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Progress in Culture and Subculture of Spheroplasts and Fastidious Acid-Fast Bacilli Isolated from Intestinal Tissues

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The efficiency of culture media was compared for the culture and subculture of very slowly growing acid-fast bacilli and spheroplast forms obtained from intestinal tissues of patients with Crohn's disease and ulcerative colitis and from controls without inflammatory bowel disease. Media were developed by modifying a nutrient broth medium based on veal infusion broth and yeast extract. We evaluated the effects of pH and the addition of Tween 80, Dubo oleic albumin complex, an extract from intestinal tissue from a patient with Crohn's disease, horse serum, sucrose, magnesium sulfate, ferrous ammonium sulfate, and sodium citrate. All media contained mycobactin J (2 μg/ml). We developed a medium (MG3) which was highly successful in promoting the growth of very fastidious organisms and promoted reversion of spheroplasts to acid-fast rods. MG3 contained veal infusion broth, 1% yeast extract, 10% horse serum, 0.3 M sucrose, 0.2% $MgSO_4$, 0.1% ferrous ammonium sulfate, 0.1% sodium citrate, and 2 mg of mycobactin J per liter. We were able to obtain quantities of organisms sufficient for examination of the organisms by molecular techniques. Successful cultivation of all isolates and reversion of spheroplasts to acid-fast forms encourage further studies of the possibility of a complex association of mycobacteria and Crohn's disease.

Mycobacteria have been proposed as possible etiologic agents of Crohn's disease since 1932, when Crohn's disease was first differentiated from intestinal tuberculosis (5). Successful isolation and culture of spheroplast forms of mycobacteria and detection of mycobacterial DNA in tissues have been recently reported as a result of studies concerning the possible mycobacterial etiology of Crohn's disease (1, 3, 4, 6, 14). That mycobacteria are present as spheroplast forms in Crohn's disease tissues could explain previous failures to consistently isolate or visualize mycobacteria in these tissues, because mycobacterial spheroplasts do not stain with traditional acid-fast or Gram stains and require very fastidious growth conditions. This hypothesis is supported by a number of studies; for example, Burnham et al. (1) isolated cell wall-defective forms containing acid-fast material from 22 of 27 Crohn's disease tissue samples, 7 of 13 ulcerative colitis tissue samples, and 1 of 11 control tissue samples. Additionally, Chiodini et al. (3, 4), using methods designed for the isolation of a fastidious, mycobactin-dependent mycobacterium, *Mycobacterium paratuberculosis*, isolated a strain of *M. paratuberculosis* (14) from four patients with Crohn's disease. These organisms were initially isolated as mycobactin-dependent spheroplast forms which later reverted to normal mycobacteria in subculture. Chiodini et al. also reported the isolation of 12 additional spheroplast forms (4), but subculture of these organisms has not yet been reported. We were also able to isolate very slowly growing, fastidious mycobacteria and spheroplast forms from intestinal tissues (6). In our series, isolation was not limited to tissues from Crohn's disease patients. We recovered similar-appearing organisms from patients with ulcerative colitis and from patients without inflammatory bowel disease. Subculture of our isolates proved difficult or impossible with standard mycobacterial media; this difficulty prevented attempts to establish whether a specific organism might be responsible for Crohn's disease. We report here our progress in developing a medium capable of supporting growth of the very slowly growing acid-fast bacilli and permitting growth and reversion of spheroplasts to acid-fast bacteria (AFB).

Isolates. Isolates were fastidious, very slowly growing acid-fast bacilli and spheroplasts isolated from intestinal tissues by methods designed to maximize isolation of *M. paratuberculosis*, as previously described (6). The isolation methods were based on those in use at the Animal Mycobacterioses Laboratory of the National Animal Disease Center, Ames, Iowa.

During the course of medium testing, isolates were maintained in subcultures on Herrold egg yolk medium (HEYM) containing mycobactin J, at 37°C.

Culture media tested. Initial subcultures of all very slowly growing fastidious AFB and spheroplasts were made on HEYM with and HEYM without 4.1 g of sodium pyruvate per liter (Allied Laboratories, Ames, Iowa, and Otis Biofarms, Otisville, N.Y.), Middlebrook 7H10 and 7H11 agars, and 7H9 broth. Cultures in 7H9 broth contained 15 ml of medium and were incubated on their sides for a final medium depth of 5 mm. Synthetic media contained 100 ml of Dubo oleic albumin complex per liter and 0.05% Tween 80. They were prepared at pH 5.9. All media contained 2 μg of mycobactin J per ml (Allied Laboratories).

