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Immunologic Identification of *M. leprae* Immunofluorescence and Complement Fixation [1]

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The lepromin test, particularly as represented by the late response (Mitsuda) to the whole bacillus preparation, has long been accepted as specific for *M. leprae* and as a valid immunologic identification method for this pathogen. Since the determination requires about one month and the availability of an adequate number of cooperating tuberculoid and lepromatous patients, this technic is not suitable for monitoring the development of an *in vitro M. leprae* culture method. It certainly should be included in the eventual identity determination of claimed specific culture, at least until such a time as other specific and accepted identification procedures are evolved.

The problem of identifying and monitoring the identity of alleged cultures of *M. leprae* ([34]) has been discussed previously ([33]). The only practical approach seemed to be the development and utilization of specific immunologic technics other than those usually mooted. A preliminary communication on the use of FITC coupled antibody fluorescence has been reported ([16]) and some problems associated with the method were discussed.

Having worked with the immunofluorescent technic, it seemed desirable and also feasible to develop a second immunologic means of identification as a check against and also as possibly supportive of the former.

TABLE 1. *Significance of phospholipids as* Mycobacterium leprae *specific antigen.*

	Ogata *et al* 1952 ([29])	Abe, 1953 ([2])	Honda & Oshima 1955 ([12])	Ogata *et al* 1956 ([28])	Present Study
Methodology	Agglutination	Agglutination	Complement fixation	Complement fixation	Complement fixation
Antigens & Ratio Cardiolipin Lecithin	 Cardiolipin: 1 Lecithin: 1	 Cardiolipin: 1 Lecithin: 10	 Cardiolipin: 1 Cholesterol: 5 Cephalin: 10	 Cardiolipin: 1 Lecithin: 1 Cholesterol: 20	 Cardiolipin: 1 Lecithin: 1 NE
Anti-serum	Patient's sera	Patient's sera	Patient's Sera	Patient's sera	IgG separated from pooled lepromatous leprosy patient sera
Tests for:	Leprosy	Syphilis	Leprosy	Leprosy	Leprosy
Method to remove anti-complement	Dilution	Dilution	Dilution	Dilution	Dilution
Positive rate according to type of leprosy	Nodular 95% Macular 100% Neural 80%		Nodular 94.2% Macular 73.9% Neural 86.4%	Nodular 85% Macular 14% Neural 14%	
Positive rate in nonleprosy diseases			TB 0% Cancer 0% Normal 0% Syphilis 7.9% Pregnancy 20%		

[1] Received for publication 5 February 1976.

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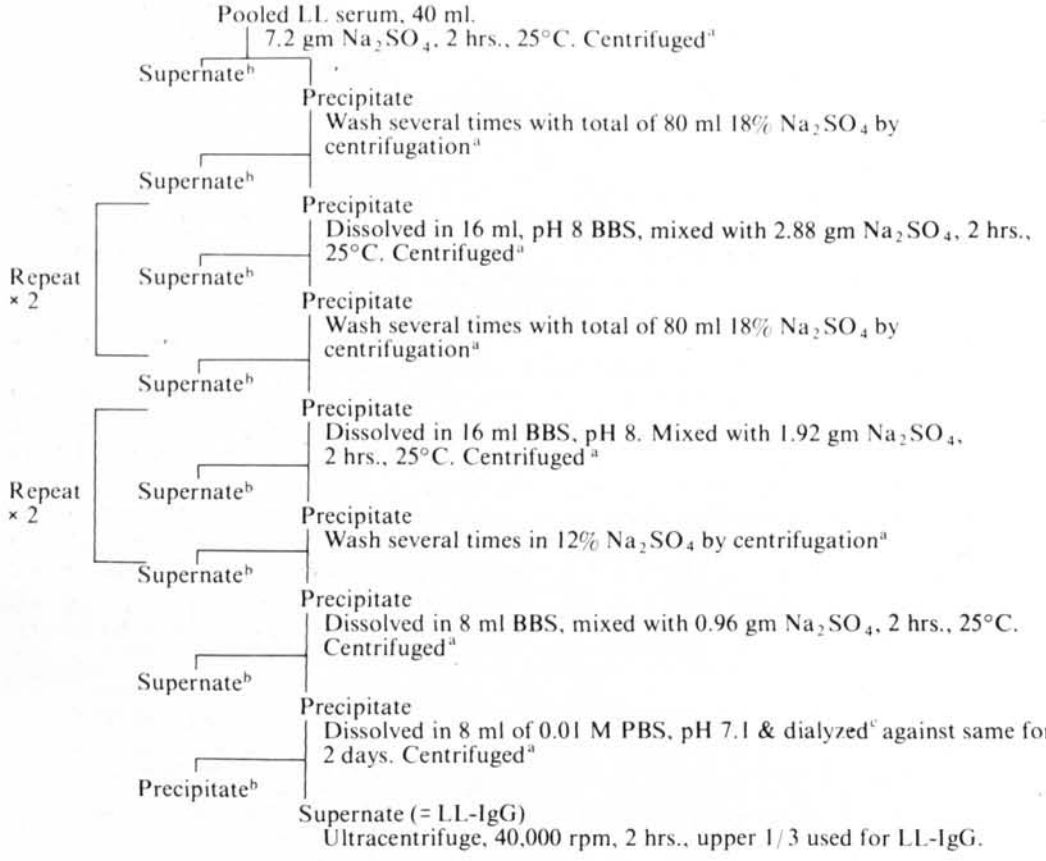
TABLE 2. *Preparation of LL-IgG* ([13]).



