Specific Direct Fluorescent Antibody Identification of *Mycobacterium leprae*[1]

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In attempting to cultivate *M. leprae in vitro*, a major problem is the identification of the cultivated bacillus as *M. leprae*. Some of the inherent problems associated with mooted technics are outlined elsewhere ([18]). In monitoring the development of cultures, it is highly desirable to utilize a method which is applicable to the presence of relatively few bacilli, that is rapid enough to follow progress from culture to subculture, and that is so specific as to lend confidence in its results. There seemed available only one possible approach that would satisfy these requirements, and that lay in the technic of immunofluorescence.

The early work of Dharmendra ([6, 7]) and more recent work, particularly that of Abe ([1, 2, 4]) and of Navalkar ([14–17]) and their associates, strongly indicated that specific identity of *M. leprae* probably lies with its protein or protein-complexed antigens rather than in the polysaccharide moieties. Abe's indirect fluorescence technic appeared promising but presented a possible problem in application as to its specificity. A direct method was desirable but presented another problem in that the resulting fluorescence was weak and, therefore, difficult to evaluate with certainty and, in the same tenor, was too weak to record photographically with available microscopic equipment, even with film speed boosted to ASA 1200.

In the face of these problems, the direct immunofluorescence technic described herein was developed and, after its specificity was determined, was reinforced with indirect, secondary, fluorescence coupled serum, for photographic purposes.

The procedures used, as here described, were dictated essentially by two factors. The first was the limited amount of lepromatous serum available in our laboratory bank since we had not anticipated a need for becoming involved in the development of this technic. The second factor lay in the fact that the newness of the approach required trial and error possibilities. The method now described is, therefore, presently being refined, but as it stands was the method utilized for monitoring the alleged cultures of *M. leprae* as described elsewhere ([19]).

MATERIALS AND METHODS

For ease in understanding, a simplified schema is presented in Figure 1.

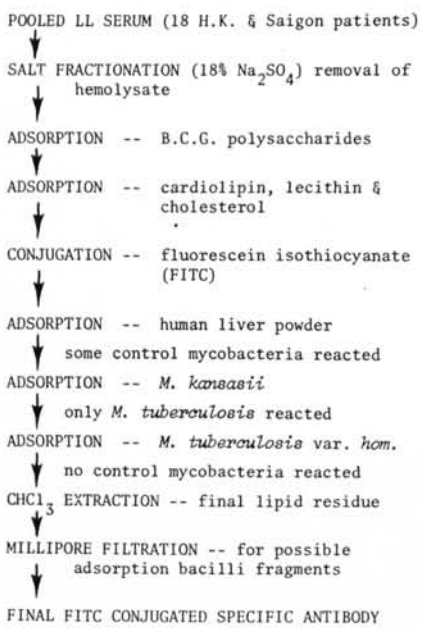

FIG. 1. Specific FITC conjugated antibody preparation.

Salt fractionation of leprosy patient serum. One half to three milliliters each of 18 lepromatous leprosy patient sera obtained in Hong Kong, Saigon and locally in Honolulu, and stored at 0°C for 7 to 14 months, were pooled after inactivation at 57°C for 30 minutes followed by the addition of thymerosal (1/10,000). Ten milliliters of the pooled serum (LL serum) were partially purified to a crude globulin fraction (LL globulin) by a modification of Kekwick's procedures ([10]), as shown in Figure 2.