In an attempt to identify media that would better support the growth of spheroplasts and fastidious bacilli, we modified a nutrient broth medium based on veal infusion broth and yeast extract (7). In all preparations, veal infusion broth (Difco Laboratories, Detroit, Mich.) was made according to the specifications of the manufacturer. Yeast extract (Difco) was added as a separately autoclaved 10% solution to a 1% final concentration. Modifications included combinations of pH (adjustment to 5.0, 5.5, and 6.0 by dropwise addition of HCl), Tween 80 (added to an 0.05% final concentration before autoclaving), Dubo oleic albumin complex (added as a sterile solution to autoclaved, cooled broth at 100 ml/liter), and tissue extract (1% final concentration) prepared as a filtered (0.2-μm pore size) homogenate (10% [wt/vol] in

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phosphate-buffered saline) of normal-appearing resected ileum from a patient with Crohn's disease. Further modifications included horse serum (GIBCO Laboratories, Grand Island, N.Y.) which was heat inactivated (56°C for 30 min) and added to a 10% final concentration. Tonicity was increased with 0.3 M sucrose (final concentration), and $MgSO_4$ was included at 0.2% (13; R. Samuel, Ph.D. thesis, University of Delhi, Delhi, India, 1975). Ferrous ammonium sulfate and sodium citrate were also added, each to a 0.1% final concentration. All modifications contained mycobactin J (2 μg/ml).

Medium was divided into 20-ml volumes in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) which were incubated upright; final medium depth was 2 cm. The effect of the depth of the medium was examined by incubating some of these cultures on their sides (final medium depth, 5 mm). Inoculated cultures and uninoculated medium from each medium lot were incubated at 37°C concurrently. We also examined the effect of 5% CO_2 on growth in standard mycobacterial media and in modified veal-yeast broths.

Inoculation of cultures. In general, loopfuls (3-mm diameter) of growth from HEYM slants were transferred directly to the medium to be tested. When quantitation or equivalency of inocula was desired, a single-cell suspension was prepared by suspending the organisms in 0.9% saline–0.02% Tween 80. The suspension was vortexed with a few sterile glass beads and then centrifuged at low speed to sediment larger clumps. Subculture in broth media was performed by pelleting a sample at 17,000 × *g* for 15 min, resuspending the pellet in fresh medium, and transferring samples of this suspension to the medium to be tested.

Detection of growth. Solid media were examined with a dissection microscope for growth of small colonies. Broth cultures were compared with uninoculated media for qualitative indicators of growth, e.g., sediment formation and increased turbidity. Because of the nature of the isolates, dilution plating, direct counts of stained organisms, and changes of optical density could not be used to assess bacterial growth. Growth was scored by visual inspection and confirmed by a measured increase in DNA concentration of the culture. The DNA assay was based on the fluorescence properties of Hoechst H33258, which selectively binds to DNA (8). Briefly, a sample of broth culture was pelleted, washed in phosphate-buffered 0.9% saline, and suspended in 50 μl of assay buffer (2 M NaCl, 0.1 M Na_2HPO_4 plus NaH_2PO_4 [pH 7.4]). The resuspended sample was incubated in disposable acrylic cuvettes (four sides clear; Fisher Scientific Co., Pittsburgh, Pa.) with 3 ml of 1-μg/ml H33258 prepared in assay buffer. Fluorescence was measured on an LS-5 fluorescence spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.), and micrograms of DNA per milliliter were determined from linear regression analysis established by a series of mycobacterial DNA standards. Background fluorescence of pelleted, washed, uninoculated media was subtracted from the sample measurements before calculation of DNA content. The validity of measuring intracellular mycobacterial DNA by using purified DNA standards was confirmed in experiments which compared the results of measuring the DNA in known numbers of mycobacteria (10^5, 10^6, 10^7, 10^8, and 10^9 organisms per ml). The conversion factor used was 1 mycobacterium = 0.01 pg of DNA. The limit of detection was 10^5 organisms (0.01 μg of DNA). The correlation coefficient for comparing the standard pure DNA and the experimental values was excellent ($r = 0.9976$).

Growth was also detected by uptake of [^{3}H]uracil into trichloroacetic acid-precipitable material. Mycobacteria have been shown to incorporate this precursor when it is added to the medium (12). Briefly, 1.25 μCi of [^{3}H]uracil (TRK-408; Amersham Corp., Arlington Heights, Ill.) was added to cultures and incubated at 37°C. On a weekly basis, a sample of each culture was pelleted and analyzed for trichloroacetic acid-precipitable counts. Results were expressed as the percentage of increase in total counts per minute of the pellet.

TABLE 1. Results of culture of mycobacteria from intestinal tissues

Source of tissue sample (no. in culture)	No. of isolates		
	Slow growers	Spheroplasts	Reversion to AFB
Crohn's disease			
Resections (55)	0	13	7
Biopsies (32)	6	5	5
Ulcerative colitis			
Resections (3)	0	1	1
Biopsies (22)	4	2	0
Controls[a]			
Resections (16)	1	3	1
Biopsies (25)	7	2	0

[a] Without inflammatory bowel disease.