[a] 10,000 rpm, 20 minutes, 25°C.
[b] Discard.
[c] 2 hours, 25°C. Thereafter in refrigerator.

The development of a complement fixation test seemed feasible and of probable validity in identification and this concept was reinforced by Abe ([3]).

Review of the literature relating to the possibility of developing a complement fixation test for the *M. leprae* specific antigen revealed that previous work along these lines had established the importance of phospholipids in the specific antigen of *M. leprae* and had established that the addition of a proper ratio of cardiolipin and lecithin is important to the reaction ([24, 27, 28, 37]). The ratio of cardiolipin to lecithin, designated the L-ratio phospholipid (L-ratio PL), is 1:1 ([29]). These relationships are tabulated in Table 1 and have precedent in serologic work ([2]) where lecithin and cholesterol have been used in addition to cardiolipin in the serodiagnosis of syphilis. This concept was therefore incorporated into the approach used in the development of a complement fixation reaction.

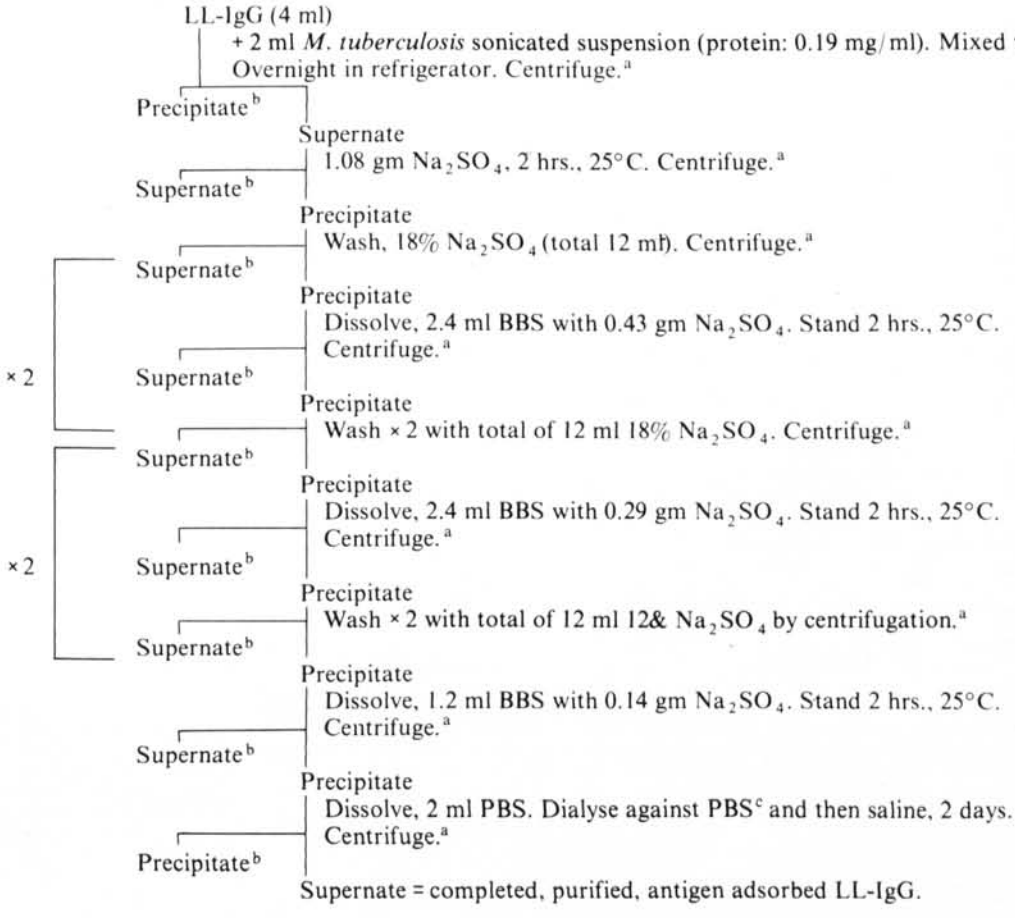
The present report combines the related studies in improving the fluorescent technic and in developing and utilizing a complement fixation technic for the identification of *M. leprae*.

MATERIALS AND METHODS

Immunofluorescence. Fluorescein isothiocyanate (FITC) labelled lepromatous leprosy serum derived IgG (FITC-LL-IgG) was prepared as detailed in Tables 2, 3 and 4. The original serum used was that previously reported ([16]).

Essentially the preparation of the FITC-LL-IgG serum consisted of fractionation of the pooled serum to the IgG segment by salt fractionation, adsorption against *M. tuberculosis* antigen, repurification to the IgG

TABLE 3. *Removal of common mycobacterial antigens by antigenic adsorption of LL-IgG.*



[a] 10,000 rpm, 20 minutes, 25°C.
[b] Discard.
[c] 2 hours, 25°C. Thereafter in refrigerator.

fraction and final conjugation of the IgG with FITC.

To achieve specificity, the LL-IgG was adsorbed against *M. tuberculosis* since prior experience ([16]) had demonstrated that this removes the nonleprosy-specific antibodies common to mycobacteria. The strain of tuberculosis bacilli utilized had been isolated from the sputum of a patient at this hospital and was maintained on Ogawa's medium. The adsorption antigen was prepared from a quantity of bacilli grown on Dubos medium, separated by centrifugation at 10,000 rpm for 20 minutes, suspended in normal saline and sonicated for five minutes by 30 second bursts of sonication separated by 30 second cooling intervals. Both the resultant precipitate and supernatant were used for adsorption. The protein concentration of the supernatant after centrifugation was used as a guide to the ratio of LL-IgG and tuberculosis antigen to be used in adsorption. It had previously been determined that the optimum ratio is 152 mg IgG to 0.38 mg TB-protein.

