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Separation of *M. leprae* from Human Leproma and the Development of a Cytoplasmic Skin Test Antigen from Purified Bacilli [1, 2]

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The primary aim of these investigations was to develop an antigen (leprolin) from leprosy bacilli which would be more useful in testing for skin sensitivity than lepromin. Though lepromin may be a useful tool for clinical determination of the prognosis of a case of leprosy, it is of little value in epidemiologic studies where the purpose is to determine whether an individual has been exposed to *Mycobacterium leprae*. The development of purified protein derivative (PPD) from tuberculosis culture filtrate provided a primary tool for the epidemiologic study of tuberculosis and other mycobacterial infections where the etiologic agent could be grown *in vitro*. Similarly, a more specific skin test antigen for leprosy might help to identify factors involving individual and population susceptibility or resistance to disease, mode of transmission, carrier states and relationship to other mycobacterial infections. Until such an epidemiologic tool is available progress toward understanding these relationships, especially in populations where other mycobacteria abound, will continue to be slow. Since the organism cannot be grown *in vitro,* the source of specific antigens must be human or animal infections. In recognition of emerging work on the intracellular components of other mycobacteria, it was considered possible that such materials, proteins in particular, might prove more specific than PPD.

This paper reports a technic to separate and clean *M. leprae* from human tissue. Antigens were isolated by disruption and fractionation methods. The sensitivity reactions were tested in guinea pigs and humans.

MATERIALS AND METHODS

Preparation of suspensions. Human lepromata from untreated cases of lepromatous leprosy in Nepal were collected in two times the volume by weight of 2.5% acetic acid.[4] These fresh tissues containing unkilled *M. leprae* were sent by air to the Baltimore laboratory. By the time they made the trip from Nepal to the U.S., the tissue had digested to become quite soft and easily ground to a homogenous suspension ([11]). Bacterial contamination was rare. The diluent used throughout the purification procedure was 0.01% Tween 80 at pH 3.5.

After digestion in acetic acid for varying lengths of time, the epidermis was separated from the leproma, discarded, and the tissue was removed for grinding. Free mycobacteria were collected from the acetic acid by centrifugation at 17,300 × g for 30 minutes. These were washed twice with diluent and stored for enzyme digestion.

The tissue was chopped fine with scissors, homogenized in an omnimix for ten seconds at a concentration of 3 grams of tissue in 15 ml diluent.

The homogenate was diluted 50% and spun at 480 × g for 10 minutes. The supernatants were further diluted and spun at 1,800 × g for 20 minutes. The resulting supernatants were again combined and diluted further to be spun at 17,300 × g for 15 minutes. Mycobacteria which rose to the surface of the suspension at this speed (floaters) were collected and put aside for enzymatic digestions as were all collections of acid-fast bacteria (AFB) considered optimally clean of tissue. The sediments were assessed for AFB content and were dealt with in several ways. If microscopic examination showed:

1. Few AFB, the sediments were discarded.

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[4] Collected by Dr. J. R. Harris, Anandaban Leprosy Hospital, Kathmandu, Nepal.

2. Many free AFB (sinkers) and solubilized tissue, the sediments were further washed.

3. Heavy clumps of organisms and solid chunks of tissue organisms were separated from tissue by oil extraction ([10]). Olive oil, 1/2 the volume of the homogenized tissue to be extracted, was added and shaken vigorously by hand. The emulsion was allowed to stand several hours, then spun for 10 minutes at 480 × g. The oil phase and interphase were removed and spun at 20,000 × g for 20 minutes. Clear oil was removed, the sediment from this spin was quickly washed with ether to remove residual oils and spun at 3,020 × g for 10 minutes. Ether was decanted and discarded, the tube was placed in an ice bath and vacuum was applied for one hour to remove gas in solution.

The resulting suspension was stored for enzyme digestion. This oil emulsion procedure was performed on tissue homogenate until it was optimally free of AFB. Figure 1 summarizes the steps taken.

Microscopic examination was performed on all preparations at each step and a judgement was made about how to proceed.

The staining procedure which proved most useful in estimating the ratio of acid-fast bacilli to other materials in media or tissue homogenate involved formalin fume fixation, decolorization with 5% sulfuric acid for two minutes and counterstaining with .13% nile blue sulfate in 33% alcohol for 15 seconds ([5]). This method maximized the nonmycobacterial elements in the film and provided a good basis for judging the bacteriologic purity of the suspension.

Enzymatic digestion. All batches of partially purified organisms were combined for enzyme digestion. The first stage was 1/4 hour in 1% trypsin, pH 8, at 40°C. Trypsin was made up in a 2% solution, sterilized by passage through a .45 μ filter and added in equal volume to the homogenate. The suspension was then brought from its acid state to pH 8 by the dropwise addition of 1 N NaOH while being agitated on a magnetic stirrer. Timing of the digestion was started when pH 8 was attained. After 15 minutes the suspension was spun at 20,200 × g and refrigerated for 1/2 hour. Floaters were lifted from the surface with a pipet, trypsin solution was removed to be spun again to further collect floaters which resuspend quickly, and sediment was washed twice in 0.01% Tween 80. Each time the sediment was washed or the diluent was spun more floaters appeared. They were collected in one tube and concentrated by spinning and removing fluid from under them, a laborious procedure. The sediment, after washing free of trypsin, was resuspended in 0.01% Tween 80. It became progressively more difficult to keep the floaters from sticking to glass.

