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Immunological determination of *Mycobacterium leprae* by means of cytoplasmic antigens

J. B. G. KWAPINSKI,[1] J. O. DE ALMEIDA,[2] & E. H. KWAPINSKI [3]

Mycobacterium leprae *was isolated and purified from lepromas, the spleen, and the liver of leprosy patients. An immunodiffusion analysis of the cytoplasms obtained from four lots of* M. leprae *and* M. lepraemurium, *295 strains of different actinomycetales, and 12 other bacteria was performed with the use of the cytoplasm antisera. Immunological relationships were revealed between the cytoplasms of* M. leprae, M. lepraemurium, M. avium, M. gallinarum, M. tuberculosis, M. simiae, M. kansasii, M. chitae, M. capsulatum, Actinomyces israelii, A. naeslundii, *and some strains of saprophytic mycobacteria. These studies led to the proposed concept of the immunological evolution of* M. leprae *and* M. lepraemurium *and an* Actinomyces-*like progenitor through* M. avium–M. gallinarum *and to a proposal for the polyvalent vaccine currently being developed by this research group.*

Most of the past immunological research on leprosy dealt with the skin or serum reactions of leprosy patients with different mycobacterial antigen preparations. Almost all of these data were critically reviewed by Bechelli (1971) and by de Almeida.[4] More recently, the cross-reactions given by polysaccharide–protein complexes purified from *M. leprae* with the sera obtained from human leprosy, tuberculosis, and nocardiosis were revealed by Estrada-Parra (1970). Investigations related to our research (Fukui et al., 1966) have shown that some antigens isolated from mice infected with *M. lepraemurium* shared an immunological factor with the extra-cellular antigens of *M. avium* and *M. kansasii*.

The main purpose of the research reported in the present paper was to characterize immunologically the cytoplasm antigens prepared from purified *M. leprae*, isolated from biopsy and autopsy materials, in relation to those of other actinomycetales. It was envisaged that the data thus obtained would help to elucidate the immunogenicity of *M. leprae* and would be useful for the preparation of an anti-leprosy vaccine.

MATERIALS AND METHODS

Sources of M. leprae and M. lepraemurium

The materials from which *M. leprae* was isolated were: (1) three different batches of lepromas, each collected from 12–20 cases of lepromatous leprosy, obtained from Ayer's Leprosarium, São Paulo, and from Santa Clara Leprosarium, Goyana, Brazil, and (2) the spleen and a part of the liver obtained from an autopsy of a 59-year-old female, who had suffered from lepromatous leprosy and who had not been treated for 10 years because her illness did not respond to sulfone drugs (Santa Clara Leprosarium). *M. lepraemurium* in a partly purified form was recovered from infected rat tissues and was supplied by L. Kató.[5]

The following cultures (a total of 310) of bacteria were employed: 164 *Mycobacterium*, 62 *Nocardia*, 57 *Streptomyces*, 7 *Actinomyces*, 2 *Mycococcus*, 4 *Dermatophilus*, 10 *Streptococcus*, *Pneumococcus*, and *Staphylococcus*, as well as 4 *Candida* strains.

[1] Professor of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada.

[2] WHO Reference Laboratory for Serology of Leprosy Departamento de Microbiologia e Imunologia Faculdade de Medicina, Universidade de São Paulo, Riberão Preto, S.P., Brazil.

[3] Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada.

[4] Almeida, J. O. de (1967) *Serology in leprosy*, unpublished WHO document LEP/69/2.

[5] Dr L. Kató, Institute of Hygiene and Microbiology, University of Montreal, Canada.

Isolation and purification of M. leprae

The isolation of *M. leprae* from the tissues was preceded by the detection of large aggregates of the acid-fast bacteria in smears and ultrathin sections stained by the method of Fite (1940). The materials were then treated with 1% formol at 20°C for 16 hours, after which the tissues were freed from formol by repeated washing in saline and dialysis against distilled water overnight. After dialysis, the tissues were placed in a steel chamber held in crushed ice and were homogenized in a Sorvall electric omnimixer at the highest speed for 5 min. The homogenates were centrifuged at 4 000 *g* for 10 min in an International Refrigerated Centrifuge, model B-20. The sediment was washed 5 times in PBS, pH 7.2, and was finally resuspended in distilled water and redialysed against distilled water overnight at 4°C.

