# Double Blind Trial on the Effect of Certain Soluble Cytoplasmic Mycobacterial Antigens on the Late Reactivity to Lepromin<sup>1</sup>

Nagib Haddad, Luiz Marino Bechelli, J. B. G. Kwapinski, and Emília Tavares Simão<sup>2</sup>

It is known that most people have the potential to react to lepromin and that this lepromin reactivity becomes manifest with age, through exposure to *Mycobacterium leprae*, *M. tuberculosis* and possibly to nonpathogenic mycobacteria, and some unknown factors. Thus it is interesting to study the influence of mycobacterial antigens on the Mitsuda reaction. The subject has practical importance and may help in the interpretation of findings of field epidemiological trails to determine the preventive effect of certain antigens against leprosy.

Close immunological relationships between the cytoplasmic antigens of M. *lep*rae and those of M. avium, M. gallinarum, M. simiae, and other mycobacteria have been demonstrated by Kwapinski, *et al.* (<sup>4, 5</sup>). These observations led us to study the effects of inoculations of different mixtures of cytoplasmic antigens from these mycobacteria in eliciting or reinforcing lepromin reactivity, this reactivity indicating the relative degree of resistance to leprosy (<sup>6</sup>).

In our first study in 1975 (<sup>6</sup>), an unexpected result became evident. The incidence and intensity of the late response to lepromin were significantly reduced in children preinjected with certain soluble cytoplasmic antigens obtained from mycobac-

teria found to be immunologically closely related to, or identical with, *M. leprae* (antigen *X*: *M. avium* and *M. gallinarum*; antigen *Y*: *M. simiae*, *M. gallinarum*, and *M. avium*; antigen *Z*: *M. leprae*, *M. simiae*, and *M. borstelense*). In contrast, there was increased lepromin reactivity in the BCG groups and in the placebo (saline-injected) group. About one year later, 50 negative and doubtful lepromin children were retested with lepromin, and the results still revealed a probable inhibition of the reaction in 56% of the children and positive conversion in only 44% of them (<sup>1</sup>).

The present trial was undertaken in order to test other soluble cytoplasmic antigens obtained from mycobacteria found to be immunologically closely related to, or identical with, *M. leprae*. We tested other groups of children, using the same methodology of the first trial, and compare the results of both investigations.

## SUBJECTS AND METHODS

Two hundred fifty-four children, between six months and 59 months of age and of both sexes, from nursery schools and playgrounds in Ribeirão Preto, São Paulo, Brazil, were studied. Authorization for testing the children was obtained from their parents. Most of the children had probably not been exposed to *M. leprae*, and none showed signs of leprosy. The children were divided into five different groups by a randomized stratified sampling method according to age and sex.

The study was carried out as a double blind trial, in which only one of the investigators (N.H.) was aware of the identity of all the factors involved in the investigation. The readings of the lepromin reactions were performed by only one of the investigators (L.M.B.).

<sup>&</sup>lt;sup>1</sup> Received for publication on 26 February 1981; accepted for publication in revised form on 23 March 1982. These findings were reported at the XI International Congress of Leprosy.

<sup>&</sup>lt;sup>2</sup> N. Haddad, M.D., Ph.D., Professor and Head, Department of Social Medicine, L. M. Bechelli, M.D., Ph.D., Professor and Head, Chair of Dermatology, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil; J. B. G. Kwapinski, Ph.D., Department of Microbiology, Kirksville College of Medicine, Kirksville, Missouri, U.S.A.; E. T. Simão, M.D., Ph.D., Chair of Dermatology, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil.

Group	Age in months	Sex		a 1
		Male	Female	Total
Antigen G	6-35	7	5	12
	36-47	8	7	15
	48-59	9	10	19
Total		24	22	46
Antigen F	6-35	8	7	15
	36-47	9	7	16
	48-59	10	10	20
Total		27	24	51
BCG	6-35	11	6	17
	36-47	9	4	13
	48-59	14	12	26
Total		34	22	56
Saline	6-35	7	7	14
	36-47	11	5	16
	48-59	11	12	23
Total		29	24	53
Antigen H	6-35	4	7	11
	36-47	9	8	17
	48-59	11	9	20
Total		24	24	48

 TABLE 1. Distribution of children by age

 and sex for each of the randomized groups.

Soluble cytoplasmic antigens (0.1% solids) were obtained from ultrasonically lysed cultures ( $^{3,4,5}$ ) of the following mycobacteria: antigen F: M. tuberculosis (strain 202), M. aquae (strain 23422), and M. lepraemurium; antigen G: M. simiae (strain 3016), M. piscium (strain 13936), and M. balnei (strain 11565); and antigen H.: M. leprae. A BCG vaccine (1 mg/ml), from Connaught Medical Research Laboratory, Toronto, Canada, was injected, and a sterile 0.85% sodium chloride solution was used as the placebo.

The cytoplasmic antigens (F, G, H) and saline were injected subcutaneously in a volume of 0.1 ml, and 0.1 ml of BCG was injected intradermally in the deltoid region. During the ensuing week, all the children were observed as to their behavior, appetite, temperature, and local and general symptoms.