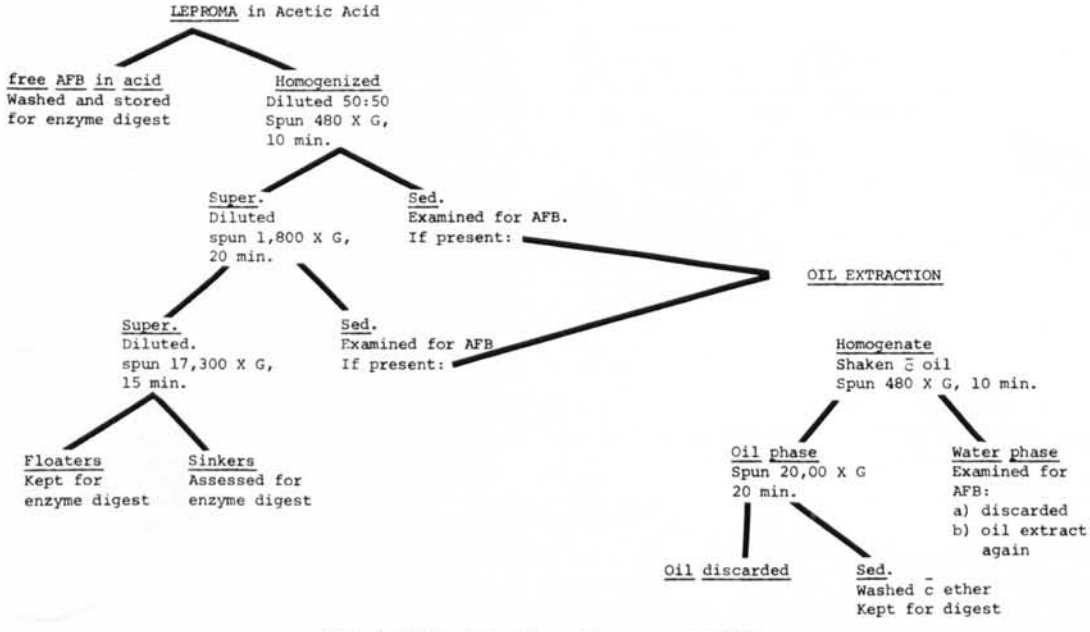

FIG. 1. Flow chart for antigen preparation.

After washing to remove trypsin the homogenate was digested with 1 mg/ml lipase for one hour at pH 7.3 and 37°C. Again, the pH was adjusted after the addition of the sterilized enzyme to the bacillary suspension.

The digest was washed as before, separating and washing the floaters and sinkers independently in preparation for pronase digestion, 100PU/ml, pH 8 at 37°C for three hours.

Final washing with 0.01% Tween 80 yielded two grades of purified acid-fast organisms.

Floaters. Occurred sometimes singly, sometimes in massive waxy balls, completely free of any blue staining material. Acidified washes of these suspensions were free from precipitate when heated. These were judged to be pure mycobacteria.

Sinkers. Masses of acid-fast organisms intimately associated with the small matrix of blue fibrous tissue residue amounting to less than 15% of the total content. Acidified washes from these suspensions were also free of precipitate when heated. These were judged to be adequately pure for use in antigen preparation. At no time were the bacteria exposed to heat above 40°C. The above steps are summarized in Figure 2.

Fractionation of bacterial cells. *M. leprae.* The pH of the purified suspension was adjusted to 3.5 and the suspensions were subjected to sonic oscillation at 4°C in a Raytheon model DF 101 for 40 minutes. On microscopic examination, the resulting liquid was uniformly granular with no intact acid-fast organisms visible. It was felt that the lack of viability of any possible remaining organisms was ensured by the successive chemical treatment by ether, enzymes and the dialysis which followed. The sonicate was spun at 10,000 × g for one hour. An aliquot of the resulting supernatant was stored for protein assay. The supernate was termed IC_1 (intracellular component) and the sediment $shell_1$ (see Fig. 3). The remainder of the supernatant and the sediment were dialysed against distilled water at 4°C for three days. The volume of the dialysate was reduced further by dialysis against carbowax 20 M for 10 hours, a reduction of half the volume.

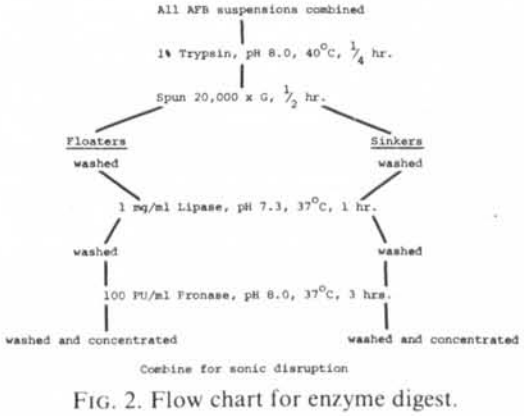

FIG. 2. Flow chart for enzyme digest.

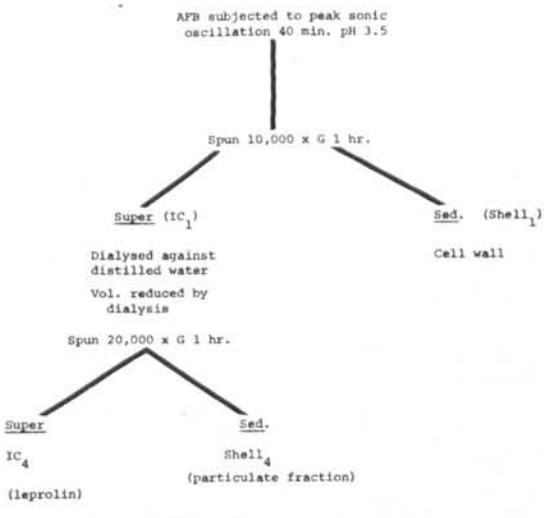

FIG. 3. Flow chart for fractionation.

The dialysed sonicate was spun at 20,000 × g for one hour, the resulting supernatant termed IC_4 (leprolin), a preparation that could be considered free of cell wall material, and $shell_4$ (particulate fraction) composed of materials such as fragments of cell membrane. All antigens were assayed for protein content and adjusted to a predetermined level. The fractions were then lyophilized and stored at 4°C until used.

R_1R_v. A thick suspension of three week old R_1R_v cells grown on Sauton's ([20]) synthetic medium was subjected to sonic oscillation for one hour, washed three times and used for sensitizing animals and fractionation. Fractions IC_1 and $shell_1$ were prepared as described above. Each was assayed for protein content and used as a test antigen in guinea pigs.