After adsorption against the tuberculosis bacilli sonicate, the preparation was again purified to the IgG level, coupled with FITC, and then purified by molecular sieving, ion exchange chromatography, and final ultracentrifugation at 40,000 rpm for two hours.

For fluorescence, the unit of FITC-LL-IgG is taken as the maximum dilution at which fluorescence occurs with the bacillary antigen. This is determined by twofold stepwise dilution, and with this serum occurred at a

TABLE 4. *FITC labeling of LL-IgG.*

LL-IgG, adsorbed, purified..... 1.06 ml (total protein 24.6 mg[a])
Saline..... 2.36 ml
Carbonate buffer, pH 9.1..... 0.41 ml
FITC..... 1.0 mg

Mixed by stirring, 25°C, 30 mins.

Dialyze 2 hrs. against 0.0175 M phosphate buffer containing 0.125 NaCl, pH 6.3 ([29]) (= PBS, pH 6.3).

Pass through a 10 ml pipet, by gravity, packed with Sephadex G-25 equilibrated with PBS, pH 6.3 FITC conjugated LL-IgG collected.

FITC conjugated LL-IgG passed, by gravity, through a 10 ml plastic syringe column packed with DEAE cellulose equilibrated with PBS, pH 6.3. FITC-LL-IgG eluate collected.

Concentration in negative pressure dialysis ([16]) and dialyzed against PBS, 0.01 M, pH 7.1.

Ultracentrifugation at 40,000 rpm, 2 hrs. Uppermost 1/3 of supernate utilized. Protein conc. = 1.4 mg/ml.

Ratio FITC/protein (F/P) = 1.28

1 unit = 16× dilution.

Staining titer = 4× dilution.

[a] Protein concentration, measured by Biuret method ([35]).

TABLE 5. *Comparative immunofluorescent and complement fixation technics.*

Immunofluorescent Microscopy

Treatment of antigen	Abe ([1])	Present
1. Affixatives	-	Bioden mesh cement ([21])
2. Sterilization	-	UV light ([16])
3. Fixation	CCl_4	CCl_4
4. Pretreatment of bacilli	trypsin	Lysozyme ([16])

Antibody	Abe	Present
1. Primary antibody	LL-serum	LL-IgG FITC tagged
2. Secondary antibody	Anti-human IgG FITC tagged	-
3. Diluent	PBS + LL ratio phospholipid & albumin	PBS

Complement Fixation

Treatment of antigen	Abe ([4])	Present
1. Molecular sieving	Sephadex G-200	Minicon S-125
2. Pretreatment of antigen	-	CCl_4
3. Addition of supportive phospholipid	-	L ratio phospholipid ([1, 29])

Antibody	Abe	Present
1. Adsorption	-	Sonicated *M. tuberculosis*
2. Ultracentrifugation	-	+
3. Antibody	Rabbit antibody against NE	Lepromatous leprosy patient IgG

TABLE 6. *Immunofluorescent microscopy methodology.*

Bacillary smears on Bioden mesh cement coated slides ([21]).
↓
Sterilized by UV light irradiation (40 cm), 15 min. ([16]).
↓
Wash with 0.066 M PBS, pH 6.3, 15 mins. total (3× for 5 min. each).
↓
Lysozyme, 20 mg/dl (0.02%) in 0.066 M PBS, pH 6.3, 1 hr. at 37°C.
↓
Wash in PBS, 0.01 M, pH 7.1; 5 min. ×3.
↓
Stain with FITC-LL-IgG diluted with L-ratio PL ([1]), 1 hr. at 25°C ([11]).
↓
Wash with PBS, 0.01 M, pH 7.1, 5 min. ×3.
↓
Cover slip with phosphate buffered glycerine (PBS:glycerine::1:9) and seal with nail polish.
↓
Observe for fluorescence with Zeiss Axiomat microscope, reflected UV blue excitation ([11, 20]), ×500 and ×1000 magnification checked against phase contrast.

dilution of 1:64. For practical use dilution is taken as two steps downward in the dilution, i.e., a dilution of 1:16. In the use of this serum for the checking of its specificity against other mycobacteria, a dilution of 1:4 was used. Thus the specificity of the *M. leprae* determination is protected by its presence at a higher dilution with *M. leprae,* and its absence with higher concentration as applied to other mycobacteria.

Fluorescence determinations were made on cryostat cut lepromatous skin sections, bacillary smears from lepromatous nodules, smears from livers of *M. leprae* infected mice which had been receiving hyaluronic acid infections during the course of their infection, and on bacillary smears from LA-3 cultures of *M. leprae* (strain HI-75). The preparation of fluorescent specimens and fluorescent microscopy is detailed in Tables 5 and 6. Phase contrast microscopy was resorted to in association with the FITC observations to give an indication of the proportion of bacilli reacting with FITC-LL-IgG.

Dilution of the FITC-LL-IgG preparation was in phosphate buffered saline (PBS) containing 0.2 mg/ml each of cardiolipin and lecithin.

TABLE 7. *Preparation of NE modified from Abe* ([4]).