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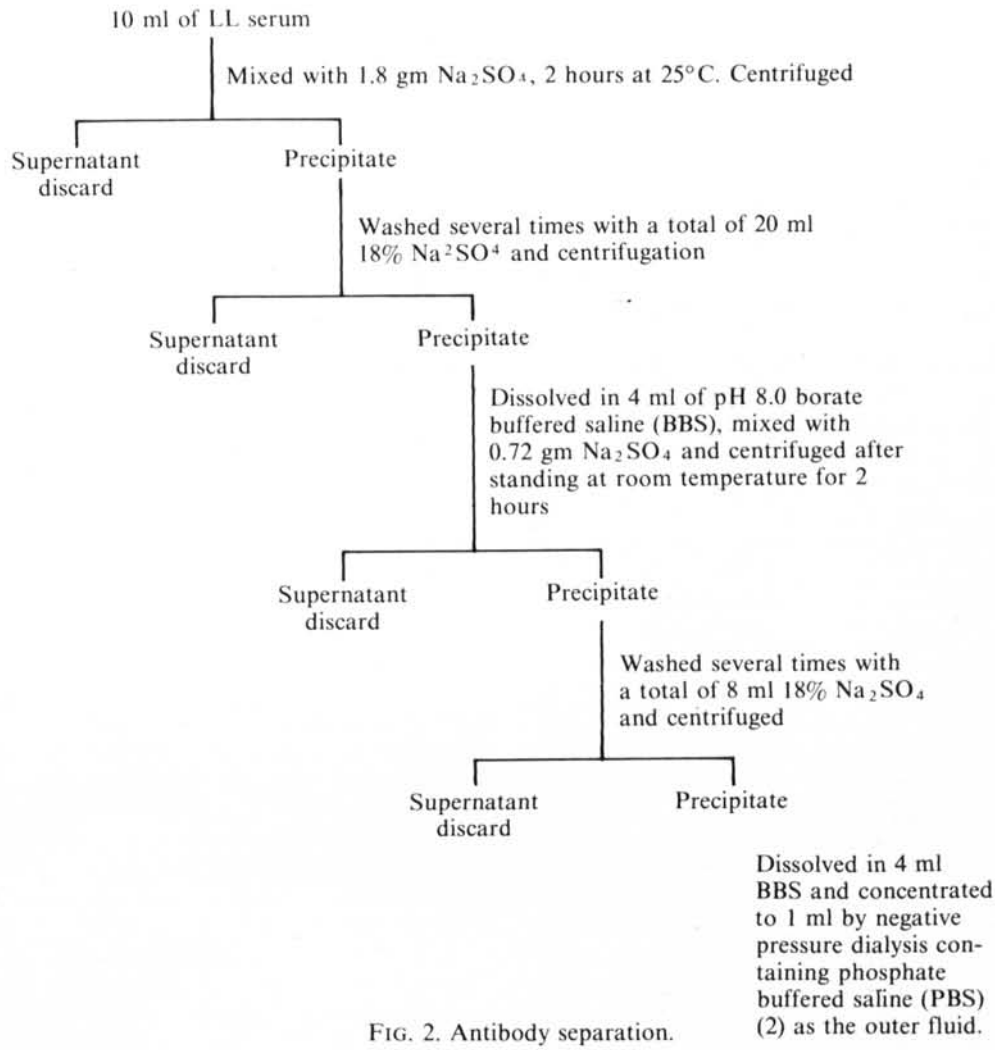

FIG. 2. Antibody separation.

Adsorption of the LL globulin with BCG polysaccharides. Polysaccharide fractions were recovered from a boiled culture filtrate of BCG, which had been maintained in Middlebrook-Dubois 7H-9 medium for one year, by precipitation with 20% trichloracetic acid followed by dialysis against tap water. The preparation was further treated with 50% ethanol to yield precipitate (AP) and supernatant (AS) fractions. The two fractions were separated and each evaporated to dryness and then each was suspended in phosphate buffered saline (PBS). Agglutination titers of both these polysaccharide fractions against the LL globulin preparation before adsorption were $2^{11} \times 10$. The determination was made by microtitration passive hemagglutination ([20]), employing formalinized tanned sheep RBC suspended in PBS and coated with 90.0 μg per ml each of the AS and AP polysaccharide fractions respectively. The diluent for the test was PBS containing 0.125% albumin as a stabilizer. Then, microsamples of LL globulin were adsorbed respectively against 8 mg of AP and 32 mg of AS polysaccharide for one hour at 37°C. Subsequently, the microtitration titers against the same polysaccharide fractions were $2^3 \times 10$ and $2^6 \times 10$ respectively, indicating incomplete adsorption of anti-polysaccharide antibody. Calculation indicated that BCG polysaccharide was inadequate for total antibody adsorption but the available quantities were utilized to adsorb the LL globulin preparation as far as possible. Microtitration of the final adsorbed preparation likewise yielded titers of $2^3 \times 10$ and $2^6 \times 10$ respectively against AP and AS polysaccharide.