Skin tests. Twelve female guinea pigs weighing about 500 grams were maintained on a stock diet of Purina guinea pig chow. The animals' backs were shaved with electric clippers every four days. The 12 animals were numbered consecutively. Three were removed at random to form an unsensitized group. The nine experimental animals were

sensitized to $R_1 R_v$ by means of 0.1 cc intradermal inoculations of 6.5×10^7 organisms into the left shoulder at day one, and the right shoulder at day twenty-one. Animals were tested with the $R_1 R_v$ fractions one month after the second sensitizing inoculation. At the time of testing the three control animals were replaced into the order. Test antigens were delivered intracutaneously along the right and left sides of the spine. Readings were taken of induration at days 1, 2, 3, 9, 13, 16 and 22. The shape of each area of induration was viewed as equivalent to an elipse with measurements taken in millimeters of the major and minor diameters. The mean of these two measurements was used for analysis. The antigens and the sites were coded so that both the administration of the test and the readings were conducted blind.

Human volunteers with typical lepromatous and tuberculoid leprosy were recruited from the Christian Leprosy Mission in Karigiri, Madras and from the leprosy village of Simonpur, Purulia District, West Bengal.

Twelve tuberculoid and twelve lepromatous leprosy patients being treated in Karigiri agreed to receive tuberculin (5 TU), whole sonicate of *M. leprae* (70 μg/dose), IC_1 (66 μg/dose), and $shell_1$ (4 μg/dose). Antigens were given in the volar surface of the forearm and were read blind daily as described above at days one through thirteen and again at three weeks.

Fourteen tuberculoid and 14 lepromatous cases of leprosy from the village of Simonpur participated in testing the following fractions of *M. leprae:*

IC_1	8.0 μg/dose
leprolin	5.1 μg/dose
particulate fraction	4.0 μg/dose

The antigens were injected and read as described above at days one through eighteen. In order to establish a dosage level, the 14 tuberculoid patients mentioned above, established as "strong reactors," were tested with leprolin at doses of 10 μg, 20 μg, 25 μg, and 50 μg; and with particulate preparations of 8 μg and 16 μg protein concentration.

To measure skin test response in individuals not exposed to leprosy, cooperation was sought from members of classes 4 and 9 of a Ludhiana Khalsa school. All the boys were examined for BCG scars. Thirty-seven class 4 members (ages 9-10) and 38 class 9 members (ages 15-18) were given tuberculin PPD-S (5 TU), leprolin (20 μg) and *M. leprae* particulate fraction (5 μg) intracutaneously in the forearm. Readings were made 12 times between days one and twenty, as described above.

RESULTS

Purification of M. leprae. During attempts at removing tissue antigens from leproma homogenates, it was found that maintaining the suspensions in an acid state, as they were received from the collection site, to the point of enzyme digestion avoided repeated protein and collagen floculation that occurred whenever pH changed from acid to basic or vice versa. Bacteria were then more easily washed free of tissue elements and not trapped in fibrous masses.

As the purification method proceeded the phenomenon of floaters occurred. Through successive washings an increasing "scum" of milky suspension appeared at the meniscus. Increased speed of centrifugation only served to concentrate the milky layer at the top. On microscopic examination, this milky suspension proved to be composed of strongly acid-fast organisms, usually suspended singly. This characteristic of remaining in suspension without clumping, however, seemed to change under certain conditions.

1. If the original leproma was somewhat fatty, releasing lipids into the system, the floaters seemed to clump. When this happened, they also tended to stick to the glass of test tubes and pipets at the level of the highest meniscus.

2. Standing in suspension over a period of time frequently resulted in the adherence of the "free" floaters to the glass.

3. Freezing and thawing of lepromas before extraction resulted in an increased lipophilic:hydrophobic tendency of the organisms. Clumping and sticking to utensils then became a severe problem in the attempt at purification.

4. Each enzymatic digestion produced more floaters and the tendency to clump and stick increased. It was postulated that as the purpose of each digestion was achieved, i.e., removal of tissue proteins adhering to the waxy coats of the mycobacteria, the organisms were rendered more hydrophobic and harder to maintain in an aqueous system. The addition of Tween 80, 0.01%, did little to "wet"

these waxy organisms or conglomerates. The only treatment that was really successful in removing the aggregations from utensils was sonic oscillation of the equipment. Sonication freed the organisms, eventually breaking them into unrecognizable particles. As the process progressed, Tween 80 seemed to be liberated into the system as evidenced by a reappearance of foaming in the suspension.

When working with aliquots of the same tissue, some of which had been heated, and some of which had not, it was observed that the bacilli in the unheated suspensions tended to clump and stick more than those in the heated suspensions. This perhaps contributes to the explanation of why floaters and sinkers have not been described in the literature. Both floaters and sinkers released protein into solution on sonic disruption. They were combined for final antigen preparation.

Fractionation of mycobacteria. The most successful method for cell disruption was found to be the Raytheon sonic oscillator by the technic described above. After breaking the cells the first concern was to separate the waxy mycobacterial cell walls from what was termed cytoplasmic components. The differential centrifugation procedure was ultimately developed by field testing preparations as they were produced.

Skin tests. The first antigens tested were made from actively growing R_1R_v and used in guinea pig skin tests. Test antigens were those tabulated in Table 1. As can be seen in Figure 4, fraction IC_1 produced a greater difference between control and sensitized animal mean reaction size at 48 hours, than the whole organism preparation or $shell_1$. $Shell_1$ (cell wall) also seemed to produce a reasonably good differentiation but the standard deviation from the mean in the control and experimental groups indicated that the two populations overlapped considerably. The observation of greatest interest was that separation of the waxy mycobacterial coat from smaller and soluble components seemed to enhance the antigenicity of each fraction. Another apparent difference that appeared in this comparison was that while the response to the whole cells seemed to be following the typical bimodal pattern with a later second peak, the sizes of reactions to the two fractions maintained a significant level for three weeks, only slowly decreasing.

