day	65×10^4	32×10^4	77×10^4	31×10^4	59×10^4	34×10^4
	1 week	60×10^4	37×10^4	71×10^4	35×10^4	51×10^4	37×10^4
	16 weeks	58×10^4	52×10^4	63×10^4	47×10^4	41×10^4	54×10^4

[a] Number of viable units per milliliter of the original suspension before freezing: no additive, 60×10^4; glycerol, 82×10^4; and dimethyl sulfoxide, 60×10^4.

[b] Refers to rate of freezing.

Viability of H37Rv cells. After certain intervals, indicated in Table 2, ampoules of the H37Rv suspension were removed from storage and thawed quickly, and the number of viable units was determined. During storage for 11 months at −70 C, H37Rv cells suspended in Dubos Broth showed no loss of viability. No change in growth characteristics of H37Rv cells was observed during this period.

Infectivity of H37Rv cells. After storage at low temperatures for long periods of time, cultures of some microorganisms contain cells which, although viable, were demonstrated by Straka and Stokes (11) to exhibit varying degrees of metabolic injury. If extended storage at −70 C injured H37Rv cells metabolically, their infectivity might be lowered. This could result in an unpredictable level of infection.

To examine this possibility, suspensions stored from 2 to 11 months were thawed quickly for infection of guinea pigs under conditions described previously (7). At each interval, groups of 5 to 20 guinea pigs were infected by the respiratory route with the undiluted suspension or with a suspension diluted 5- or 10-fold in Dubos Broth. The amount of infection was determined 5 weeks after exposure by counting primary pulmonary lesions found on the surface of the lungs at autopsy (7). Results (Table 3) indicate that infectivity of the suspension was constant during the period of the study. The mean numbers of primary pulmonary lesions developing in two groups of guinea pigs infected with undiluted suspension were 50 and 55. The mean number of lesions developing in one group infected with a 5-fold dilution was 11, and, for two groups exposed to a 10-fold dilution, the mean numbers of lesions were 5 and 8. The values in the last column in Table 3 were obtained by dividing the number of bacilli per milliliter of nebulizer fluid by the number of lesions that resulted. The data indicate that one lesion develops for each 5,000 to 7,000 organisms present in the nebulizer.

TABLE 2. *Viability of H37Rv cells after storage at −70 C*

Duration of storage	No. of viable units per ml ±SD
Original suspension, not frozen	$35 \pm 10 \times 10^4$
2 weeks	$39 \pm 6 \times 10^4$
1 month	$34 \pm 7 \times 10^4$
2 months	$38 \pm 6 \times 10^4$
4 months	$38 \pm 4 \times 10^4$
5 months	$33 \pm 3 \times 10^4$
8 months	$38 \pm 6 \times 10^4$
11 months	$41 \pm 8 \times 10^4$

TABLE 3. *Infectivity of H37Rv cells after storage at −70 C*

Duration of storage (months)	Dilution of frozen suspension	No. of guinea pigs	No. of primary pulmonary lesions ±SD	No. of viable units per ml of nebulizer suspension per lesion
2	Undiluted	5	55 ± 19	6,300
4	Undiluted	5	50 ± 10	7,500
5	1:5	14	11 ± 5	5,000
9	1:10	20	5 ± 2	4,700
11	1:10	14	8 ± 3	5,100

DISCUSSION

Species of microorganisms differ greatly in the extent of damage they sustain from storage at low temperatures. For example, Bridges (1) found that 93.9% of *Staphylococcus aureus* survived the same fast-freeze, fast-thaw procedure which reduced the viability of *Achromobacter fischeri* to less than 0.1%. Species of microorganisms differ also in the conditions required for survival of a maximal number of cells. Important relationships between the rates of freezing and thawing and the suspending fluid have been pointed out (1, 6). For example, survival of a *Pseudomonas* sp. ranged from 4.7 to 82%, depending upon the conditions used (1).

The protective action of glycerol is quite general, permitting freezing and thawing of some microorganisms with nearly 100% survival. When glycerol is used, the rates of freezing and thawing become less critical (1).

Although low temperatures have been used to preserve mycobacteria, there are few published data to indicate conditions which permit survival of the maximal number of cells. Effects of low temperatures on the preservation of biological properties of mycobacteria have received little attention. In the initial study with BCG cells, a concentration of 5% glycerol or 5% DMSO as protective additives did not prevent a reduction of viable units after rapid freezing, but, with suspensions frozen slowly, the additives were sufficient to prevent a loss after storage at −20 C. The observed loss of viability of BCG cells suspended in Dubos Broth without additives is consistent with the generalization that for many cells the most damaging range of storage is −10 to −40 C (1). At these temperatures, the cell contents may not be frozen entirely, and the concentration of electrolytes may become very high in the unfrozen parts. Lovelock (5) suggests that this harmful concentration of electrolytes is averted by glycerol, which binds substantial proportions of cell

water and prevents its freezing. Further study on the need for additives or the rate of freezing in relation to viability appeared unnecessary, because washed BCG cells suspended in Dubos Broth, frozen slowly and stored at −70 C or lower, resulted in nearly 100% survival.