Skin nodules of lepromatous leprosy (−70°C up to three years) 3 gm total.
Ground with Potter-Elvehjem homogenizer with 7% sucrose for ten minutes and centrifuged at 1000 rpm for ten minutes.

Precipitate — Supernate

Centrifuged at 7000 rpm for 30 minutes.

Precipitate — Supernate

Dialyzed against distilled water for six to seven days concentrated by negative pressure dialysis and centrifuged at 10,000 rpm for 30 minutes.

Precipitate — Supernate

After further concentration with minicon S-125, treated with equal amount of CCl_4 by flash-mixing, followed by centrifugation at 10,000 rpm for 30 minutes. Procedure repeated two to three times until the brown colored water layer became completely clear.

Precipitate
+
CCl_4 layer
↓
discard

NE (Supernate)

TABLE 8. *Characteristics of NE disclosed by complement fixation tests.*

Antibody	Purpose of experiment	Antigen	Well number / Dilution of Ag / Diluent	1	2	3	4	5	Meaning of result
				½	¼	⅛	1/16	1/36	
LL-IgG-tb adsorbed (cf Tables 2, 3)	Resistance of NE to 5 min. sonication (cf Tables 11, 11a)	Sonicated NE	Mg-saline + L-ratio PL	0	0	2	2	3	NE can resist sonication
		NE	Mg-saline + L-ratio PL	0	0	2	2	3	
LL-IgG-tb adsorbed (cf Tables 2, 3)	Increase of antigenicity of NE by L-ratio PL	NE	Mg-saline	0	3	3	3	3	1. L-ratio PL increase the antigenicity of NE. 2. L-ratio PL itself does not show antigenicity in the present complement fixation test (cf Table 8).
		NE	Mg-saline + L-ratio PL	3	0	0	2	2	
		L-ratio PL	Mg-saline	3	3	3	3	3	
LL-IgG-tb adsorbed	Other antigens in NE besides the *M. leprae* specific one	NE	Mg-saline	0	0	0	0	0	NE contains antigens common to *M. tuberculosis*.
LL-IgG		NE	Mg-saline	0	3	3	3	3	
LL-IgG-tb adsorbed	Effect of TCA treatment on NE	NE	Mg-saline + L-ratio PL	0	0	0	2	2	TCA denatures the antigenicity of NE[a]
		TCA treated NE	Mg-saline + L-ratio PL	3	3	3	3	3	
LL-IgG-tb adsorbed saline	Weak anticomplement effect of NE	NE	Mg-saline + L-ratio PL	0	0	1	3	3	Weak anticomplementary effect of NE is present but it can be definitely identified from immune reaction.
		None	Mg-saline + L-ratio PL	0	2	3	3	3	

Ag = Antigen
NE = nodular extract
PL = phospholipid
TCA = trichloroacetic acid

[a] NE mixed with equal amounts 20% TCA for 20 minutes and centrifuged to obtain supernatant: dialyzed against distilled water and then concentrated to 1/5 original volume.

Complement fixation. The procedure is derived from, and is essentially similar to Ogata's qualitative complement fixation test ([25]) save that the quantities of reactants are reduced to 1/10 and microtitrator trays (Linbro 15-MRC-9G) are used in place of test tubes.

Abe's "nodular extract" (NE), prepared by a slight modification of his method as detailed in Table 7, was developed as the standard specific antigen for *M. leprae* since it has been shown to develop a specific precipitative line against anti-*M. leprae* antibody developed in the rabbit ([4]). Its characteristics as related to preparative procedures required for the complement fixation were studied and they are recorded in Table 8.

Antigens from other mycobacteria were prepared as indicated in Table 9.

Protein concentrations of the NE and bacillary antigens were determined by the Lowry-Folin method ([15]) and serial dilutions up to elevenfold were made in Mg-saline containing 1/20 L-ratio PL before complement was added.

Procedure for the complement fixation test was essentially according to Ogata's method ([25]) but simplified and modified as illustrated in Tables 5 and 10, utilizing NE as the test antigen and either purified LL-IgG

or antigen adsorbed LL-IgG as the antibody. The LL-IgG preparation used was the same as that used for FITC conjugation for fluorescence staining and was set aside before FITC conjugation. It was diluted ten times in Mg-saline (physiologic saline containing 20 γ of Mg-ion as $MgCl_2$-$6H_2O$ per ml).

Since the preparation of NE antigen and antigens from cultivated bacilli differ, the durability of NE antigen under the conditions of sonication used in bacterial antigen preparation was tested and Table 8 indicates that sonication is not disruptive of the reacting antigens.

RESULTS

Fluorescent microscopy. The *in vitro* grown strain of *M. leprae,* HI-75 ([34]), selected for detailed experimental study and maintained in LA-3 medium over a period of 18 weeks, showed strong fluorescence (Fig. 1). The strongest fluorescence appeared to be about the periphery of the bacilli. Concomitant examination by phase contrast of the same specimen preparations indicated that not all bacilli present showed fluorescence. This suggested either that degenerating forms may have lost antigenicity or that developing forms require growth time to develop antigenicity. The question is under investigation.

Positive fluorescence of similar type was found with fresh smear impressions from LL nodules brought in from Mexico, with frozen cryostat sections cut from the same specimens and with impression smears from livers of mice infected with *M. leprae* and receiving weekly injections of hyaluronic acid ([17]).