The first human trials of these antigens were performed on a group of leprosy patient volunteers. These tests were an attempt at determining whether what was termed the cytoplasmic extract from purified *M. leprae* would produce reactions in lepromatous and tuberculoid cases of leprosy that paralleled the lepromin reaction. It was assumed that little or no reaction in lepromatous cases and significant reaction in tuberculoid cases

TABLE 1. *Skin test reactions in guinea pigs sensitized to* R_1 R_v (Mycobacterium sp.) *to 5 antigens made from the sensitizing organism measures by x diameter in mm.*

Antigen	μg/dose	Sensitized Animals 1	2	3[a]	9[b]	13	16	22
Whole R_1R_v	82	12	10	9	—	8	7	7
(SD)		(2.0)	(1.3)					
IC_1	58	17	14	11	10	—	7	6
(SD)		(2.1)	(1.8)					
$Shell_1$	12	14	13	11	9	8	8	6
(SD)		(2.3)	(2.5)					
		Controls						
Whole R_1R_v	82	5	6	6	5	6	7	9
(SD)		(0.8)	(2.6)					
IC_1	58	8	7	6	7	8	8	6
(SD)		(1.3)	(1.3)					
$Shell_1$	12	7	7	6	8	8	8	6
(SD)		(1.7)	(1.3)					

[a] All sites ulcerated. Pus sterile on culture.
[b] Most ulcers healed.

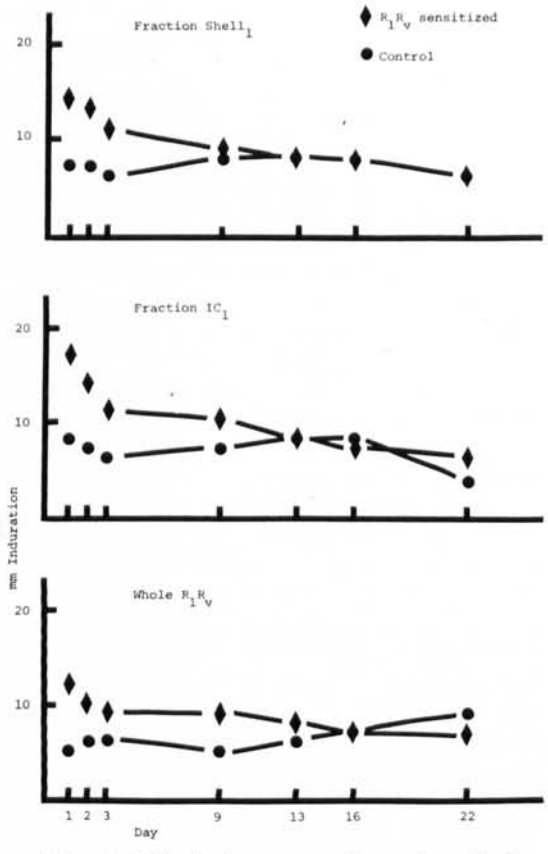

FIG. 4. Skin test responses to various R_1R_v antigens in guinea pigs.

would be expected, since these are the typical responses to the lepromin test.

Figure 5 shows the mean reaction size for whole sonicate, IC_1, and $shell_1$ in the two types of patients. With a size of 3 mm diameter defined as a positive reaction, 75% of the tuberculoid patients produced positive 48 hour reactions to the IC_1, with a mean response of 6.08 mm induration.[5] Sixty-six percent of the tuberculoid patients had positive reactions to the cell wall preparation ($shell_1$) while 42% were positive to the whole sonicate. A slight increase in reaction size appeared between five days and two weeks in all mycobacterial antigens used. Reactions to the cell wall persisted longer in both lepromatous and tuberculoid patients. The most interesting observation in this trial, however, was the increased antigenicity of the sonicated *M. leprae* after the cell walls had been removed.

The next series of tests were undertaken as part of an attempt to identify a group of tuberculoid cases who would agree to being tested at various intervals with different antigens. Fourteen individuals with tuberculoid leprosy and 14 with lepromatous leprosy were located.[5] The antigens used in this series were *M. leprae* IC_1, leprolin, and the particulate fraction. The small bimodal profile with an early and slight second peak previously mentioned can be seen in Figure 6. The antigen containing the largest amount of particulate material produced reactions of the longest duration. The tuberculoid pattern of response to each of the antigens was very similar. Though it was at a low level, the size of reaction and pattern of time profile was clearly different from that of lepromatous individuals. With an induration having a 3 mm diameter or more considered positive, 79% of the tuberculoid cases of leprosy were positive reactors to IC_1, 86% were positive to the leprolin and 93% were positive to the particulate antigen ($shell_4$). Lepromatous reactors were 0%, 18.8% and 12.5%, respectively, to the above antigens.

Other series of tests of the two *M. leprae* antigens, leprolin and particulate fraction, were undertaken with the cooperation of the "good reactor" group of tuberculoid patients. Figure 7 shows different reaction levels to the above two antigens associated with increasing protein content. The level chosen for a standard dose of leprolin was 20 μg primarily because it was the lower level at which 100% of the test individuals gave reactions above 5 mm and the standard deviation indicated that "good reactors" would produce such a reaction 95% of the time. Though 25 μg would seem to produce the next greatest increment in reaction size, with a smaller standard deviation, the material was so hard to produce that the decision was made to use the lower dosage in order to increase the number of tests available for use.

Five micrograms of protein per dose was chosen as the appropriate level for testing the small particulate fraction. Since clear differences in response to the antigens thus far tested were observed in people with varying types of leprosy it became necessary to determine the reaction in people never exposed to the disease. Specificity was also of interest in distinguishing between BCG vaccinated participants and those not sensitive to PPD-S, since large numbers of people in leprosy endemic areas of India have been vaccinated

[5] Skin tests read by Dr. Helen Gideon.