Stains. Ziehl-Neelson and Kinyoun acid-fast staining methods were used. In addition, heat-fixed (2 h at 65°C) bacterial smears were oxidized with 10% periodic acid for 4 h before acid-fast staining by a method described by Nyka (10). Acridine orange stains were performed on initial spheroplast cultures to verify the presence of organisms (2).

Results. To date we have obtained 26 spheroplast and 18 very slowly growing AFB isolates (Table 1), an increase of 11 spheroplasts since our previous publication (6). Only two of the slowly growing AFB isolates were fully cultivable and could be identified by standard biochemical methods. Spheroplasts grew in subculture only on HEYM and appeared as small, clear, moist colonies after 3 or more months in incubation. Although the primary isolation of 16 fastidious AFB isolates was achieved without difficulty, repeated subculture became increasingly difficult. Initial subcultures in synthetic media were successful for only 2 of the 16 very slowly growing AFB isolates. After two or three subcultures, these organisms could be cultured only on HEYM. Fourteen isolates never grew in synthetic media. After several subcultures on HEYM, some of these fastidious AFB cultures began to show spheroplastlike growth, acquiring the clear, moist colony appearance common to mycobacterial spheroplasts, and grew as mixed cultures. Subculture of both spheroplasts and fastidious AFB isolates was more often successful when larger inocula were used. Because of these difficulties, we evaluated a number of media for their ability to support the growth of both types of isolates.

Veal infusion broth (25 g/liter, final volume) supplemented with 1% yeast extract (pH 5.0) (designated medium MG1), was found to support the growth of spheroplasts and fastidious AFB. Growth was evidenced by the formation of a sediment which swirled upward in strands when the medium was disturbed. Maximum growth was obtained at 3 months. However, growth was still at levels insufficient for identification of the organisms, and inoculation with visibly turbid

TABLE 2. Composition of variations of MG2 medium tested[a]

Medium	pH	Component		
		0.05% Tween 80	0.1% FAS[b]	0.1% Sodium citrate
V1	5.3			
V2	5.3	+		
V3	5.3		+	+
V4	5.3	+	+	+
V5	6.7			
V6	6.7	+		
V7	6.7		+	+
V8	6.7	+	+	+

[a] All media contained veal infusion broth, 1% yeast extract, 10% horse serum, 0.3 M sucrose, 0.2% $MgSO_4$, and 2 mg of mycobactin J per liter.
[b] FAS, Ferrous ammonium sulfate.

suspensions or loopfuls of organisms was still required to ensure successful subculture.

A number of medium variations were tried. The addition of Dubo oleic albumin complex, Tween 80, or tissue extract (in any combination) had no noticeable effect, whereas the addition of 10% horse serum (designated MG2 medium) resulted in increased sediment formation and medium turbidity. Maximum growth with MG2 was at 2 months. MG2 medium was compared with HEYM and synthetic media by using measured samples of a single-cell suspension of organisms from subcultured isolates and from the primary slants (which had been incubated at room temperature for more than 1 year). Fourteen fastidious AFB and two spheroplast isolates were used, and all cultures were incubated in an atmosphere of 5% CO_2. Visually detectable growth was obtained from subcultures and primary isolates in MG2 broth after 2 and 4 weeks, respectively. Examination of cultures by Kinyoun acid-fast stain showed that MG2 encouraged the reversion of spheroplast organisms to acid-fast bacilli. Growth on synthetic medium or HEYM was less successful, e.g., BACTEC 12A medium supported the growth of 10 of 14 isolates tested, but it required 3 months for increased turbidity to be present, and only four isolates could be subcultured to BACTEC. Primary isolates failed to grow on HEYM; only one subcultured fastidious AFB grew on HEYM during the 3-month course of examination (which was not different from our previous experience). A 5% CO_2 atmosphere did not contribute to subculture in HEYM or synthetic media.

[^{3}H]uracil uptake by organisms growing in MG2 medium was examined in two spheroplast cultures and four cultures of fastidious AFB. The ^{3}H incorporation increased during 3 weeks of incubation, although actual counts were low. After 3 weeks of incubation, four cultures showed increases ranging from 270 to 650%. Incorporation in two fastidious AFB cultures decreased by 55 and 63% at this time.

Four variations of MG2 (V1 to V4) were prepared at pH 5.3, and four (V5 to V8) were prepared by buffering with sodium phosphate at pH 6.6 to 6.8 (Table 2). Inocula were prepared from five spheroplast cultures and five fastidious AFB cultures (by pelleting and resuspending in MG2) which had been incubating in MG2 medium. Cultures were examined for growth by weekly visual inspection. DNA assay was performed at the outset, at 1 month for cultures with visible growth and at 2 months postinoculation for all cultures. The DNA concentration in each culture was below detectable limits at the outset. Cultures were monitored periodically to examine the morphology of the organisms by Kinyoun acid-fast staining and by Kinyoun stain with and without prestaining oxidation at the end of the experiment.

