JOURNAL OF BACTERIOLOGY, Dec. 1999, p. 7626–7628 Vol. 181, No. 24
0021-9193/99/$04.00+0

Spent Culture Supernatant of *Mycobacterium tuberculosis* H37Ra Improves Viability of Aged Cultures of This Strain and Allows Small Inocula To Initiate Growth

ZHONGHE SUN AND YING ZHANG*

Department of Molecular Microbiology and Immunology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland

Received 19 May 1999/Accepted 5 October 1999

Spent culture supernatant from early-stationary-phase *Mycobacterium tuberculosis* H37Ra cultures increased the viability of bacilli from aged cultures of this strain and allowed small inocula to initiate growth in liquid culture. The resuscitation factor was acid labile and heat stable, with a mass of less than 1,375 Da.

When a batch culture grows to stationary phase, bacterial growth halts because of the exhaustion of essential nutrients and accumulation of toxic products (7). Upon extended incubation in stationary phase, bacteria begin to die and viability of the culture decreases. The number of CFU of an aged stationary batch culture is often orders of magnitude less than the total number of bacteria in the culture (1, 3, 8). The nonculturable bacterial population consists of dead cells as well as injured or dormant cells which may be resuscitated with factors present in the culture supernatant from the late-log-phase batch culture in the case of *Micrococcus luteus* (8). In this study, we tested the effect of spent culture supernatant of *Mycobacterium tuberculosis* H37Ra on the viability of tubercle bacilli of this strain from aged cultures. We found that early-stationary-phase culture supernatant (ESPSN) improved the viability of aged cultures of H37Ra by allowing the formation of more colonies on plates and allowed small inocula to initiate growth in liquid culture. These observations may have implications for understanding mycobacterial dormancy and development of a better cultivation method for improved diagnosis of tuberculosis (TB).

Resuscitation phenomenon in *M. tuberculosis*. *M. tuberculosis* H37Ra was cultivated in 7H9 Tween 80 (0.05%) albumin-dextrose-catalase liquid medium (Difco) for various times, ranging from a few weeks to several months and sometimes up to 2 to 3 years as standing batch cultures at 37°C with occasional agitation. The aged standing batch cultures settled to the bottom of culture tubes with no apparent surface growth. The ESPSN from a 3- to 4-week-old *M. tuberculosis* H37Ra standing culture (the optical density at 600 nm was about 1.2) grown in 7H9 medium was collected following centrifugation at 6,000 × *g* and sterilized by filtration through a 0.22-μm-pore-size filter. The sterilized ESPSN did not form colonies on 7H11 agar plates. Portions (100 μl) of bacterial cell pellets (about 10^7 to 10^8 bacilli per 100 μl) prepared from an 8-month-old H37Ra standing batch culture were resuspended in the same volume (100 μl) of supernatant of the same aged culture, fresh 7H9 medium, or filtered ESPSN (C). Upon incubation at 37°C for 3 days without shaking, the number of CFU in each medium was determined (the numbers given below are the average results of samples plated in triplicate). The bacterial suspensions were serially diluted in 7H9 liquid medium containing Tween 80, and portions of the diluted suspensions were plated on 7H11 agar plates, followed by incubation for 4 weeks at 37°C. The bacilli from the 8-month-old aged culture yielded almost 1,000-fold fewer colonies in supernatant of the same aged culture than in fresh medium (2.0×10^3 versus 1.2×10^6 CFU/ml). This suggests that the aged culture supernatant contained growth inhibitory activity. On the other hand, the aged bacilli incubated in ESPSN produced about 20-fold more colonies than the fresh medium control (2.4×10^7 versus 1.2×10^6 CFU/ml). This indicates that ESPSN allowed a population of nonculturable bacilli (injured or dormant bacilli) to form colonies. The above phenomenon has been reproduced many times with different batches of *M. tuberculosis* H37Ra cultures of varying ages (up to 2 to 3 years) with the same ESPSN. Depending on the age and viability of the bacterial culture, the results varied somewhat, but the trend of results showing that ESPSN contained resuscitation activity and that aged culture supernatant contained growth inhibitory activity remained. The resuscitation or growth stimulation phenomenon was also found with the bacilli grown in Sauton's simple salt medium (data not shown), indicating that the type of medium is not important for production of the resuscitation activity by tubercle bacilli.