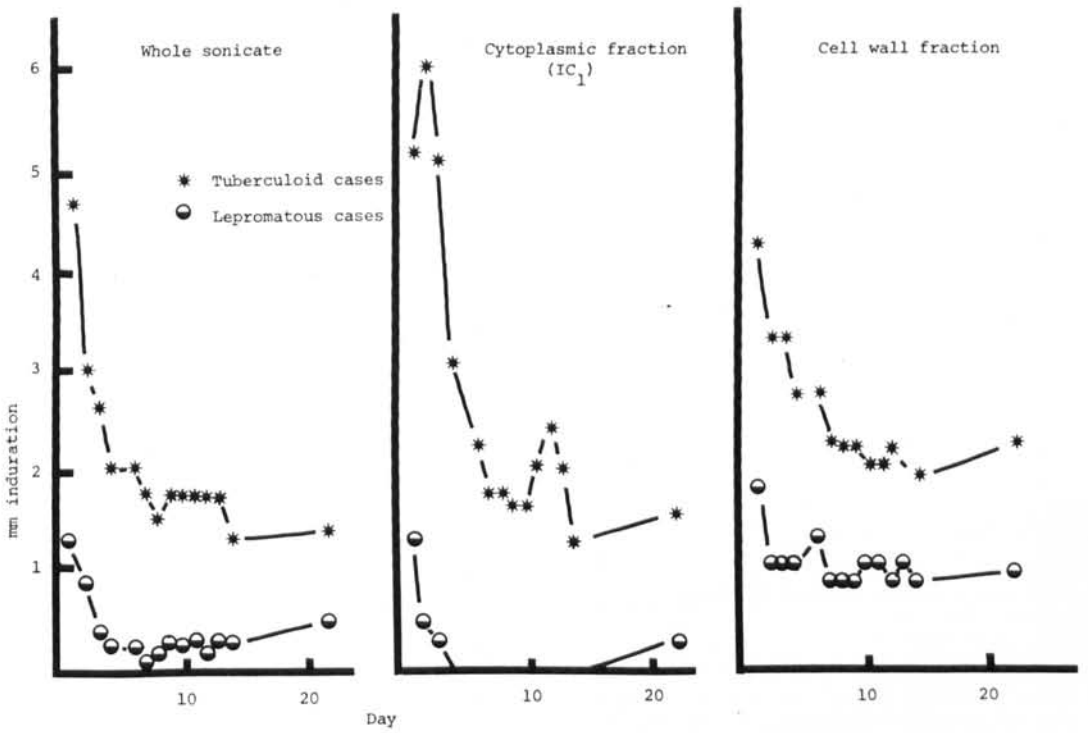

FIG. 5. Time profiles comparing mean skin reactions to three *M. leprae* antigens in 12 tuberculoid and 12 lepromatous cases of leprosy.

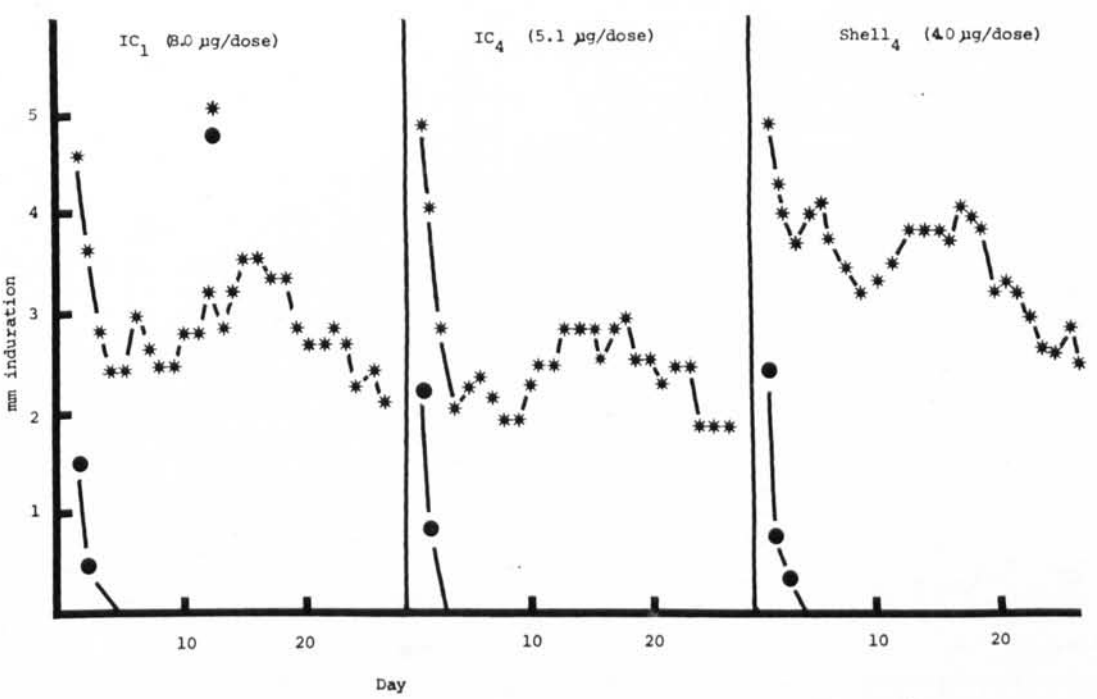

FIG. 6. Time profiles comparing mean skin reactions to three *M. leprae* antigens in 14 tuberculoid and 14 lepromatous cases of leprosy.