The dialysed materials were concentrated by centrifugation at 4 000 *g* for 10 min, and the sediment was resuspended in 15 parts (w/w) of Hanks' buffered saline solution (HBSS) and adjusted to pH 7.2. In order to liberate cells from the connective material, the tissues were treated for 2 hours at 37°C with hyaluronidase, added at a final concentration of 3.0 units/ml. The digested material was treated ultrasonically for 10 min at 9 kHz in a Raytheon sonic oscillator, and then centrifuged at 6 000 *g* for 10 min in an International Refrigerated Centrifuge. The sediment was collected, washed 5 times with HBSS, resuspended in 15 parts (w/w) of 0.01M phosphate buffer, pH 7.8, containing trypsin (0.1 mg/ml), and placed in a shaking water bath at 37°C for 3 h. Subsequently, the digested material was centrifuged at 6 000 *g* for 10 min and the sediment was washed as above.

In order to release *M. leprae* from cells that might remain undisrupted, the tissue–cell mixtures were suspended in 10 parts (w/w) of a 10^{-5}M solution of edetic acid, pH 7.5, homogenized for 2 min on a high-speed omnimixer and subjected for 2 min to 20 kHz oscillation in the Biosonik III Ultrasonicator, in the cold. The homogenate was examined microscopically, after Ziehl–Neelsen staining, for the presence of mycobacteria and cell debris.

In order to free the mycobacteria from cell debris, the homogenates were subjected to two cycles of sucrose-gradient centrifugation, and 1–2 cycles of continuous particle electrophoresis. In this procedure, after the ultrasonic treatment, the coarse cell debris was removed by centrifugation at 118 *g* for 5 min. The supernatant material was collected and placed on a sucrose gradient made in 50-ml plastic centrifuge tubes by sequential layering of 7 ml, 5 ml, and 5 ml of 30%, 10%, and 5% solutions of sucrose; 5 ml of the homogenate containing *M. leprae* were placed on the top of the 5% sucrose layer. The materials were centrifuged in the cold at 1 000 *g* for 45 min to separate two grey bands; the upper band, which contained partly purified mycobacteria, was collected. Upper bands collected separately from each lot of the material were pooled and subjected to another run of the same sucrose-gradient centrifugation. To obtain further purification of the mycobacteria, the material obtained after the second sucrose-gradient centrifugation was subjected to continuous particle electrophoresis in Brinkmann's Electrophoretic Separator Model FF-3, using a 0.01M, pH 7.3, phosphate buffer as the liquid phase. The suspensions containing purified mycobacteria, as determined microscopically after Ziehl–Neelsen staining, were pooled and centrifuged at 20 000 *g* for 1 h to deposit the purified mycobacteria, which were finally washed 3 times in distilled water.

In order to establish that no extraneous human material was adsorbed to the purified bacteria, a slide agglutination test was employed. Thus, dense suspensions of the purified mycobacteria in PBS (pH 7.2) were mixed with a drop of an anti-human rabbit serum on a glass slide and were incubated in a moist chamber at 37°C for 1–2 min and inspected for agglutination. Controls were set up with tissue homogenates containing no bacteria. The human antiserum was produced in rabbits by immunization with a mixture of different human tissues and group A and group B erythrocytes, suspended in pooled normal human sera, by the method of Kwapinski (1969).

M. lepraemurium was purified by continuous particle electrophoresis as described above.

Preparation of cytoplasms and cytoplasm antisera

Cytoplasm preparations were obtained by the method of Kwapinski & Alcasid (1970) from the purified *M. leprae*, *M. lepraemurium*, and cultivable microorganisms. The actinomycetales were grown at 37°C in Kwapinski's KIM, KNM, or KPM media (Kwapinski & Alcasid, 1970, 1972a, 1972b), depending on the preference of the culture, until a full growth was obtained. The antisera against cytoplasms of *M. leprae*, *M. lepraemurium*, and representative strains of the cultivable microorganisms were produced in rabbits according by the immunization procedure of Kwapinski (1969).