Monitoring the resuscitation phenomenon by FDA-EB staining. Because CFU determination is time consuming (taking 4 to 6 weeks), we used fluorescein diacetate-ethidium bromide (FDA-EB) staining (4) to more rapidly assess the viability status of aged *M. tuberculosis* cultures upon treatment with ESPSN. The principle of FDA-EB staining is as follows: FDA crosses the membranes of dead and live cells, hydrolyzed into free fluorescein by both types of cell, but the fluorescein is retained only by live cells with intact membranes. On the other hand, EB enters only dead cells or cells with impaired membrane integrity and stains DNA. The FDA-EB staining was performed as described previously (4). Briefly, mycobacterial cultures (100 μl) were stained for about 20 min with 50 μl of FDA-EB working solution containing FDA and EB (diluted from stock solutions of FDA [5 mg/ml in acetone] and EB [2 mg/ml in phosphate-buffered saline]) at 2 and 4 μg/ml, respectively, in phosphate-buffered saline. The stained mycobacteria were examined under a fluorescence microscope with a fluorescein isothiocyanate filter with excitation at 490 nm and emission at 525 nm. FDA-EB staining has been shown to correlate with the viability level or number of CFU of myco-

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, School of Hygiene and Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205. Phone: (410) 614-2975. Fax: (410) 955-0105. E-mail: yzhang@jhsph.edu.

TABLE 1. Effect of resuscitation medium (ESPSN) on the resuscitation of *M. tuberculosis* cultures of varying ages, determined by FDA-EB staining

Culture age (mo)	% of green (live) cells		
	Direct staining	Fresh 7H9 medium (control)	ESPSN
8	1.6	9.4	49.8
10	1.8	2.4	49.2
15	0[a]	0.8	19.4
16	0[a]	0[a]	11.2

[a] All cells were orange-red.

bacteria (4). Using the FDA-EB staining, we examined the effect of ESPSN on bacilli from aged H37Ra standing batch cultures grown in 7H9 medium of ages ranging from 8 to 16 months. Bacilli (about 10^8 to 10^9 cells/ml) from various aged cultures were incubated with ESPSN or control 7H9 medium in a volume of 100 μl at 37°C for 2 days, followed by FDA-EB staining and fluorescence microscopy. The average percentage of green cells was determined by observing at least 5 to 10 views randomly chosen under the microscope. More green, FDA-labeled cells were found when the bacilli from various aged cultures were treated with ESPSN than with fresh medium control (5- to 20-fold increase) (Table 1). A representative experiment showing bacilli from samples of a 10-month-old H37Ra culture incubated for 2 days in medium of the same culture, 7H9 medium, or ESPSN is presented in Fig. 1. More green cells are visible in Fig. 1C. These results suggest that membrane injury in the bacilli became repaired in the presence of ESPSN. As a working hypothesis, we assume that FDA-EB staining is identifying the same resuscitation phenomenon with viable counts, and we used FDA-EB staining as a quick assay of resuscitation. Culture supernatants from early-stationary-phase cultures of *Mycobacterium smegmatis* (in 7H9 medium) or *Escherichia coli* (in Luria broth) had no effect on FDA-EB staining or the increase in the number of CFU of aged cells of H37Ra (data not shown).

Expression and localization of the resuscitation activity. To determine the presence of resuscitation activity in relation to growth phase, portions of filter-sterilized culture supernatant (100 μl) taken at different growth stages of an H37Ra standing culture (up to 8 weeks at 37°C) were assayed on about 10^7 to 10^8 bacilli from the same 8-month-old culture as described above, by FDA-EB staining. The resuscitation activity was present mainly from early stationary phase (3 to 4 weeks old) to 2 months. There was hardly any resuscitation activity in the log-phase (1 to 2 weeks old) culture supernatant (data not shown). To determine where the resuscitation activity is located, the culture supernatant and bacterial lysate of a 3- to 4-week-old *M. tuberculosis* H37Ra culture were prepared (9) and assayed for resuscitation activity similarly. The lysate was reconstituted to the original volume of the culture with 7H9 medium and sterilized by filtration through a 0.22-μm-pore-size filter before use. The lysate was found to have a level of activity about 1/40 of that in the supernatant (data not shown), indicating that the resuscitation activity is mainly present in the culture supernatant and that only a small amount of this activity is present in the cell.