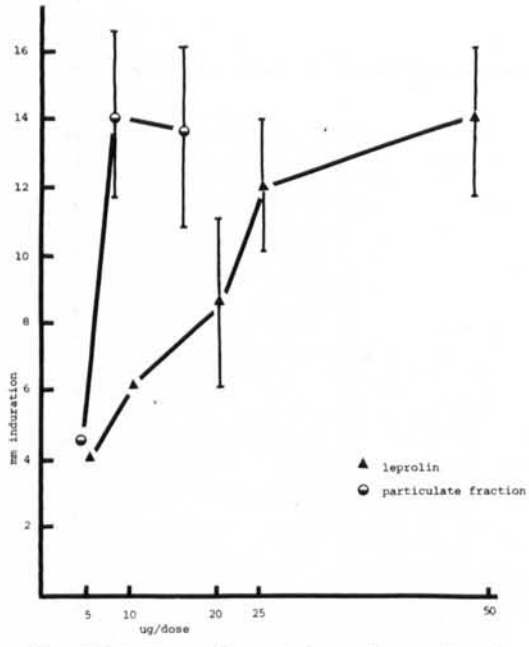

FIG. 7. Mean reactions (48 hrs) to increasing dosages of two *M. leprae* antigens in tuberculoid leprosy patients identified as "good reactors." Standard deviations plotted around points.

against tuberculosis. Boys enrolled in the Ludhiana Khalsa school seemed to be an appropriate group of volunteers after permission was obtained from their parents. All the members of class 9, boys aged 15-18 years, had received BCG vaccination two years prior to this study. They were given 5 TU PPD-S and vaccination was confirmed by locating BCG scars. Since leprosy is very rare in Punjabi plains dwellers, it was assumed that if a young man had spent his entire life in a village near Ludhiana, he had never been exposed to leprosy. On careful history taking from each boy, however, it turned out that the majority of them had moved to the Ludhiana area from what became West Pakistan in 1947. Twelve of them had spent extended periods at young ages living in resettlement camps under very difficult conditions and under increased likelihood of exposure to leprosy.

All the boys in class 4, ages 9-10 years, of the same school were given 5 TU PPD-S. None of these children had lived in refugee camps. All those with 48 hour reactions of greater than 5 mm were excluded from the experimental group (N = 23).

The two *M. leprae* antigens used were leprolin (20 μg/dose) and the particulate fraction (5 μg/dose). Figure 8 shows the pattern of response of each group to each antigen. The pattern of reaction to the particulate antigen shows a clear difference in level of response between groups, both groups showing a bimodal curve with the second peak around two weeks that had been observed in tuberculoid patients and guinea pigs. The size of the response at 48 hours, as well as of reaction in the BCG group may indicate a cross-reactivity with BCG. On the other hand, the relatively large response in the tuberculin negative group may have been the result of cross-reaction to another mycobacterium in the population not tested for here. The particulate antigen produced reactions of 5 mm or greater in 27/35 (77%) PPD-S positive individuals and 13/37 (35%) in those who were PPD-S negative. This also tended to implicate another organism as producing sensitization and contributing to nonspecific response.

Both groups had a low level response to the soluble component (leprolin) and the decline of that response was rapid in each group. The peak of the curve of BCG vaccinated individuals may have been indicative of some recognition of mycobacterial antigens

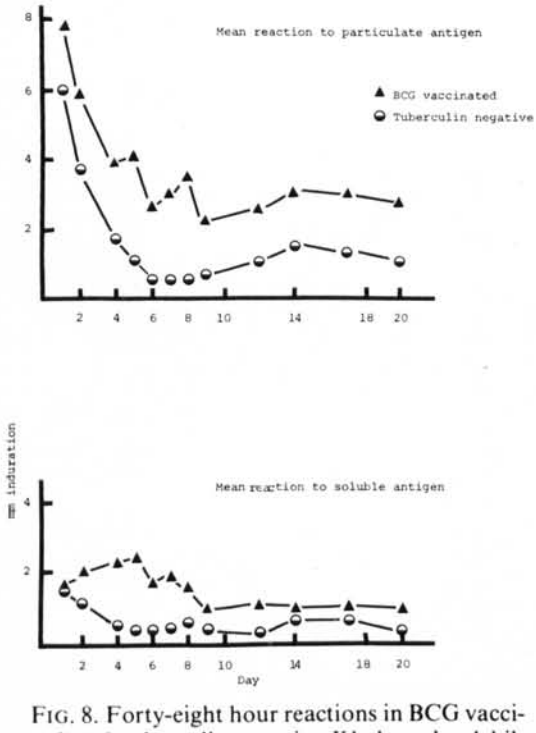

FIG. 8. Forty-eight hour reactions in BCG vaccinated and tuberculin negative Khalsa schoolchildren.

in that group. It can hardly be considered significant cross-reaction at such small sizes, however. Of the BCG vaccinated group, 4/30 (13%) had reactions of 5 mm or larger to the soluble fraction, only one of which was over 6 mm in size (Fig. 9). This individual produced a reaction to the particulate fraction significantly larger than the others and had a PPD-S reaction 12 mm larger than the mean response of the group. This boy was from a family which had moved from Pakistan Punjab in 1947 but had not spent time in refugee camps. The response of the boys in the tuberculin negative group to the soluble antigen was at a very low level, 3/35 (10%) $\geq$ 5 mm but none exceeding 6 mm.

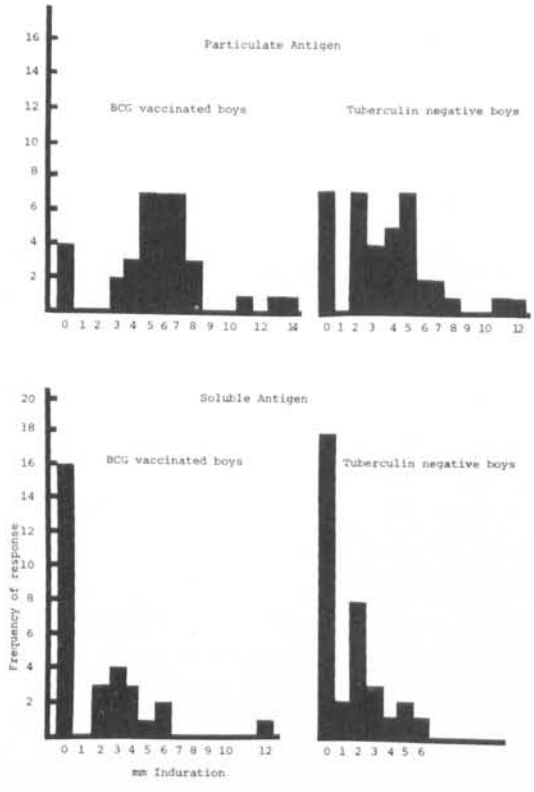

FIG. 9. Frequency distribution of 48 hour reaction sizes to *M. leprae* antigens in Khalsa schoolchildren.