The following mycobacteria, grown in LA-3 medium for four weeks, all failed to show any fluorescence: *M. tuberculosis* (also a 4 week Dubos medium culture), *M. bovis, M. ulcerans, M. kansasii, M. marinum, M. scrofulaceum, M. gordonae, M. flavescens, M. xenopi, M. triviale, M. fortuitum, M. smegmatis* and *M. phlei.*

M. avium, cultivated under the same conditions, showed some very faint fluorescence which was diffuse over the bacilli and did not show the peripheral concentration described above for *M. leprae.* The two were readily differentiated by the variant reaction and also showed distinct differences in cultural characteristics.

Impression smears, with bacilli, from mouse livers and spleens infected with *M. lepraemurium* did not present any bacillary fluorescence.

Fluorescence of *M. leprae* by the present technic was markedly improved over the method previously reported ([16]) both with respect to intensity and demonstration of whole bacillary forms. Photographic exposure time, utilizing film rated at ASA 400 rather than ASA 1200 as previously, could be exposed at 30 seconds or less as compared to the approximate 3.5 minutes previously required with the higher ASA rating. The previously described "stars in the sky" effect was no longer seen but replaced with fully fluorescent bacillary forms.

TABLE 9. *Mycobacterial antigen preparation for complement fixation determination.*

Mycobacteria (4 wk. cultures, LA-3 medium)
Centrifuge, 10,000 rpm, 20 mins.

Supernate discard

Precipitate
Suspend in 2 ml saline, sonicate at 20,000 cycles, 5 mins., with 30 second periods of sonication alternating with 30 seconds cooling (chilled with ice). Centrifuge, 10,000 rpm, 20 mins.

Precipitate discard

Supernate
Concentrate with Minicon S-125 to about 0.5 ml.
Centrifuge, 10,000 rpm, 20 min.

Precipitate discard

Supernate
Flash mix with equal quantity CCl_4.
Centrifuge, 10,000 rpm, 30 min.
Repeat procedures (2-3×) till upper layers become completely clear.

