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Separation of *Mycobacterium leprae* from Contamination with Armadillo-liver-derived "Pigment" Particles[1]

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Since the discovery that the nine-banded armadillo, *Dasypus novemcinctus* Linn., is susceptible to infection with *Mycobacterium leprae* (3), various procedures have been developed to purify the mycobacteria from host tissues (2, 4, 5, 8, 10). One widely used purification procedure, devised for production of a candidate leprosy vaccine by the IMMLEP Steering Committee of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, is that of Draper (10). It gives a high percentage recovery of *M. leprae*, largely free from contaminating host tissue.

M. leprae can most easily be recovered from the soft tissues of the armadillo—lymph nodes, spleen and, especially, the liver. However, it has been noted (7) that approximately 30% of infected liver samples (but not, apparently, spleen or lymph node) yield suspensions of *M. leprae* contaminated with a particulate brown "pigment." Such suspensions are judged unsuitable for studies in humans.

Since the production of *M. leprae* from armadillos is both expensive and time-consuming, the loss of a significant proportion of mycobacteria because of contamination with "pigment" is unsatisfactory. A procedure that could separate the contaminating "pigment" particles from the armadillo-liver-derived *M. leprae* would generate substantial further quantities of mycobacteria that could be used in man. This paper describes such a procedure.

MATERIALS AND METHODS

Bacterial suspensions. The *M. leprae* used in this study were obtained from liver tissue of infected nine-banded armadillos. The mycobacteria were purified using the WHO protocol (10). Only preparations which were a very distinct brown (as opposed to the pale cream color of pure *M. leprae*) were used. Such suspensions were seen to be contaminated with particulate material when counterstained with soluble blue (9). No other significant contamination was observed.

Counting procedure. Enumeration of acid-fast bacilli (AFB) in the original suspensions and in various fractions was carried out following the procedure of Rees (6).

Re-purification of *M. leprae*. Step gradients, prepared in 15 ml Corex centrifuge tubes, consisted of 1 ml each of 100%, 90%, 80%, 70%, 60%, and 50% (v/v) Percoll diluted with 0.15 M NaCl. Each concentration was layered onto the next higher one, using a fine Pasteur pipette. A 500 μl sample of a "pigmented" *M. leprae* suspension was taken, containing approximately 10^9 organisms per ml. A small sample (50 μl) was used for counting, and the rest carefully layered onto the Percoll gradient. The whole was centrifuged at 12,500 × *g* × 5 min in a Sorvall HB4 swing-out rotor. Visible bands were removed using a syringe and a long needle. Each fraction was centrifuged at 15,000 × *g* × 90 sec in conical plastic tubes, using a Whyteleafe centrifuge, and washed twice with 20 mM phosphate buffer, pH 7, containing 0.15 M NaCl and 0.1% Tween 80, using the same centrifugation conditions. The pellet was finally resuspended in the buffered Tween solution containing 0.02% sodium azide as a preservative.

Calibration of gradients. After centrifugation, a Percoll gradient was separated into 13 500 μl fractions. Refractive indexes of fractions were measured at room temperature (ca. 20°C) using an Abbe refractometer. The densities of a series of Percoll samples of known concentrations were measured with an electronic density-measuring ap-

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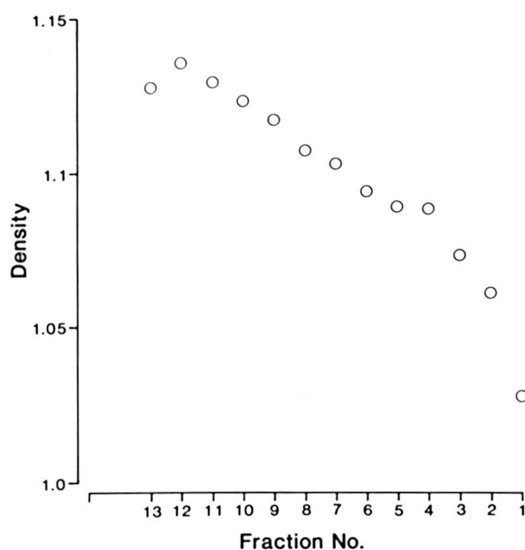

FIG. 1. Densities of fractions from a Percoll step gradient prepared and centrifuged as described under Methods. Densities were calculated from measured refractive indexes. The apparent lower density of the fraction from the bottom of the tube (fraction 1) is an experimental artifact.