The soluble cytoplasmic component of disrupted *M. leprae* was confirmed as being the most active and specific antigen examined, not producing a significant cross-reactivity with BCG.

DISCUSSION

In the search for a skin test antigen for use in leprosy investigations, much ongoing controversy over the value of lepromin as an epidemiologic tool has been generated. Kooij ([13]) and Davey ([4]) in 1956 and 1958 challenged the theory that lepromin reactivity is caused only by the presence of mycobacteria. Kinnear Brown ([1]), while he objected to the methods and conclusions of Kooij *et al,* expressed the need for an antigen from *M. leprae* which would be comparable to PPD. Failing such a specific preparation, he felt that whole killed *M. leprae* free of all human tissue detritus could be compared to whole killed tubercle bacilli.

Taylor and Hanks in 1960 and 1963 ([27, 28]) demonstrated that the human tissue components of lepromin were antigenically active when assayed in guinea pigs sensitized to lepromin and measured by skin testing with a preparation containing human tissue antigens but no mycobacteria components. In 1968, Goihman-Yahr *et al* ([7]) concluded that "the marked and persistent tissue response induced by lepromin is the result of its bacillary component" on the basis of gross and histological changes in foot pads and popliteal lymph nodes of test guinea pigs. In 1969 ([8]), the same investigators found no antibodies to human skin in pooled sera from sensitized guinea pigs either by diffusion tests of indirect fluorescent antibody tests. They did, however, find cross-reactivity between lepromin and BCG as well as other mycobacteria ([6]) which was attributed to the mycobacterial content of lepromin.

Rees ([21]), in examining the relation of sera from leprosy patients to various antigens by the Ouchterlony technic, showed precipitation bands to *M. tuberculosis* greater than *M. leprae.* Using anti-*M. leprae* serum, strong bands formed to fresh *M. leprae* and weak bands to normal skin antigens.

In 1967, Shepard and Saitz ([26]) demonstrated lepromin positivity in 73 inmates of the Atlanta Georgia Penitentiary, none of whom had been exposed to *M. leprae.* Slight positive correlation was noted between positive Mitsuda and positive PPD response, but no correlation was found between Fernandez and PPD responses. The authors reviewed the literature and concluded that a positive lepromin reaction is due to previous exposure to *M. leprae, M. tuberculosis,* or some other mycobacterium, though they pointed out that in this study lepromin positivity was not due to tuberculosis. They quoted Doull,

Guinto and Mabalay as saying that BCG converts 33% of children to lepromin positivity after 100 days and theorized that a potent antigen would cause more conversion.

Schuppli [24] in 1971, observed that though the great majority of healthy adults in large populations of middle Europe were lepromin negative, there was no significant difference between people from endemic or nonendemic areas. He also observed that active tuberculosis patients had stronger lepromin test responses than tuberculins.

Leiker [16] in 1968, discussed the role of human tissue antigens in relation to the ability of people, both healthy and with leprosy, to react to normal skin suspensions. He observed that mycobacteria and normal tissue seem to have one or more common components.

The interpretation of the leprosy history of a population through use of lepromin is clearly less than satisfactory. It is for this reason that these investigations have focused on development of an adequate antigen purification technic. Lepromin, early in preparation is boiled or autoclaved. Since it is well known that heat denatured protein has an altered antigenicity [29], this purification technic has been applied to unheated *M. leprae*. Numbers of *M. leprae* sufficiently large to yield a workable amount of material after fractionation were until recently only available from lepromatous leprosy cases, also the source of lepromin. It is interesting that most investigators, in attempts to purify the mycobacteria, start the process by boiling or autoclaving as though preparing lepromin. In view of the altered antigenicity of heat denatured protein, one would expect greater attempts at nontraumatic separation of tissue and mycobacterial substances. Henderson [10] in 1942, developed a technic based on the lipophilic nature of *M. leprae* which has proven useful in these investigations. Navalkar [19] in 1971, demonstrated five or more antigens in a suspension of disrupted *M. leprae* cells which were never heated during purification. Most investigators when looking for common antigens of mycobacteria do so by using test preparations from *in vitro* grown organisms; they sensitize animals to lepromin [6-9], if they include *M. leprae* as one of those organisms under scrutiny at all.

Laszlo Kato [12] in 1971, described a purification procedure directed at removing "growth inhibitors" from culture inocula of *M. leprae* and *M. lepraemurium*, two noncultivable mycobacteria.

Experience in the attempts reported here to purify *M. leprae* has brought recognition of several problems not apparently observed by Kato. The first of these was the extensive floculation of tissue materials producing a new solid mass each time a homogenate was passed from acid to basic pH and back. For this reason the pH was kept around 3.5 until enzyme digestion was undertaken. At that point the enzyme in acid state was mixed with the homogenate and the pH adjusted appropriately for each enzyme while the solution was being mechanically stirred at the appropriate temperature. When the bacteria were being washed after enzyme digestion the pH of the Tween 80 solution used was neutral. It was during these washes that another phenomenon not mentioned by Kato was most apparent, that of the increasing numbers of floaters, or hydrophobic organisms.

It has long been known that mycobacteria consist of many antigens and that the more species specific of these seem to be located in the protoplasm of the organisms [15, 22]. It has also been known that antigens specific to mycobacterial strains can be precipitated from the culture filtrate in which the organism was grown [25]. Much concern about specificity vs. cross-reactivity among mycobacteria has, however, developed since the time when it was considered that a skin test reaction of larger than 5 mm, or 8 mm, or 10 mm to PPD-S was indicative of tuberculosis infection. Since the PPD method of precipitating a specific antigen from media was obviously impossible in the pursuit of a specific antigen of *M. leprae* the problem of fractionating the organism itself was undertaken. It was also postulated that a specific antigen obtained from the mycobacterial cell would be more sensitive in detecting previous experience with the organism than an antigen isolated from culture media.