The ESPSN allowed smaller bacterial inocula to start culture growth than fresh medium. To start an *M. tuberculosis* culture frequently requires a relatively large inoculum, and a small inoculum often fails to initiate the growth of *M. tuberculosis* in liquid culture (2). The reason for this is unknown. We tested whether the ESPSN could influence the size of bacterial inoculum required to initiate growth of tubercle bacilli in liquid culture. To do this, a 6-month-old *M. tuberculosis* H37Ra batch culture that had been kept at 37°C without shaking was 10-fold serially diluted (0.4 into 3.6 ml) into filter-sterilized ESPSN (prepared from a 4-week-old H37Ra standing culture grown in Sauton's medium) and into a control Sauton's medium. The Sauton's medium contained the detergent Triton WR1339 at 0.025% to reduce bacterial clumping. The various dilutions were incubated at 37°C for 2 weeks when the visibility of bacterial growth and number of CFU were determined. ESPSN allowed smaller inocula (10^{-3} to 10^{-4} dilutions) to form visible growth in liquid subcultures, whereas the inocula failed to yield any visible growth in Sauton's medium alone (Table 2). The growth-stimulating or resuscitation effect was also reflected in the increased CFU over the medium control (Table 2). Strikingly, no CFU was demonstrable for 10^{-3} to 10^{-5} dilutions when incubated for 2 weeks in medium control and then directly plated, whereas 10^4 to 10^5 CFU/ml were detected for the same dilutions in ESPSN. Neither ESPSN alone nor the 10^{-6} dilutions in ESPSN or fresh medium gave any bacterial growth in liquid culture or on agar plates.

Characterization of the resuscitation activity. To determine the effect of physicochemical factors on the resuscitation activity and the nature of the activity, we subjected the ESPSN to various treatments and then assayed the resuscitation activity using FDA-EB staining and fluorescence microscopy. In the following experiments, the 8-month-old culture described above and a positive control ESPSN were used. Heating ESPSN at 100°C for 10 min had no effect on the resuscitation

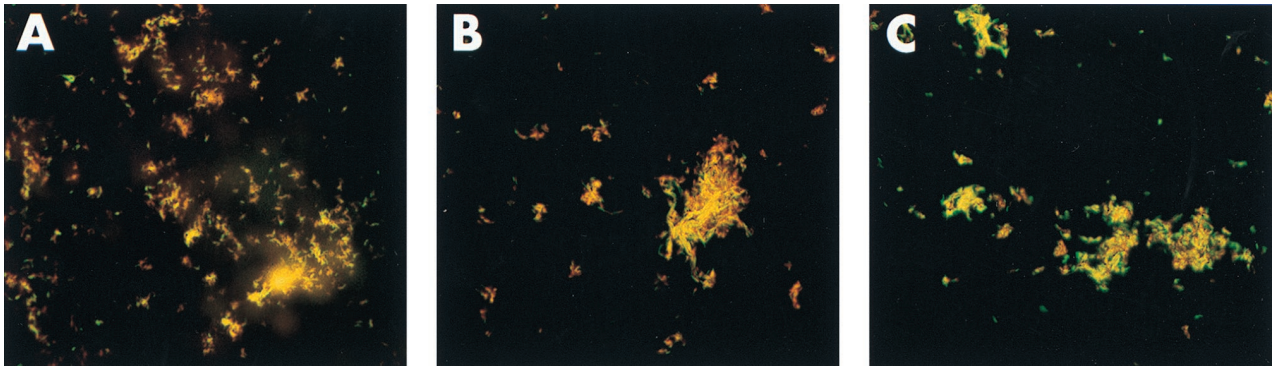

FIG. 1. FDA-EB staining of bacilli from a 10-month-old H37Ra culture in various media. About 10^7 to 10^8 bacilli from the 10-month-old culture grown in 7H9 medium as a standing culture were incubated in 100-μl volumes of supernatant of the same culture (A), 7H9 medium (B), and ESPSN (C), respectively, for 2 days, at which point the status of the bacterial cells was assessed by FDA-EB staining and fluorescence microscopy.

TABLE 2. Growth stimulation of 6-month-old *M. tuberculosis* cultures by ESPSN with small bacterial inocula

Dilution	Visible growth[a]		CFU/ml[b]	
	Sauton's	ESPSN	Sauton's	ESPSN
10^{-1}	+	+	5×10^6	$>10^7$
10^{-2}	+	+	10^6	10^7
10^{-3}	−	+	ND	3×10^5
10^{-4}	−	+	ND	2×10^5
10^{-5}	−	−	ND	3×10^4
10^{-6}	−	−	ND	ND

[a] +, visible growth; −, no visible growth.
[b] Determined from 2-week-old subcultures which had been inoculated with various dilutions of the 6-month-old culture diluted and grown in Sauton's medium alone or in ESPSN. Data are the averages of the results from triplicate platings of undiluted and diluted (10^{-2}) samples on 7H11 agar plates. ND, no CFU detectable when directly plated on agar plates.

activity, indicating this resuscitation activity is heat stable. This experiment also rules out the possibility that any residual bacilli or filterable form of the bacilli in the filtered culture supernatant may have contributed to the increased number of viable bacilli. In addition, repeated freezing (−70°C) and thawing (37°C) for 10 to 15 cycles had no significant effect on the resuscitation activity (data not shown). However, acid treatment (with 1 N HCl for 1 h at room temperature followed by neutralization with the same molar concentration of alkali) completely abolished the resuscitation activity and alkali treatment reduced the activity but to a lesser extent. Treatment of ESPSN with RNase T_1 (2,000 U/ml), DNase I (100 U/ml), exonuclease III (150 U/ml), and proteinase K (20 μg/ml), pronase (250 μg/ml), or trypsin (25 μg/ml) in appropriate buffers did not appear to affect the activity, indicating that the resuscitation factor does not appear to be a nucleic acid or polypeptide.