The cytoplasms were examined with the cytoplasm antisera using an immunodiffusion method (Kwapin-

Table 1. Cytoplasms and cytoplasm-antisera of actinomycetales reactive with the cytoplasm-antisera or cytoplasms of *M. leprae*, determined by the immunodiffusion test

Name	Number	Cytoplasm	factor [a]
M. lepraemurium	LK-1		
M. avium	801	III	*a*bcdf*h*i
M. avium	701	III	*a*bcdef*h*ijkl
M. avium	19077	III	*a*bcdfik
M. avium	15769	III	*a*ik
M. gallinarum	19711	III	*a*bfj
M. gallinarum	19710	III	*a*fk
Mycobacterium sp.	23396	III	*a*jno
M. intracellulare	13209	III	g*h*jk
M. simiae	3055	I	*g*f
M. simiae	3056	I	*g*f
M. simiae	3016	I	*g*
M. tuberculosis	201 ($H_{37}Ra$)	III	*c*dh
M. tuberculosis	206 (R_1Ra)	III	*c*dj
M. kansasii	P-24	II	*c*fgh
M. smegmatis	23019	IV	*a*b*c*
M. smegmatis	31003	IV	*a*c
M. abscessus	23003	IV	*a*cd
M. borstelense	19235	IV	*a*cd
M. borstelense	19237	IV	ab (*c*)
M. fortuitum	23010	IV	bcd
M. chitae	19627	IV	b*c*
M. chitae	19628	IV	b*c*
M. chitae	19629	IV	b*c*
M. capsulatum	13556, 13557		
Actinomyces israelii	236, 271, 285, 337		
Actinomyces naeslundii	45, 599A, X600		
Streptomyces abikoensis	5025	V	*a*bcde
Streptomyces halstedii	5068	V	*a*bcde
Streptomyces cellostaticus	5189	IV	*a*bcd
Streptomyces fradiae	5063	IV	*a*bcd
Streptomyces goshikiensis	5190	II	*a*b
Streptomyces roseus	5076	I	*a*
Streptomyces exfoliatus	5060	I	*a*

[a] The antigenic factors have been named according to reactions of the cytoplasm with a standard cytoplasm-antiserum (consult references for details).

ski & Alcasid, 1972a, 1972b). Each test was set up in triplicate.

RESULTS

It was found that the cytoplasms prepared from *M. leprae* recovered from all four sources of human tissue cross-reacted with each other's cytoplasm antiserum as well as with the *M. lepraemurium* antiserum, producing two precipitation lines. Reactions between the cytoplasms of *M. leprae* and the cytoplasm antisera of certain actinomycetales, and the reactions given by combinations between the *M. leprae* cytoplasm antisera and cytoplasms of some actinomycetales, are presented in Table 1. Since the reactions of all four *M. leprae* cytoplasms with the actinomycetales were practically identical, the data are presented jointly.

Thus, the *M. leprae* cytoplasms were found to share a single antigenic factor with the following actinomycetales: the factor III*a* with *M. avium* and *M. gallinarum*, the determinant I*g* with *M. simiae*, the factor III*c* with *M. tuberculosis* and an irregular *M. kansasii* strain, and the determinant IV*c* (and probably IV*a*, IV*b*) with *M. chitae* and some strains designated as *M. smegmatis*, *M. borstelense*, *M. abscessus*, and *M. fortuitum*. Cross-reactions were also revealed between the *M. leprae* antisera and the main antigenic factor *a* of the streptomycetes and with the determinants of *Mycococcus* and *Actinomyces*, not identified so far.

No immunological reactions were observed when *M. leprae* cytoplasms and antisera were investigated with the corresponding antisera or cytoplasms of the nocardiae, dermatophili, corynebacteria, candidas, pneumococci, streptococci, and staphylococci.

DISCUSSION

Although the cytoplasm antigens of *M. leprae* have been found to be immunologically related to a few different actinomycetales, the most significant immunological relationships seem to be those between *M. leprae* and *M. avium*, *M. gallinarum*, *M. simiae*, *M. chitae*, *Mycococcus*, *Actinomyces*, and *M. lepraemurium*. The finding of cross-reactions between *M. leprae* and *M. avium* are in line with the data of Fukui et al. (1966) on the relationship between *M. lepraemurium* and *M. avium*. The exact immunochemical nature of these immunological relationships is the subject of our follow-up studies; but it may be assumed that these antigenic connections depend on the presence in the *M. leprae* cytoplasm of a supramolecular component endowed with a number of determinants identical to those occurring in the actinomycetales bearing a close immunobiological relationship to *M. leprae*.