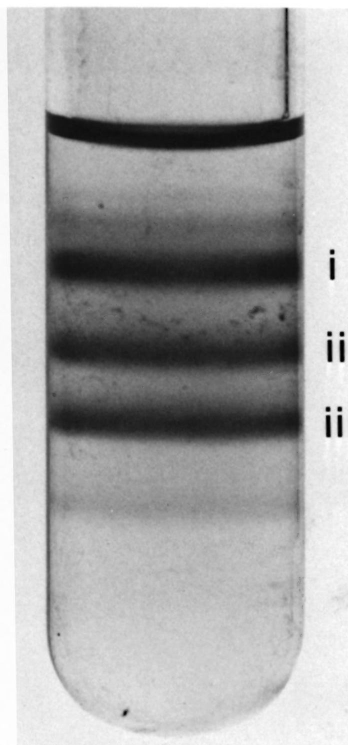

FIG. 2. Separation of a suspension of *M. leprae* contaminated with "pigment" in a Percoll step gradient after centrifugation at 12, 500 × *g* × 5 min. The three major bands discussed in the text are indicated.

paratus (Anton Paar AG). A standard curve was prepared by plotting measured densities against refractive indexes of the standard Percoll samples; using this curve the densities of the gradient fractions could be calculated.

Electron microscopy. Suspensions containing "pigment" were examined, unstained or negatively stained with 0.5% potassium phosphotungstate, on carbon- and collodion-coated grids in a Philips EM300 electron microscope.

An X-ray microanalysis of the pigment "particles" was carried out using a Link System 860 series 2 X-ray analyzer operating with a JEOL 1200EX electron microscope.

RESULTS

It was found that the density gradients generated as described in Methods were approximately linear (Fig. 1). Centrifugation of "pigment"-containing *M. leprae* suspensions on such gradients generated three distinct bands (Fig. 2).

Microscopic examination of the bands showed that they had distinctly different compositions of particles. Figure 3a shows an unseparated "pigment"-contaminated suspension. Many particles staining strongly with soluble blue could be seen along with the *M. leprae.* Band i, the least-dense band from the gradient, was composed of almost pure *M. leprae,* virtually no "pigment" particles being observed (Fig. 3b). The bulk of the "pigment," which was more dense than the bacilli, was present in band ii (Fig. 3c). Only a few bacilli were present in this fraction. Band iii contained a residue of "pigment" particles (Fig. 3d).

The actual recoveries of *M. leprae* from the bands in the gradient, in three separate experiments, are shown in The Table. The bulk of the bacilli were recovered in band i; these were seen by microscopy to be almost pure. Only relatively trivial amounts of bacilli remained associated with the "pigment" particles in bands ii and iii.

Electron microscopy (Fig. 4) showed extremely electron-dense particles, either single, roughly spherical units or lobed, larger particles which seemed to be clumps of par-