Storage at low temperatures alters the subsequent growth of some microorganisms (9, 12). Stern and Tompsett (10) recorded altered colony morphology of *M. tuberculosis* after storage for 31 months at −20 C on Oleic Acid Albumin Agar. The first subcultures of altered colonies, however, appeared to have the same rate of growth and colony morphology as their stock strain of H37Rv cells. In our studies, the increased size of BCG colonies was marked in those cells which had been frozen rapidly and stored at −20 or −70 C, but not in those stored at −196 C. There was no change in growth of BCG cells frozen slowly and stored at −70 or −196 C. This change was associated with rapid freezing and storage at −20 C, conditions which reduced the viability of BCG cells. No increase in colony size was observed in H37Rv cells frozen slowly and stored at −70 C.

A suspension of H37Rv cells stored at −70 C for a year showed no loss of infectivity, as assessed by the number of primary pulmonary lesions caused in guinea pigs. In other experiments, Swiss mice have been infected reproducibly with the same suspension under conditions which resulted in three to five primary pulmonary lesions. This requires a nebulizer suspension with 10-fold more bacilli than that required for the same level of infection in guinea pigs. This increase can be accounted for entirely by the 10-fold difference in minute respiratory volumes for mice and guinea pigs (4).

Advantages of storage at low temperatures of a suspension of mycobacteria for infection of animals include the following: it avoids the risk of gradual attenuation which follows serial transfer on laboratory media; it eliminates the technical inconveniences and problems associated with preparing inocula from fresh cultures; and it makes possible the use of an inoculum containing the desired number of viable units. When the number of viable units is known prior to use of the inoculum, a much lower level of infection can be produced with a certainty which is lacking when fresh cultures are used. Constant viability and infectivity of a suspension stored under these conditions assures the reproducibility of the amount of infection and facilitates comparisons between experiments.

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LITERATURE CITED

1. BRIDGES, B. A. 1966. Preservation of microorganisms at low temperatures. Lab. Pract. **15**:418–422.
2. FENNER, F. 1951. Enumeration of viable tubercle bacilli by surface plate counts. Am. Rev. Tuberc. **64**:353–380.
3. GLOVER, R. E. 1946. The effects of (a) freeze-drying and (b) low temperature on viability of mycobacterium tuberculosis. J. Pathol. Bacteriol. **58**:111–114.
4. GUYTON, A. C. 1947. Measurement of respiratory volumes of laboratory animals. Am. J. Physiol. **150**:70–77.
5. LOVELOCK, J. E. 1953. The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochim. Biophys. Acta **11**:28–36.
6. MERRYMAN, H. T. 1963. Preservation of living cells. Federation Proc. **22**:81–89.
7. SMITH, D. W., E. WIEGESHAUS, R. NAVALKER, AND A. A. GROVER. 1966. Host-parasite relationships in experimental airborne tuberculosis. I. Preliminary studies in BCG-vaccinated and nonvaccinated animals. J. Bacteriol. **91**:718–724.
8. SOKOLSKI, W. T., E. M. STAPERT, AND E. B. FERRER. 1964. Liquid nitrogen freezing in microbiological assay systems. I. Preservation of *Lactobacillus leichmannii* for direct use in vitamin B_{12} assay. Appl. Microbiol. **12**:327–329.
9. SQUIRES, R. W., AND S. E. HARTSELL. 1955. Survival and growth initiation of defrosted *E. coli* as affected by frozen storage menstrua. Appl. Microbiol. **3**:40–45.
10. STERN, K., AND R. TOMPSETT. 1951. Preservation of cultures of *M. tuberculosis* by freezing. Am. Rev. Tuberc. **64**:696–697.
11. STRAKA, R. P. AND J. L. STOKES. 1959. Metabolic injury to bacteria at low temperatures. J. Bacteriol. **78**:181–185.
12. TANGUAY, A. E. 1959. Preservation of microbiological assay organisms by direct freezing. Appl. Microbiol. **7**:84–88.
13. TSUJI, K. 1966. Liquid nitrogen preservation of *Saccharomyces carlsbergensis* and its use in a rapid biological assay of vitamin B_6 (pyridoxine). Appl. Microbiol. **14**:456–461.
14. TSUJI, K. 1966. Liquid nitrogen preservation of *Saccharomyces carlsbergensis* and its use in a rapid biological assay of pantothenic acid. Appl. Microbiol. **14**:462–465.