In view of these data and the scheme of Kwapinski (1972) for the phylo-immunogenic evolution of microorganisms, it is postulated that *M. leprae* and its closest relative, *M. lepraemurium*, are derived from an *Actinomyces*-like microorganism. Through the association with birds, this progenitor microorganism acquired immunobiological properties of the *avium*-type mycobacteria. Later in the process of evolution, as a result of the association of birds and man in nature, individual microorganisms of the *M. avium* or *M. gallinarum* type might have been transformed into *M. leprae* and *M. lepraemurium* in competent human and rat cells, respectively. This postulated sequence of biological events would then have been instrumental in the establishment of the strict parasitic associations between *M. leprae* and susceptible human cells and between *M. lepraemurium* and rat cells. On the basis of the present research, it is postulated that a vaccine prepared from the cytoplasms of *M. avium*, *M. gallinarum*, *M. simiae*, and *M. chitae*, or from a combination of these microorganisms sharing certain antigens with *M. leprae* might be immunologically effective against human leprosy, and that conversely, immunization with the *M. leprae* cytoplasm may be protective against infections with *M. avium*, *M. gallinarum*, and *M. simiae*. These antigens, as well as a vaccine prepared from *M. leprae* isolated from human tissues and a vaccine obtained from *M. lepraemurium*, are currently being investigated by this research group.

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RÉSUMÉ

CARACTÉRISATION IMMUNOLOGIQUE DE *MYCOBACTERIUM LEPRAE* AU MOYEN D'ANTIGÈNES CYTOPLASMIQUES

L'objectif principal de la présente recherche était d'étudier les relations immunologiques entre les antigènes cytoplasmiques de *Mycobacterium leprae* et ceux d'autres actinomycétales.

On a préparé les antigènes cytoplasmiques de *Myco. leprae* à partir de trois lots de lépromes prélevés chez des malades lépromateux et de fragments de tissus hépatique et splénique recueillis à l'autopsie d'une autre malade. *Myco. lepraemurium* a été obtenu à partir de tissus de rat infecté. Les autres actinomycétales et un certain nombre de bactéries (310 souches au total) ont été récoltés à partir de cultures.

Après une purification très poussée du matériel d'étude, on a procédé à des épreuves d'immunodiffusion à l'aide des préparations cytoplasmiques des différents antigènes et des antisérums correspondants obtenus par immunisation du lapin.

On a observé des réactions croisées entre les antigènes et les antisérums de *Myco. leprae* de différentes origines ainsi qu'entre *Myco. leprae* et l'antisérum *Myco. lepraemurium*. Les épreuves ont aussi fait ressortir une parenté immunologique entre les antigènes de *Myco. leprae* et ceux de *Myco. avium*, *Myco. gallinarum*, *Myco. simiae*, *Myco. tuberculosis*, *Myco. kansasii*, *Myco. chitae*, *Actinomyces israelii*, *A. naeslundi* et les streptomycètes possédant le facteur antigénique *a*. On n'a par contre pas constaté de réaction entre antigènes et antisérums de *Myco. leprae* et antigènes ou antisérums de *Nocardia*, *Dermatophilus*, *Candida*, *Pneumococcus*, *Streptococcus* et *Staphylococcus*.

Ces résultats amènent les auteurs à formuler une hypothèse selon laquelle *Myco. leprae* et *Myco. lepraemurium* auraient pour ancêtre un micro-organisme du type *Actinomyces*. Au cours de l'évolution, ce dernier se serait d'abord adapté aux oiseaux, acquérant les caractéristiques immunologiques de *Myco. avium* ou *Myco. gallinarum*, puis se serait transformé en *Myco. leprae* et *Myco. lepraemurium*, adaptés à l'homme et au rat, et agents de la lèpre chez ces espèces. On étudie actuellement la possibilité de préparer un vaccin contre la lèpre humaine en utilisant les antigènes cytoplasmiques de *Myco. avium*, *Myco. gallinarum*, *Myco. simiae* et *Myco. chitae* qui présentent une parenté immunologique avec *Myco. leprae*.

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