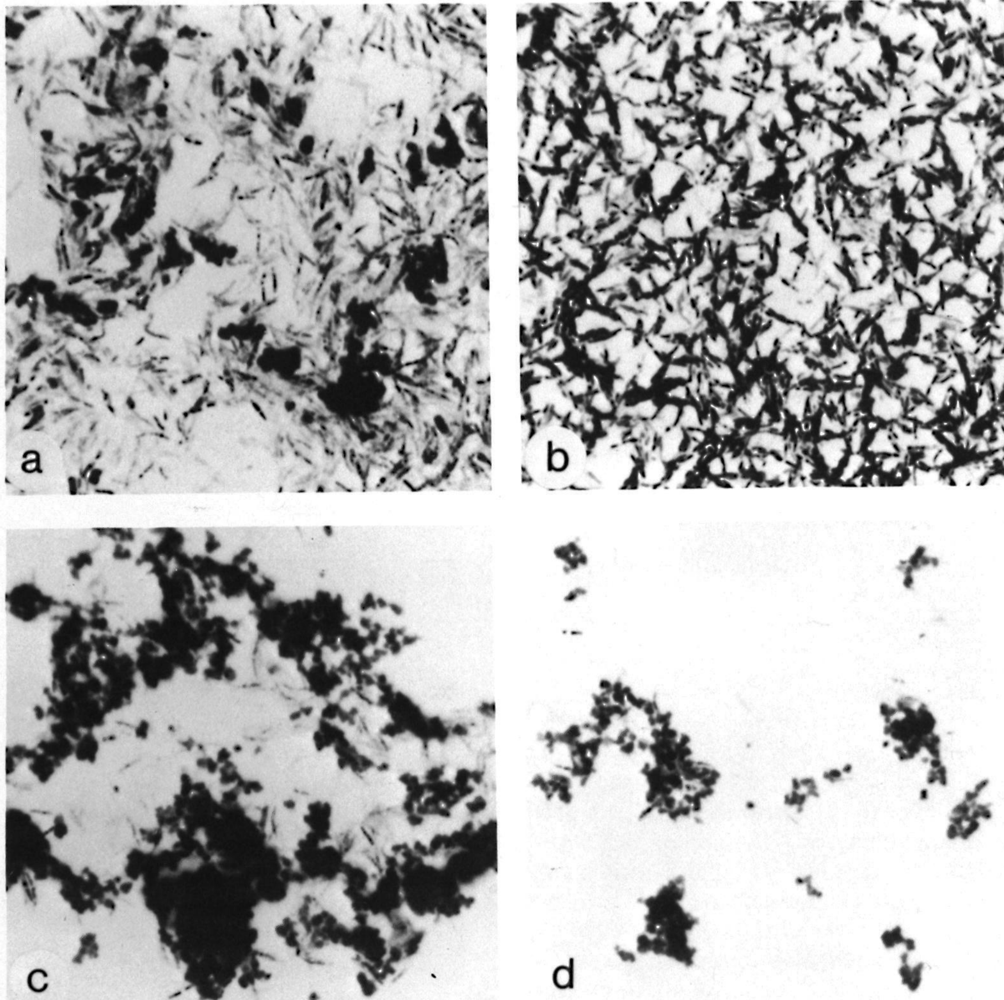

FIG. 3. Light micrographs of "pigment"-contaminated *M. leprae* stained by the Ziehl-Neelsen procedure and counterstained with soluble blue: a = suspension before purification; b, c, and d = bands i, ii, and iii, respectively, from a Percoll gradient after centrifugation (×1000).

ticles. The X-ray analyzer makes use of secondary X-rays emitted by atoms exposed to an electron beam: elements of moderate-to-high molecular weight produce characteristic X-rays. The "pigment" particles contained no large amounts of X-ray emitting atoms (carbon, oxygen and nitrogen were not detected), but detectable quantities of sulfur and calcium were present.

DISCUSSION

Since there is, as yet, no confirmed culture system for *M. leprae*, and certainly none that can yield large quantities of bacteria, the nine-banded armadillo is an immensely important source of bacterial cells. It is also convenient in that the bacteria occur in soft tissues and so are simpler to purify than they are from human skin.

The liver of the nine-banded armadillo is quite substantial, weighing about 150 g, and is the most important source of *M. leprae*. Levels of infection can exceed 10^{10} bacilli per g of liver tissue, yielding 150 mg or more from a single liver. Unfortunately, contamination with "pigment" particles renders an important fraction of suspensions from this source useless for studies in humans. "Pigment" has not been noted in suspensions obtained from the spleen or lymph nodes.

The actual color of the "pigment" has

been found to vary: sometimes the particles were quite pale in color; in other samples, they were a deep chocolate brown. Their microscopic appearance and ability to stain with soluble blue were uniform, however. The X-ray analytical results were consistent with the particles being proteinaceous. Unpublished results obtained in our laboratory, and light- and electron-microscopical data obtained by Drs. A. C. McDougall and P. R. Millard (personal communication), suggest that the particles may be "age pigment" or lipofuscin, but further data need to be accumulated to prove this point.

There had been several earlier attempts, in our laboratory and elsewhere (unpublished results), to separate bacteria from "pigment" particles. The methods included sucrose density gradients (which are capable of a partial separation); columns of Ballotini beads (Dr. P. Novotny, personal communication) or Dowex 50 (both of which bind the particles but also, unfortunately, the *M. leprae*); gel filtration with Sephacryl S1000 (which can handle particles as large as these but which also binds to them in this case); and various aqueous two-phase polymer systems. Separation was incomplete or yields of bacteria were unacceptably low in every case.