Larson and Ribi [14, 22, 23] described the production of two fractions of *M. tuberculosis* and *M. butyricum* suspensions by use of a Mickle Tissue disintegrator and a pressure cell. In these studies the cell walls and protoplasm were separated by differential centrifugation and tested in rabbits. The antigen preparations were rid of any remaining via-

ble organisms by heating at 75°C for 45 minutes and ether saturation. They showed that 20-50 μg of protoplasm in animals sensitized to cell walls, killed whole cells or live cells produced leage erythematous lesions within 24-48 hours which then disappear within 48 hours. Cell wall preparations at 20 μg produced lesions with necrotic centers and scarring as well as hypersensitivity.

Various investigators have used different technics for disrupting mycobacteria. Castelnuovo [2] froze the cells and broke them in a ball mill. She centrifuged this material at 6,000 × g to remove cells and cell walls. Common and specific antigens were demonstrated in the protoplasm by immunodiffusion. Morisama *et al* [18] produced a tuberculin active peptide from whole $H_{37}R_v$ cells by extraction of acetone dried cells with 0.1 N HCl at 37°C for 72 hours and subsequent purification of the material extracted. They, however, killed the virulent tubercle bacilli by heating at 100°C for 30 minutes before extraction.

These present studies applied sonic oscillation which produced good disruption of cells as evidenced by microscopic evaluation. Experience with protoplasm and cell walls was similar to that of Larson in that the protoplasmic fractions of mycobacteria produced distinct hypersensitivity reactions in sensitized guinea pigs (Figs. 4, 5, and 6). The mean reaction to protoplasm was higher than that for whole cells. The cell wall fractions ($shell_1$ and $shell_4$) produced larger reactions in controls thus obscuring detection of the sensitized group. The dose levels necessary to produce adequate skin response were much higher for the protoplasmic fractions than the cell wall fractions (Figs. 4 and 7).

Contrary to Larson's [14] experience with other mycobacteria, those reactions produced by the fraction comparable to his protoplasmic fraction did not disappear in 48 hours. The protoplasmic fractions prepared in this study gave evidence of a slight second peak of response at 10-14 days both in guinea pigs and humans, sensitized and unsensitized, for all mycobacteria used. The necrosis mentioned by Larson in the cell-wall induced lesions was confirmed. Whatever differences were observed could have been due to differences in procedures. Larson fractionated cells by a press, measured content by dry weight, and measured specificity of response by the minimum dose necessary to produce a reaction 10 × 10 × .4 mm of erythema in rabbits while in these studies disruption was caused by sonic oscillation, content measured by protein determined by Lowry's method [17] and specificity measured in millimeters of induration in guinea pig skin.

The observation of increased specific antigenicity as protein purification improved (Fig. 5) confirmed the observations of Counts and Kubica [3] in their investigations of protoplasmic mycobacterial extracts as a source of skin test antigen.

A final comment relating to the eventual usefulness of this procedure is that the preparation of leprolin was previously sharply restricted by the difficulty of obtaining fresh lepromatous tissue. The possibility of using an armadillo source now needs to be explored.

SUMMARY

A successful method for purification of *M. leprae* from human leproma without subjection to heat has been developed.

The "floater" phenomenon has been described which consists of bacillary tendency to float in the supernate when bacilli which are not autoclaved are separated from tissues by enzymatic digestion.

A method for preparing cytoplasmic fractions from purified *M. leprae* has been developed for the production of a skin test antigen for leprosy. The cytoplasmic fraction of *M. leprae* elicited positive skin test responses in people with tuberculoid leprosy and negative responses in lepromatous leprosy.

Cytoplasmic preparations from purified *M. leprae* had little cross-reactive relationship with the organism BCG.

The small particulate fraction elicited positive reactions in PPD-S negative as well as BCG vaccinated individuals.

RESUMEN

Se ha desarrollado un método exitoso para la purificación de *M. leprae* de lepromas humanos sin necesidad de calentamiento.

El fenómeno de "flotamiento" descripto consiste en la tendencia bacilar de flotar en el sobrenadante cuando los bacilos no sujetos a la esterilización en el autoclave, son separados de los tejidos mediante la digestión enzimática.

Se ha desarrollado un método para preparar fracciones citoplasmáticas de *M. leprae* purificados para la producción de una prueba antígeno cutanea de lepra.

La fraccion citoplasmática de *M. leprae* produce respuestas cutaneas positivas en pacientes con lepra tuberculoide y negativa en aquellos con lepra lepromatosa.

Preparaciones citoplasmaticas de *M. leprae* purificadas tuvieron poca reactividad cruzada con el organismo BCG.

La pequeña fracción particulada produce reacciones positivas en PPD-S negativos y en individuos vacunados con BCG.

RÉSUMÉ

On a développé une méthode qui permet de purifier avec succès *M. leprae* obtenu de lépromes humains, et ceci sans les soumettre à la chaleur. Le phénomène de "floater" a été décrit. Ce phénomène consiste en la tendance que montre les bacilles à flotter dans le liquide surnageant, lorsque des bacilles qui n'ont pas été autoclavés sont séparés des tissus par digestion enzymatique.

On a developpé une méthode qui permet de préparer des fractions cytoplasmiques de *M. leprae* purifié en vue de produire un antigène pour épreuves cutanées dans la lèpre. La fraction cytoplasmique de *M. leprae* a entraîné des réponses positives de l'épreuve cutanée chez des individus atteints de lèpre tuberculoïde, alors que les individus lépromateux réagissaient négativement.

Des préparations cytoplasmiques de *M. leprae* purifié ont présenté une relation d'immunité croisée faible avec le bacille du BCG.

La petite fraction particulée a induit des réactions positives chez des individus négatifs pour le PPD-S, aussi bien que chez des individus vaccinés par le BCG.

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