Bacillary antigen

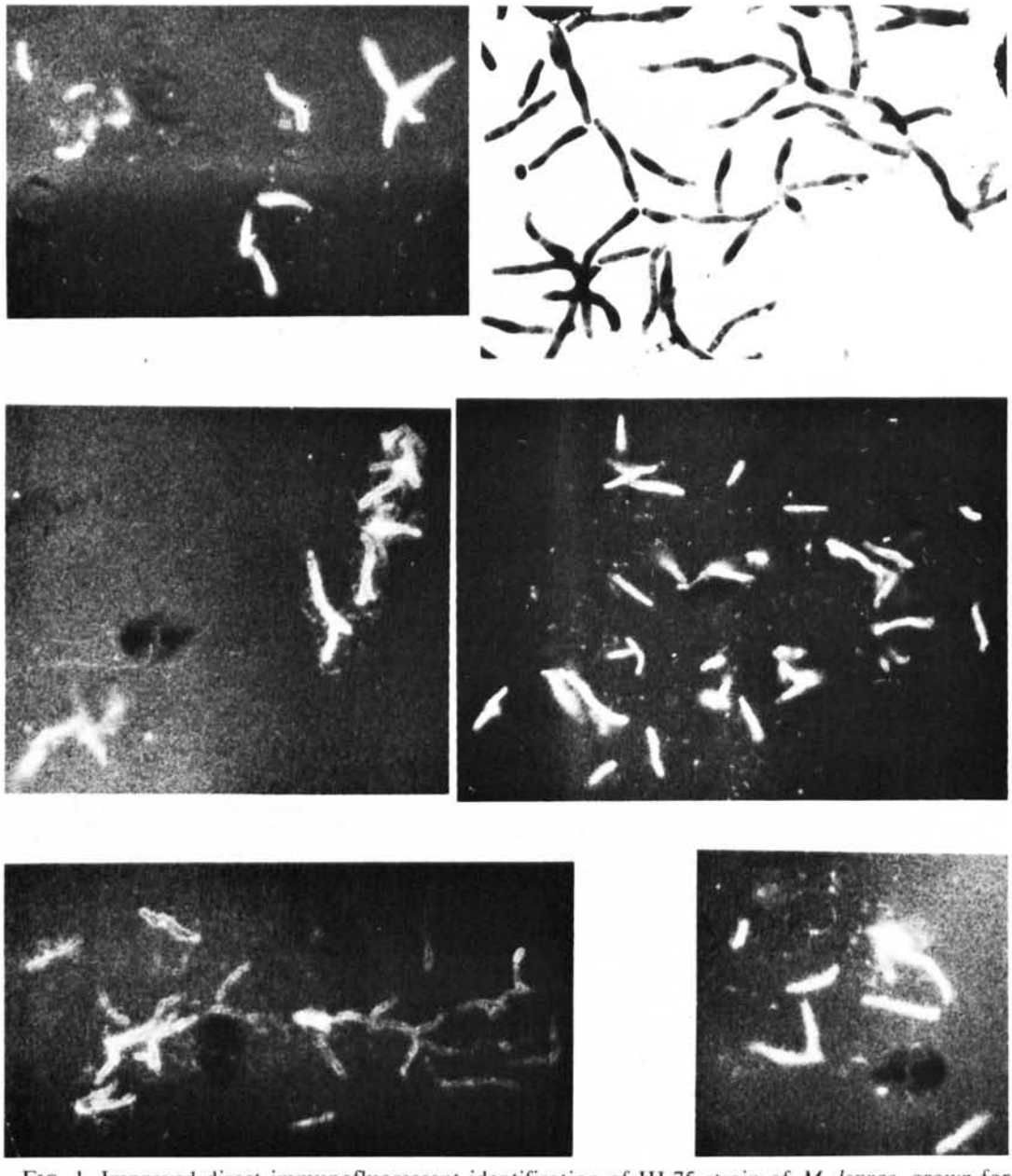


FIG. 1. Improved direct immunofluorescent identification of HI-75 strain of *M. leprae*, grown for eight days in LA-3 medium, against FITC coupled lepromatous leprosy serum IgG. Common mycobacterial antibodies removed by appropriate adsorption, controlled by immunofluorescent determinations against a battery of other known mycobacteria. Electron microscopic appearance of same culture strain, upper right. Apparent branching chiefly due to mutually adherent bacilli.

Complement fixation. As recorded in Table 8 the NE antigen increases in antigenic reaction strength when in combination with L-ratio PL, resists disruption by sonication, has antigens common to *M. tuberculosis*, is TCA precipitable and has weak anticomplementary effect. Nevertheless, it presents clear immunologic activity against LL-IgG which has been adsorbed against *M. tuberculosis* and this effect can be differentiated from anticomplementary effect.

M. leprae, cultured *in vitro* in LA-3 me-

TABLE 10. *Complement fixation microtitration technic.*

Reagent mixing sequence	Well numbers[a] 1	2	3	4	5	6	7	8	9	10	11	12
1. Mg-saline + L-ratio PL, 20:1, 0.025 ml well	+	+	+	+	+	+	+	+	+	+	+	+
2. Antigen, serial dilution ½n[b]	1	2	3	4	5	6	7	8	9	10	11	12
½n	↓ Mix well by vibration before adding complement.											
3. Complement, 2 units/0.025 ml/well (Difco)	+	+	+	+	+	+	+	+	+	+	+	0
4. Test antibody LL-IgG diluted ×10 in Mg-saline, 0.025 ml/well	+	+	+	+	+	+	+	+	+	+	+	+
	↓ Mix well by vibration. Incubate, 37°C, 1 hr.											
5. Sensitized sheep RBC, 2% in Mg-saline, 0.025 ml/well	+	+	+	+	+	+	+	+	+	+	+	+
	↓ Mix well by vibration. Incubate, 37°C, 30 min.											
Read results[c]												[d]

[a] Linbro, IS-MRC-96
[b] n = well number
[c] Record as: 0 = no hemolysis; 1 = slight hemolysis; 2 = moderate hemolysis; 3 = complete hemolysis
[d] Should always show complete hemolysis

TABLE 11. *Fractionation of* M. leprae *for specific antigen localization by complement fixation.*

M. leprae culture (H1-75 on LA-3)
Centrifuge, 10,000 rpm, 20 min.

- Supernate Fraction I[a,b]
- Precipitate-Bacilli
  Ground in Potter-Elvehjem homogenizer with 5 ml distilled H_2O for 10 min.
  Centrifuge, 10,000 rpm, 20 min.
  - Supernate Fraction II[a,b]
  - Precipitate-Bacilli (wet wt. 0.5 gm)
    Sonicated at 20,000 cycles for 5 min. for 30 sec. alternate intervals of sonication and cooling.[a]
    - Precipitate Discard
    - Supernate Fraction III[a,b]

[a] Chilled with wet ice.
[b] Each fraction dialyzed overnight against distilled water and then concentrated in Minicon S-125 serum concentrators. Thereafter each fraction flash mixed with CCl_4 as in Table 6.

TABLE 11a. *Complement fixation of* M. leprae *fractions against LL-IgG.*

M. leprae fractions	Antigen dilution ½	¼	⅛	1/16	1/32
Fraction I	3	3	3	3	3
Fraction II (protein 3.9 mg/ml)	0	0	0	0	3
Fraction III	3	3	3	3	3
NE (control from leproma) (protein 3.9 mg/ml)	0	0	3	3	3

For reading of results see Table 10.
Antibody used was LL-IgG purified and *M. tuberculosis* adsorbed.

dium, showed complement fixation comparable to NE only in the fraction released by grinding the bacilli (Tables 11, 11a).

Only NE and the antigen extracted from *M. leprae* cultured in LA-3 medium showed complement fixation in combination with *M. tuberculosis* adsorbed LL-IgG prepared from pooled lepromatous serum. Results with antigens derived from other mycobacteria seem to be negligible (Table 12).

DISCUSSION

Fluorescence identification. *Antibody loss.* In previous studies ([16]) the LL serum was fractionated to a crude globulin fraction only; a not unusual practice in immunofluorescence microscopy ([20]). In the present study the LL antibody was fractionated to the IgG level, as also suggested by Abe ([3]). However, the determinations by Ogata *et al* ([27, 28]) as summarized in Table 13 suggest that the *M. leprae* specific antibody is not a pure IgG but involves also β-globulin. In the interest of improving specificity, we also applied sephadex and DEAE cellulose column purification, though this also is not always required in immunofluorescence work ([20]), and these procedures may well result in some loss of antibody. On the other hand, ultracentrifugation at 40,000 rpm to help avoid the problems of anticomplementary effect, may have been antibody concentrating, if, as suggested by the work of Ogata *et al* ([27, 28]), the *M. leprae* specific antibody is in combination with a lipid fraction since we finally employed only the upper third of the ultracentrifuged prep-