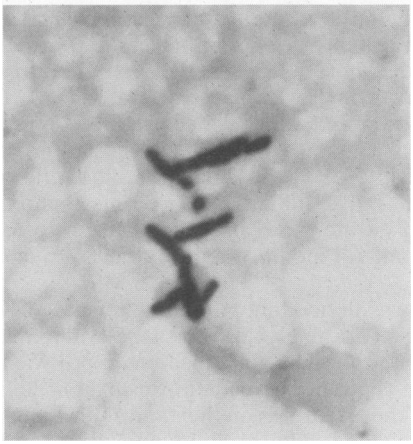

FIG. 1. Kinyoun acid-fast stain of spheroplasts that reverted to acid-fast bacilli in MG3 medium. Magnification, ×5,700.

After 2 months of incubation, growth was obtained in all media tested, indicating that the previous requirement for large inoculum size had been overcome. We compared media by several factors to select the best medium. Tween-containing media were eliminated because a turbid and particulate artifact formed, and Tween 80 produced a high fluorescence reading under the conditions of the DNA assay.

The three culture media with the highest DNA values (V1, V3, and V7) differed in pH and in the addition of ferrous ammonium sulfate and sodium citrate. The amounts of DNA (in micrograms per milliliter) with media V1, V3, V5, and V7 were 0.34, 0.41, 0.24, and 0.34, respectively, or 2×10^6 to 4×10^6 organisms per ml. Medium V3 consistently produced the best results, including reversion of spheroplasts to acid-fast bacilli (Kinyoun acid-fast stain). More acid-fast rods were found in V3 than in the other media tested. AFB from V3 could be stained by the Kinyoun method, whereas in the other media, bacilli required oxidation by the Nyka method before acid-fast staining in order to be detectable in numbers comparable to those in V3 (Fig. 1). Even then, the morphology of the organisms was not as healthy as those found in V3 medium (i.e., organisms were of irregular size and shape). V3 was redesignated MG3.

Mycobacteria and mycobacterial spheroplasts can be isolated from tissues obtained from patients with Crohn's disease. However, similar-appearing organisms can also be isolated from tissues from patients with ulcerative colitis and from those without inflammatory bowel disease. An etiological association would be more convincing if the same organisms were isolated from patients with Crohn's disease but not from patients with the other conditions. To compare organisms requires the ability to grow the organisms in subculture. Successful subculture has now been achieved and should allow further examinations of the role of mycobacteria in Crohn's disease.

Starvation is one possible explanation for the increasing difficulty experienced during attempts to subculture the fastidious AFB and spheroplast isolates. One or more essential factors may be missing from the HEYM and synthetic media, thus preventing adaptation to growth in vitro. This hypothesis is strengthened by the observation of the eventual decline of the organisms into cell wall-defective and poorly staining or chromophobic forms. Nyka and O'Neill

recognized chromophobic mycobacteria in studies of organisms in prolonged room temperature incubation (presumably starvation conditions) (11) and related this chromophobic nature to decreased respiration. Improvement in the culture of our isolates, with reversion of spheroplasts to AFB and return of acid-fastness to fastidious AFB, may be a result of overcoming respiration deficiencies and subsequent cell wall manufacture defects by the inclusion of essential nutrients under appropriate growth conditions.

MG3 medium contains horse serum, which has been used for many years in spheroplast, pleuropneumonia like-organism, and mycoplasma research. It has been theorized that horse serum serves as an absorbant of toxins (9). It is also of nutritive value, as it contains free amino acids, free fatty acids, triglycerides, cholesterol, albumin, and numerous vitamins and trace elements. It may also contain yet unidentified factors which encourage growth.

Increased tonicity, attained in the MG3 medium by the addition of 0.3 M sucrose, has been frequently recommended for spheroplast cultures, where it serves to stabilize the tonically sensitive cell wall-defective organisms. It may also provide a carbon source. Yeast extract is a highly nutritive additive which is known to be metabolized by mycobacteria. Ferrous ammonium sulfate and sodium citrate provide iron and citrate in solution. The requirement for $MgSO_4$ in defined media has been recognized for many years; its presence may be significant for cell wall synthesis and membrane stabilization (13).

Experiments now under way involve further refinements of the MG3 medium, determination of its suitability for continued subculture, and definition of essential components. We are now obtaining quantities of organisms sufficient for examination of the organisms by molecular techniques (e.g., DNA-DNA hybridization in solution) and hope to be able to culture these organisms in the large quantities needed for examination by serological and biochemical techniques. Successful cultivation of all isolates and reversion of spheroplasts to acid-fast forms encourage further studies of the possibility of a complex association of mycobacteria and Crohn's disease.

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