Adsorption of the LL globulin with cardiolipin. One ml of BCG adsorbed LL globulin was mixed with 16.5 ml of 1/150 antigen (0.03% cardiolipin, 0.5% lecithin, 0.3% cholesterol in ethanol; Sylvania Chemical Co., Orange, New Jersey) suspended in NaCl-tris buffer ([21]) and left four hours at 37°C and then overnight at 4°C. The precipitate was removed by centrifugation. The ratio of cardiolipin antigen needed for adsorption was estimated from the highest complement fixation titer obtained by microcomplement fixation ([21]) with 1/10,000 hemolysin, 1/200 dilution of LL globulin and serial twofold dilution of antigen from 1/150.

FITC conjugation of the LL globulin. After fractionating once more with 18% Na_2SO_4 and concentrating to 2.4 ml, the adsorbed LL globulin was conjugated with fluorescein isothiocyanate (FITC) by the method of Hamajima ([8]) utilizing a combination of 2.3 ml LL globulin (34.5 mg protein by Folin-Ciocalteau method), 2.415 ml saline, 0.58 ml PBS and 0.86 mg of FITC isomer I (Sigma). FITC was the fluorescent compound of choice since it is one of the better ones for protein conjugation. The conjugation was performed at room temperature for 30 minutes instead of six hours in a cold room as described in the original method. The conjugated LL-globulin (FITC-LL-globulin) was separated from free FITC by passing through a column containing three grams of Sephadex G-25. The eluate was concentrated by negative pressure dialysis. The FITC/globulin ratio was 3.20 at this stage. FITC-LL-globulin was further adsorbed with acetone liver powder ([8]) produced from human liver (prepared in this laboratory) in a ratio of 0.1 gm (dry weight)/ml.

Further adsorption of FITC-LL-globulin with M. kansasii and M. tuberculosis. The total 2.55 ml of the FITC-LL-globulin was divided into 0.75 and 1.8 ml and diluted to 5.25 ml (FITC-LL-globulin 1) and 14.4 ml (FITC-LL-globulin 2) with PBS respectively and respectively adsorbed with 3.0 and 6.3 gm (wet weights) of *M. kansasii* grown in modified Sauton's medium ([22]) for five months. After *M. kansasii* was removed by centrifugation, both globulin fractions were then adsorbed with *M. tuberculosis* grown for two months on Ogawa's medium, separately cultured from four tuberculosis patients. The cultured bacilli were scraped off and boiled in PBS and centrifuged. The wet weight of the recovered tuberculosis bacilli was 1.9 gm which was added to the first 1.8 ml of the adsorbed FITC-LL-globulin 1, left for one hour at 37°C and then centrifuged. The precipitate thus obtained was added to the 14.4 ml of FITC-globulin 2, left one hour at 37°C and 4°C overnight and then centrifuged. After removal of the precipitates each supernatant was cleared of remaining slight turbidity by flash-mixing with equal volumes of chloroform at room temperature for one minute followed by centrifugation to remove the chloroform and precipitates (bacilli and denatured proteins) which formed between the chloroform and FITC-LL-globulin layers. The procedure was repeated and each globulin fraction was mixed with 1% of sodium axide and passed through 0.22 μ pore size millipore filters.

Adsorptions were performed at 37° for one hour unless otherwise specified. Centrifugations were performed at room temperature at 12,000 rpm for 15 minutes unless otherwise specified.

Fluorescent microscopy. A wire-loopful of bacillary suspension derived from tissue suspension or culture fluid, as the case might be, was smeared together with a drop of the affixative solution on precleaned glass slides. The affixative solution ([11]) had the following composition:

5% EDTA ..... 8 ml
20% Bovine serum albumin..... 20 ml
Phosphate buffered saline ([8]) ... 60 ml
Penicillin G .....10,000 unit
Concentrated $NaHCO_3$ to bring the above solution to pH 6.8.
Distilled water to adjust the total volume to 100 ml.

The slide was then dried for 15 minutes at room temperature and irradiated for 15 minutes at a distance of 40 cm under a 15 watt GE ultraviolet light. Subsequent processing was as follows:

1. Fixation with 7% formol-phosphate buffered saline (0.01 M PO_4, 0.15 M NaCl, pH 7.0) for 15 minutes ([5]).
2. Washed with PBS ([8]) two times, seven minutes each.
3. Digestion with 4 mg lysozyme from egg white (grade I, Sigma) dissolved in 20 ml of 0.066 M phosphate buffer pH 6.24, one hour, 37°C.
4. Washed with PBS for 15 minutes.
5. Stained with FITC LL globulin-2 (frac-

tion 1 preserved for further purification and use) for one hour at room temperature.
6. Washed with a large volume of PBS several times within 15 to 20 minutes.
7. Secondary staining only in some selected cases with FITC conjugated antihuman globulin produced in rabbit (Cappel Laboratories, Inc., Downington, Pa.) for one hour followed by washing as in procedure 6.
8. Mounted in PBS-glycerine ([8]).
9. Observation and photomicrography was with a Zeiss IF microscope equipped with 12.5 V tungsten halogen lamp and FITC filter.