TABLE 12. *Complement fixation determinations on* M. leprae *and other mycobacteria.*

Runyon Group	Antigens[a]	Well number / Antigen dilution / Protein concentration per ml[b]	1	2	3	4	5
			1	½	¼	⅛	1/16
	NE (cf Table 7)	0.41 mg	0	0	3	3	3
	HI-75 (cf Table 11)	0.43 mg	0	0	3	3	3
	M. tuberculosis	0.40 mg	3	3	3	3	3
	M. bovis	0.37 mg	3	3	3	3	3
	M. ulcerans	0.27 mg	3	3	3	3	3
	M. lepraemurium	0.34 mg	3	3	3	3	3
I	*M. kansasii*	0.26 mg	3	3	3	3	3
	M. marinum	0.40 mg	3	3	3	3	3
II	*M. scrofulaceum*	0.25 mg	3	3	3	3	3
	M. gordonae	0.6 mg	2	3	3	3	3
	M. flavescens	0.5 mg	3	3	3	3	3
III	*M. xenopi*	0.24 mg	3	3	3	3	3
	M. avium	0.4 mg	2	3	3	3	3
	M. triviale	0.31 mg	3	3	3	3	3
IV	*M. fortuitum*	0.26 mg	3	3	3	3	3
	M. smegmatis	0.25 mg	3	3	3	3	3
	M. phlei	0.28 mg	3	3	3	3	3

[a] Mycobacteria cultured for four weeks from heavy inoculums into LA-3 medium.
[b] Protein concentrations of the antigen preparations were determined by the Lowry-Folin method ([15]). For complement fixation, the original antigen preparations were diluted and treated as in Table 8. Protein concentrations in this column were the lowest concentrations most nearly approximating that of the NE preparation. The degree of complement fixation serially diluted from these concentrations is listed.

aration.

Ogata *et al* also reported that *M. leprae* antibody, recovered from immune complex consisting of antibody and L-ratio PL by ether extraction, lost its activity but that activity was recovered in the presence of normal human serum. In our previous report, we included flash treatment with $CHCl_3$, finding that this brief treatment did not seem to significantly denature the protein antigens as might be expected with more prolonged treatment. In the present study, however, $CHCl_3$ treatment was omitted in view of the possible extraction of lipid in the antibody complex as suggested by Ogata's findings.

Bacillary smears. The use of Bioden mesh cement ([21]) was of distinct value in retaining bacilli on the slide. Since we did not omit UV irradiation of the bacillary smears we have no information as to any enhancing effect this treatment might have, but it did not seem deleterious to the methodology. Treatment of the smears with CCl_4, as proposed by Abe ([1]) was of distinct value. Also of significance was vibration of the slides, especially when considerable numbers were handled at the same time in plastic microslide holders. The vibration seemed to help break the water repellency of the preparations and facilitate the reaction. The rationale for the use of lysozyme has been discussed previously ([16]).

The FITC labeling procedure, washing, and the sealing of the cover slip were by usual methods ([11, 20]).

Observations of fluorescence. Observation of the fluorescent specimens was by blue excitation ([11, 20]) under UV light from a mercury vapor lamp utilizing the reflected light system afforded by the Zeiss Axiomat microscope. The FITC excitation light, under conditions of marked FITC dilution, is greenish yellow. Since *M. leprae* cultivated in LA-3 medium are chromogens having a yellowish hue, it seemed important to eliminate the possibility of the presentation of a false greenish hue by the combination of blue excitation with the bacillary color. This was accomplished by removing the blue excitation filter and viewing under UV illumination employing a UG^{-1} excitation filter. Since the objectives of the Axiomat system available to us do not transmit light waves of a lower range than 400 mμ, this checking is not possible with available objectives for this instrument which requires objectives transmitting light focused for infinity rather than for the conventional 160 cm length of microscope barrels. Resort was had to adapting to the Axiomat an infinity focus 100 × oil immersion objective manufactured by the American Optical Co. (Cat #1079). This transmits light down to the 365 mμ length level and with this arrangement it was possible to verify that the fluorescent staining was specific.

Complement fixation. After several attempts at adapting more commonly employed methods of complement fixation determination, as described, for example, by Levine ([14]), it was concluded that Ogata's method ([25]) provided greater promise since it requires smaller amounts of materials, titration is easier, the reaction time is shorter and it is readily modified to microtitration ([32, 36]).

The essential characteristic of Ogata's method is that it employs antigen dilution as contrasted with more common methods which utilize either antibody dilution or increasing complement levels. Additionally,

TABLE 13. *Complement fixation activity of* M. leprae *specific antibody in leprosy patient sera* ([27, 28]).

1. Heat denatured by 63°C for five minutes.
2. Activity of ammonium sulfate fractionations:

		Main component	Activities
G I	1.4 M	γ-globulin	-
G II	1.7 M	β & γ globulin	+
G III	2.1 M	β-globulin	+
Supernatant of the above			-

3. Activity of specific antibody purified from the immune complex formation after ether extraction of phospholipid,—none.
4. Recovery of ether extracted activity by the addition of normal human sera,—yes.

work has previously been reported regarding its adaptation to use with leprosy sera ([25]). The general value of this system has also been described ([32, 36]).

Antibody. Essentially the same considerations apply that were discussed in relation to fluorescence antibody preparation since the immunologic reaction is basically the same though its determination differs.

It was found not to be necessary to apply the higher concentrations of purified antibody since in this system it is not necessary to obtain maximal reaction in order to make the necessary *M. leprae* identification.