To determine the molecular mass of the resuscitation factor, size exclusion chromatography with Bio-Gel-P2 (Bio-Rad), which separates compounds with small molecular masses (exclusion limit, 1,800 Da), was used. A molecular mass standard (thyroglobulin, 670 kDa; bovine gamma globulin, 158 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa; and vitamin B_{12}, 1.375 kDa) was run, and a standard curve was established. After the ESPSN sample was loaded, the column was eluted with 7H9 medium without albumin-dextrose-catalase, and various fractions were filter sterilized before being tested for activity by FDA-EB staining. The resuscitation factor was found to be smaller than the vitamin B_{12} standard, which is 1,375 Da. Consistent with this finding, we found that dialysis of ESPSN using dialysis tubing with a molecular mass cutoff of 3,000 Da caused complete loss of the activity (data not shown).

In this study we identified a resuscitation activity from the ESPSN of tubercle bacilli, as judged by increased CFU formation of the aged batch culture and by a large increase in FDA-stainable cells with ESPSN compared to that with control fresh medium. One issue is whether the phenomenon we observed is due to resuscitation or regrowth (or growth stimulation) of viable bacilli present in aged cultures. The observation that a large fraction of bacilli (e.g., 50% in FDA-positive cells in ESPSN versus 2 to 10% in cells in 7H9 medium) became FDA stainable (Table 1 and Fig. 1) in 2 days is best explained by resuscitation through repair of membrane damage in injured or dormant cells, rather than by regrowth of viable bacilli. However, we cannot definitely conclude that the increase in CFU and the increase in FDA-stainable cells are due to the same bioactive molecule(s).

The release of an autocrine resuscitation activity into the culture supernatant at the onset of stationary phase may be a self-protective mechanism to prepare tubercle bacilli (and perhaps other bacteria) to survive and remain viable in extended stationary phase. Further biochemical and genetic characterization of the resuscitation activity and identification of bacterial genes that are switched on during resuscitation may help improve our understanding of dormancy in *M. tuberculosis* (5). The current definitive clinical diagnosis of TB still relies on cultivation of tubercle bacilli from clinical samples. The identification of a resuscitation or growth-stimulating activity in the spent culture supernatant that allowed smaller inocula to start culture may have implications for developing a better cultivation method for *M. tuberculosis* for improved clinical diagnosis of TB.

This work was supported by grants from the American Lung Association, Potts Memorial Foundation, and NIH (AI40584).

REFERENCES

1. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
2. **Dubos, R. J., and B. D. Davis.** 1946. Factors affecting the growth of tubercle bacilli in liquid media. J. Exp. Med. **83:**409–423.
3. **Kaprelyants, A. S., and D. B. Kell.** 1993. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. Appl. Environ. Microbiol. **59:**3187–3196.
4. **Kvach, J. T., and J. R. Veras.** 1982. A fluorescent staining procedure for determining the viability of mycobacterial cells. Int. J. Lepr. **50:**183–192.
5. **McCune, R. M., F. M. Feldman, H. Lambert, and W. McDermott.** 1966. Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. J. Exp. Med. **123:**445–468.
6. **Mukamolova, G. V., A. S. Kaprelyants, D. I. Young, M. Young, and D. B. Kell.** 1998. A bacterial cytokine. Proc. Natl. Acad. Sci. USA **95:**8916–8921.
7. **Postgate, J. R.** 1967. Viability measurements and the survival of microbes under minimum stress. Adv. Microb. Physiol. **1:**1–23.
8. **Votyakova, T. V., A. S. Kaprelyants, and D. B. Kell.** 1994. Influence of viable cells on the resuscitation of dormant cells in *Micrococcus* cultures held in an extended stationary phase: the population effect. Appl. Environ. Microbiol. **60:**3284–3291.
9. **Zhang, Y., A. Scorpio, H. Nikaido, and Z. H. Sun.** 1999. Role of acid pH and deficient efflux of pyrazinoic acid in the unique susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. J. Bacteriol. **181:**2044–2049.