M. leprae suspensions and mycobacterial controls. Suspensions of *M. leprae* were obtained from minced LL skin biopsies, ground in a Potter-Elvehjem homogenizer in 1/15 M phosphate buffer at pH 6.24, centrifuged several times at 1000 rpm and then at 10,000 rpm, treated with 1% NaOH, with the final suspension held in phosphate buffer solution.

Other mycobacterial strains (Table 2) were obtained from standard cultures maintained in this hospital.

Determination of serum preparation specificity. Following serum conjugation with FITC, all subsequent adsorption procedures were monitored against pertinent control mycobacterial strains (Table 1) and against *M. leprae.*

RESULTS

Prior to complete adsorption of polysaccharide antigens, the various mycobacteria, including *M. leprae,* whose polysaccharide antigens had not been adsorbed, showed good fluorescence of bacterial forms. The partial, comparative antigen maps of Navalkar *et al* ([14, 15]) and Abe ([4]) were of help in determining the necessary adsorptions. As each was performed the number of strains of fluorescing mycobacteria were necessarily reduced till, just prior to the final adsorption, only *M. tuberculosis* and *M. leprae* responded. After the final polysaccharide adsorption *(M. tuberculosis)* only *M. leprae* showed fluorescence, but the fluorescent image now presented only a few faint bacillary shapes and, in the main, as small, punctate images resembling "stars in the sky." It was the first postulated that these points might represent antigen marker sites but further consideration suggested that the presumed protein antigen might be masked by capsular polysaccharide and lipids of *M. leprae.* This seemed to be the case since application of the described lysozyme treatment markedly reduced the "stars in the sky" effect, permitting the appearance of punctate, bacillary-shaped, and a strange shape resembling an elongated donut (Fig. 1), the central "hole" retaining a bacillary shape in many instances.

TABLE 1. *Negative controls for LL purified serum specificity.*

M. tuberculosis, var. hominis
BCG
M. lepraemurium
Group I
M. kansasii
Group II
M. smegmatis
M. fortuitum
M. phlei
Group III
M. scrofulaceum
Group IV
M. intracellulare

Photographing these results of direct immunofluorescent coupling was virtually impossible since the image proved too weak to register on film during the period of fluorescence before fading under microscopic illumination.

Accordingly, the fluorescent image was strengthened by treatment with FITC coupled, *M. tuberculosis* adsorbed, commercially obtained FITC conjugated antihuman globulin produced in rabbits. After adsorption against *M. tuberculosis* this did not stain *M. tuberculosis* and was then used as a secondary indirect stain in conjunction with the previously described direct immunofluorescent stain. With this combination, the same fluorescing morphologic forms were seen in *M. leprae* preparations but had considerably more intensity.

By developing Kodak Tri-X pan and High Speed Ektachrome (daylight type) to a maximum film speed, in each case ASA 1200, it was possible to record the images with exposures of 2.5 to 3 minutes utilizing oil immersion objectives. This time interval represented the approximate duration of fluorescence before fading under the influence of the microscopic illuminant. Exposure

necessary for the color film was determined by trial with and immediate development of the monochrome film since the illumination was too weak to be read photometrically with available equipment.

DISCUSSION

The described immunofluorescence technic for the identification of *M. leprae* can, judging by present experience, be simplified under the following considerations.

Mycobacterial adsorption of LL globulin. Simple adsorption of the globulin fraction by *M. tuberculosis* should render it specific for *M. leprae* since Abe [1] found this adequate and since Navalkar and associates [15] found that *M. tuberculosis* possesses all antigens common to mycobacteria.

Cardiolipin adsorption. Cardiolipin adsorption was used because of the frequently reported "false positive" Wassermann reactions of LL sera. This is thought to be due to structural similarities between cardiolipin and the nucleotide and diphosphoinositide of mycobacteria with respect to the spatial distances between and arrangements of their phosphorus atoms [9]. Cardiolipin adsorption can probably be dispensed with if adequate amounts of acetone separated human liver powder are used for adsorption. Acetone liver powder also contains nuclear extract and, therefore, nucleotide.