Ultracentrifugation was necessary in order to avoid anticomplementary effect in the IgG fraction. The upper third of the ultracentrifuged preparation proved to be most suitable. Pilot study indicated that the lower third of this fraction had some anticomplementary effect. It was further determined that the upper third of the ultracentrifuged FITC-LL-IgG used in the fluorescence study could be utilized also in the complement fixation determination. The antibody preparation requires about ten drops.

Antigen. Abe's method of preparing NE was modified and simplified since NE is reported to pass through Sephadex G-200. This indicates a molecular size greater than 200,000 ([7]). Minicon S-125 serum concentrators,[3] which retain molecules larger than 125,000, were found to be convenient for the sample sizes employed.

The preparation was CCl_4 treated to remove excess, interfering lipids. Since Abe had found its use practical in treating bacillary smears for immunofluorescence it was reasoned that its use should not destroy bacillary antigen. As recorded in Table 8, CCl_4 treated NE showed relatively weak antigenicity but this was satisfactorily increased by utilizing L-ratio PL as diluent for NE. L-ratio PL alone did not present any antigenic reactivity so it is surmised that the effect results from its combination with NE to produce a more reactive molecule.

The strong antigenicity found in the fraction derived from cultured bacilli which had been ground up and not in the other fractions, including residual bacilli, as recorded in Tables 11 and 11a, suggests that the *M. leprae* specific antigen is related to the surface of the bacilli. This is compatible with the fluorescence observations noted above.

The resistance of NE to sonication lends assurance as to the adequacy of the methodology recorded in Tables 9 and 12 for the application of the complement fixation determination as used to determine its specificity for cultivated *M. leprae* as compared to the reaction with other mycobacteria.

General. It is concluded that the methodology here described demonstrates *M. leprae* specificity determinations with LL-IgG prepared from human serum without resorting to antibody preparation by animal inoculation.

The determinations strongly suggest the probable importance of L-ratio PL in combination with an unspecified component of NE in the mosaic structure of *M. leprae* specific antigen.

These determinations reinforce the previously published allegations ([34]) of *in vitro* cultivation of *M. leprae* in the hyaluronic acid based medium LA-3, since cultivated strain HI-75, now under intensive study, reacts positively by both methods.

SUMMARY

A markedly improved immunofluorescent technic employing FITC conjugated IgG antibody prepared from lepromatous serum is described as a means of specific identification of *M. leprae*.

An additional immunologic identification method for *M. leprae* is presented as a micro-complement fixation technic employing antigen rather than antibody dilution.

Studies with these technics suggest that *M. leprae* specific antigen is probably a surface antigen and has as part of its mosaic a lecithin-phospholipid component. It is not unlikely that it is a protein-glyco-phospholipid with a polysaccharide component.

These technics employed with nodular extract (NE) from lepromas, with 15 strains of mycobacteria, with *M. leprae* from human tissue as well as from previously reported *in vitro* culture, strongly reinforce the allegation that *M. leprae* is readily cultivated *in vitro* in the hyaluronic acid based medium LA-3, previously reported.

RESUMEN

Se describe una técnica inmuno-fluorescente muy mejorada empleando Ig G anticuerpos FITC

[3] Amicon Corporation, Lexington, Mass., U.S.A.

conjugados preparados de suero lepromatoso como medio de identificación específica de *M. leprae*.

También se describe un método inmunológico de identificación de *M. leprae* consistente en una micro-técnica de fijación del complemento utilizando antígeno en lugar de dilución de anticuerpos.

Los estudios de estas técnicas sugieren que el antígeno específico *M. leprae* es probablemente un antígeno de superficie y que tiene un componente lecitina-fosfolípido como parte de su mosaico. Es improbable que sea un compuesto proteina-gluco-fosfolípido con un componente polisacárido.

Estas técnicas usadas con extracto nodular (N. E.) de lepromas, con 15 variedades de micobacterias, con *M. leprae* de tejidos humanos y con cultivos *in vitro* anteriormente descriptos, fuertemente reinforzan la alegación que *M. leprae* es facilmente cultivado *in vitro* en el medio LA-3, basado en el acido hialurónico, previamente reportado.

RÉSUMÉ

On décrit ici une technique immunofluorescente fortement améliorée utilisant des anticorps IgG conjugués au FITC préparé à partir de serum lépromateux, pour identifier de manière spécifique *M. leprae*.

On présente une méthode supplémentaire d'identification immunologique de *M. leprae*, consistant dans une technique de fixation du complément au moyen d'un antigène, plutôt que par dilution des anticorps.

Des études pratiquées en utilisant ces techniques suggèrent que l'antigène spécifique de *M. leprae* est probablement l'antigène de surface, et comprend dans sa mosaïque un composé lécithine-phospholipidique. Il n'est pas invraisemblable qu'il s'agisse d'un phospholipide glycoprotéinique, avec un constituant polysaccharidique.

Ces techniques ont été utilisées sur des extraits nodulaires de lépromes, sur 15 souches de mycobactéries, sur des bacilles de *M. leprae* recueillis dans des tissus humains, et également sur des cultures *in vitro* de *M. leprae* dont il a déjà été fait état. Cette observation renforce considérablement les allégations qui ont été faites concernant la facilité de cultiver *M. leprae in vitro* dans un milieu LA-3 à base d'acide hyaluronique, ainsi qu'il a déjà été rapporté.

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