Discontinuous Percoll gradients have been utilized to purify many materials, including *Trypanosoma cruzi* ([1]) and *M. leprae* from armadillo tissue ([4]). Percoll differs from other materials used to form density gradients in that physicochemical conditions, including osmotic strength, can be adjusted independently of density, since Percoll itself is non-ionic and contributes negligibly to tonicity. So the behavior of particles in Percoll gradients may differ markedly from their behavior in other gradients. The discontinuous gradients used in the present study separated *M. leprae* quite distinctly from contaminating "pigment" particles. The bands were immediately distinguishable by color:

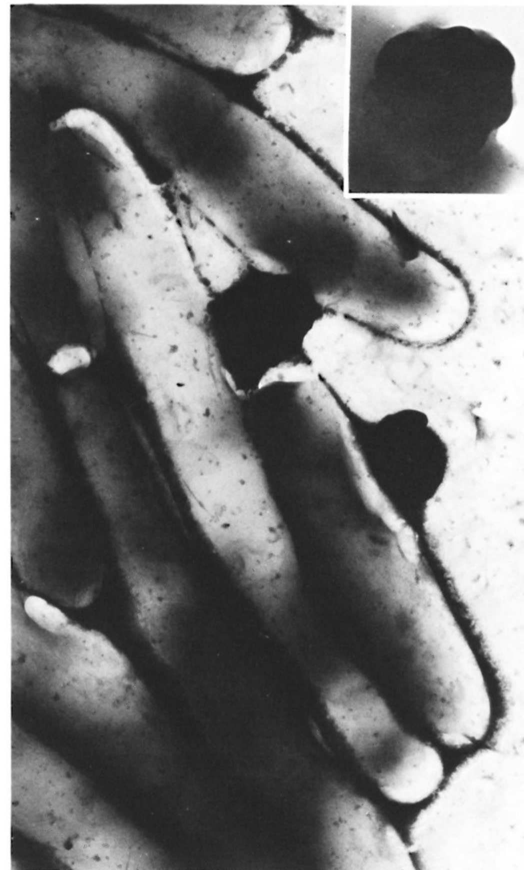

FIG. 4. Electron micrograph of "pigment"-containing suspensions of *M. leprae* negatively stained with potassium phosphotungstate. Inset = lobed particle of "pigment" in unstained preparation; contrast range of the inset micrograph has been reduced photographically by the technique of unsharp masking to reveal detail (×57,000).

THE TABLE. *Recovery of* M. leprae *from Percoll gradients.*

Fraction	Color	Counts[a] Run no. 1	2	3	Mean ± S.E.M.	Recovery % ± S.E.M.
Starting material	Brown	530	660	510	567 ± 51	100 ± 9
Fraction i (density 1.073)	Cream	430	680	480	528 ± 81	93 ± 15
Fraction ii (density 1.091)	Brown/cream	9.9	23	8.1	13.5 ± 4.9	2.4 ± 0.9
Fraction iii (density 1.105)	Brown/cream	0.22	1.4	3.6	1.9 ± 0.9	0.3 ± 0.2

[a] Numbers of bacteria in millions.

band i, containing the majority of the bacteria in a pure state, was pale cream; bands ii and iii were brown.

Unlike other procedures attempted earlier, discontinuous Percoll gradients provided high yields of pure *M. leprae*. As shown in The Table, well over 90% of the bacteria in the original suspension were present in band i. There appeared to be no morphological difference, assessed by light microscopy, between the bacteria isolated from this band and those in the original (contaminated) suspension or isolated from other armadillo tissues.

In this study, 4 to 6 $\times$ 10^8 *M. leprae* in 0.5 ml buffer were used. We have found that the method may readily be scaled up by using 4.5 ml (instead of 1 ml) of each of the concentrations of Percoll in the gradient and several milliliters of a concentrated *M. leprae* suspension. In one experiment, a 3 ml suspension containing 8.2 $\times$ 10^{10} *M. leprae* was successfully purified in one gradient. The banding in the gradients was similar to that seen in the small-scale experiments reported above. It is, therefore, possible to isolate large amounts of *M. leprae* from pooled contaminated suspensions with equal ease to the small scale originally studied.