Other proposed modifications in FITC-LL globulin preparation. Investigation was made into the possible advisability of adsorption of the LL globulin fraction with ingredients of the culture media, though results to date with bacilli from culture plates as compared to those from the liquid medium suggests that this is not necessary. Complement fixation determinations reveal no interference by the media so this adsorption is judged to be unnecessary.

The globulin fraction will be further purified by DEAE-cellulose as larger amounts of sera become available.

Additional checks for possible nonspecific immune reactions to other mycobacteria will be made by complement fixation determinations as well as by further fluorescence microscopy.

Fixation of bacilli. Adequate fixation of bacilli to the microscope slide has been a steady problem since the fluorescent staining technic requires vigorous solution vibration in the working processes. Bacilli, often few to begin with, are lost and retaining adequate numbers for visualization becomes a troublesome problem.

Of the 10 to 15 fixation methods tried the method described in this paper has been found to yield the best results. However, Nakamura [12, 13] has described the use of Bioden Mesh Cement[3] for holding *M. lepraemurium* on slides immersed in culture media for two months [12, 13].

It was found that the use of acetone, methanol and ethanol as fixatives or longer fixation with formalin are all unacceptable procedures. Abe's [3] suggestion that carbon tetrachloride be used will be tested.

Mounting of slides. During prolonged observation or exposure for photography under oil immersion objective, bacilli have been found to become detached and to float about. To minimize this problem the smear should be completely dried, as was also the precaution used by Abe [1].

Further use experience and the findings of others who will probably work on this method of identification of *M. leprae* can eventually be expected to yield further refinements and standardizations. In the meantime, the method as described has been found to be the most suitable method for identifying *M. leprae* and for monitoring their cultivation with reasonable confidence. Simplification may lead to possible diagnostic use. Such procedure should be of prime value in any leprosy investigation where surety in identification of the bacilli is essential.

SUMMARY

In vitro cultivation of *M. leprae* requires a rapid, specific identification procedure for monitoring the cultures. A method utilizing direct FITC-coupled lepromatous, specific serum globulin is described in detail with suggestions for improvement. After various purification and adsorption procedures, notably against human liver powder and *M. tuberculosis,* a fluorescent serum preparation is obtained which specifically reacts with *M. leprae* and not with other mycobacteria.

RESUMEN

El cultivo *in vitro* del *M. leprae* requiere para su control un procedimiento de identificación rápido y específico. En este trabajo se describe en detalle y con sugerencias para su mejorami-

[3] 2% Neoprene W (Dupont) in toluene.

ento un método que utiliza la sero-globulina lepromatosa específica directamente acoplada al FITC. Mediante una serie de procedimientos de purificación y adsorción, particularmente con hígado humano pulverizado y *M. tuberculosis*, se obtuvo una preparación sérica fluorescente que reacciona específicamente con el *M. leprae* pero no con otras micobacterias.

RÉSUMÉ

La culture *in vitro* de *M. leprae* exige que l'on puisse disposer d'un procédé d'identification rapide et spécifique, afin de surveiller les cultures. On décrit en détail une méthode basée sur l'utilisation directe d'un globuline spécifique du sérum lépromateux couplée au FITC. Des suggestions sont également apportées pour l'amélioration de cette méthode. Apres diverses purifications et adsorptions, surtout menées en vue d'exclure le tissu humain et *M. tuberculosis*, on obtient une préparation de sérum fluorescent qui réagit spécifiquement avec *M. leprae*, et avec aucune autre mycobactérie.

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ADDENDUM: Leproma "nodular extract" (NE), described by Abe *et al* (4) as specific for leprosy, has been prepared from new biopsies. Positive complement fixation utilizing this preparation together with the LL serum antibody utilized in the fluorescence identification of cultivated *M. leprae*, supports the concept that the LL antibody preparation used was specific for *M. leprae*. Similarly, antigen prepared from *in vitro* cultivated *M. leprae* (strain HI-75) gave positive complement fixation together with the LL serum antibody, thus giving additional confirmatory evidence of *M. leprae* identity of the cultivated bacilli. A detailed report is in preparation.