SUMMARY

Mycobacterium leprae isolated from armadillo liver by the widely used IMMLEP protocol is sometimes contaminated with a particulate "pigment." This paper describes a simple, efficient, and rapid method for purifying large quantities of contaminated bacteria, which may readily be used as an additional step added at the end of the protocol when necessary. The process involves a discontinuous Percoll gradient and generates an essentially pure fraction containing >90% of the original bacteria, and a fraction of "pigment" slightly contaminated with bacteria. Use of the system should release large additional numbers of pure *M. leprae* suitable for use in human vaccine trials.

RESUMEN

Los *Mycobacterium leprae* aislados del hígado de armadillos por el ampliamente utilizado protocolo del IMMLEP algunas veces se encuentran contaminados con un pigmento particulado. Este trabajo describe un método simple, eficiente y rápido para purificar grandes cantidades de bacterias a partir de preparaciones contaminadas, el cual puede utilizarse como un paso adicional del proceso de purificación del *M. leprae*. El proceso involucra el uso de un gradiente discontinuo de Percoll y genera una fracción esencialmente pura conteniendo más del 90% de las bacterias originales, y una fracción del "pigmento" ligeramente contaminado con bacterias. El uso del sistema podría producir grandes números de *M. leprae* altamente purificado adecuado para su uso en ensayos de vacunación en humanos.

RÉSUMÉ

Mycobacterium leprae, lorsqu'il est isolé à partir de foie de tatou par la méthode prescrite dans le projet IMMLEP, est parfois contaminé avec un "pigment" spécial. Cet article décrit une méthode simple, rentable et rapide pour purifier de grandes quantités de bactéries contaminées. Le procédé peut être utilisé comme étape supplémentaire à la fin des manipulations, si nécessaire. Le procédé est basé sur un gradient Percoll discontinu; on obtient ainsi une fraction très pure contenant plus de 90% des bactéries utilisées au départ, et une fraction de "pigment" légèrement contaminé par des bactéries. Ce système devrait permettre de recouvrer des quantités supplémentaires importantes de *M. leprae* pur, en vue de leur utilisation pour des essais vaccinaux chez l'homme.

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REFERENCES

1. CASTANYS, S., OSUNA, A., GAMARRO, F. and RUIZ-PEREZ, L. M. Purification of metacyclic forms of *Trypanosoma cruzi* by Percoll discontinuous gradient centrifugation. Z. Parasitenkd. **70** (1984) 443–449.
2. DAS, P. K. and TULP, A. Use of a unit gravity sedimentation chamber for the purification of *Mycobacterium leprae*. Ann. Microbiol. (Paris) **133B** (1982) 389–400.
3. KIRCHHEIMER, W. F. and STORRS, E. E. Attempts to establish the armadillo (*Dasypus novemcinctus* Linn.) as a model for the study of leprosy. I. Report of lepromatoid leprosy in an experimentally infected armadillo. Int. J. Lepr. **39** (1971) 693–702.
4. MORI, T., MIYATA, Y., YONEDA, K. and ITO, T. Collection method for *Mycobacterium leprae* from infected armadillo liver. Int. J. Lepr. **52** (1984) 41–43.
5. PRABHAKARAN, K., HARRIS, E. B. and KIRCHHEIMER, W. F. Binding of ^{14}C-labeled DOPA by *My-*

cobacterium leprae in vitro. Int. J. Lepr. **44** (1976) 58–64.

6. REES, R. J. W. Limited multiplication of acid fast bacilli in the footpads of mice inoculated with *Mycobacterium leprae.* Br. J. Exp. Pathol. **45** (1964) 207–218.
7. SHEPARD, C. C., DRAPER, P., REES, R. J. W. and LOWE, C. Effect of purification steps on the immunogenicity of *Mycobacterium leprae.* Br. J. Exp. Pathol. **61** (1980) 376–379.
8. UNDP/WORLD BANK/WHO SPECIAL PROGRAMME FOR RESEARCH AND TRAINING IN TROPICAL DISEASES. Sixth programme report. Abstract in Indian J. Lepr. **56** (1984) 118–147.
9. WHEELER, P. R. and DRAPER, P. Soluble blue as a counterstain in the Ziehl-Neelsen procedure. A brief communication. Int. J. Lepr. **48** (1980) 15–17.
10. WORLD HEALTH ORGANIZATION. Purification of *M. leprae.* Annex 4 of Report of the Fifth Meeting of the Scientific Working Group on the Immunology of Leprosy (IMMLEP), Geneva, 24–26 June 1980. WHO Document TDR/IMMLEP-SWG(5)/80.3.