Three weeks after injection of the antigens and placebo, the skin reactivity was determined. The children preinjected with antigens F, G, and H received intradermal injections of 0.1 ml of the corresponding antigen, diluted 1:100 with a sterile saline solution. Those who were preinjected with BCG or placebo received an intradermal injection of PPD (2 TU), and the Mantoux test was considered positive if the reactions measured 10 mm or more in diameter. The skin tests were given on the anterior forearm.

Lepromin was produced by the Instituto de Leprologia, Rio de Janeiro, Brazil, and contained 40 million bacilli per ml; 0.1 ml was injected intradermally on the anterior surface of the left arm at the same times as the antigens for the other skin tests. The late or Mitsuda reactions were read after 30 days by measuring two diameters of the infiltration or nodule at right angles and recording the presence or absence of necrosis. The reactions were graded according to Hanks, *et al.* (<sup>2</sup>).

The chi-square test was used to determine the significance of differences in proportions. Differences with a  $p \le 0.05$  were considered to be statistically significant.

### RESULTS

Table 1 shows the distribution of children by age and sex for each of the randomized groups. These were similar for each of the five groups.

The proportion of positive late (Mitsuda) lepromin reactions in the children receiving BCG vaccine was found to be significantly higher than that in children of the placebo group and those who received antigens F, G, and H (Table 2 and Fig. 1).

The percentages of negative and doubtful reactions and, on the other hand, of positive reactions (1+, 2+, and 3+) in groups 1 (antigen *G*), 2 (antigen *F*), and 5 (antigen *H*) were similar to those observed in the placebo group (Table 2 and Fig. 1). Group 5 (antigen *H*) had a slightly higher proportion of reactors; however, the differences from the control group were not statistically significant.

Mitsuda reactions were stronger (3+) in the BCG vaccinated children (26.7%) than in the groups inoculated with antigens F, G, and H, and the placebo—7.8%, 4.3%, 0.0%, and 3.8%, respectively (Table 2 and Fig. 1). In the groups that received antigens F, G, and H, the skin test reactions to the corresponding antigens were much weaker than those in the BCG group.

In the placebo group, only 4.0% of the children had positive Mantoux reactions,

suggesting that among the children included in the trial only a few would have had previous exposure to *M. tuberculosis*. Among the 48 children with negative Mantoux reactions, 23 (48%) were lepromin reactors.

50.3

# DISCUSSION

The findings of this trial show that the injection of antigens F, G, and H, containing cytoplasmic components of the mycobacteria, M. tuberculosis, M. aquae, M. lepraemurium, M. simiae, M. piscium, M. balnei, and M. leprae, had no effect on the late reactivity to lepromin. This could be due to the fact that soluble and not whole bacillary antigens were used. As was observed in the first trial (<sup>1,6</sup>), whole BCG induced a high proportion of positive Mitsuda reactions.

The children tested in both the first and the second trials were chosen from the same institutions, and have shared the same socio-economic and environmental conditions. The methodology of investigation was nearly the same for the two trials. Lepromin reactivity was lower in the placebo group in the present (second) trial than in that of the earlier (first) trial. This could be explained by a slight difference in the age composition of the two trials, since lepromin activity is known to increase with age. This could be the case, since, in fact, the proportion of older children (48 months to 59 months) in the five groups of the second trial was a few less than those in the five groups of the first trial.

The data of the present investigation can be compared with the findings in the first trial (Fig. 2). In that trial the injection of the antigens X (containing soluble cytoplasmic components of M. avium-M. gallinarum),



FIG. 1. Late lepromin reactions in groups of children preinjected with antigens G, F, H, BCG vaccine, and placebo. Shaded areas refer to the strength of the reactions. Results from the present (second) study.

Y (M. simiae-M. gallinarum-M. avium), and Z (M. leprae-M. simiae-M. borstelense) caused significantly lower positive late lepromin reactions. From these results it appears that M. borstelense and either M. avium or M. gallinarum were instrumental in this impairment, since M. simiae and M.

TABLE 2. Late lepromin reactions after injection of certain mycobacterial antigens, BCG, and saline.

Group		Tatal			
	$-$ and $\pm$	+	++	+++	Total
Antigen G	22 (47.8)	11 (23.9)	11 (23.9)	2 ( 4.3)	46 (100.0)
Antigen F	24 (47.1)	17 (33.3)	6 (11.7)	4 (7.8)	51 (100.0)
BCG	6 (10.7)	22 (39.2)	13 (23.2)	15 (26.7)	56 (100.0)
Saline	28 (52.8)	9 (16.9)	14 (26.4)	2 ( 3.8)	53 (100.0)
Antigen H	21 (43.7)	15 (31.2)	12 (25.0)	0	48 (100.0)
TOTAL	101	74	56	23	254



328

FIG. 2. Late lepromin reactions in groups of children preinjected with antigens X, Y, Z, BCG vaccine, and placebo. Shaded areas refer to the strength of the reactions. Results from the first (earlier) trial (<sup>6</sup>).

*leprae* were included in the present trial and no such impairment of lepromin reactivity was observed.

Further investigations with *M. borstelense*, *M. avium*, and *M. gallinarum* and with other mycobacteria are desirable in order to confirm these observations and to determine those mycobacteria that can cause either an impairment or an increase in lepromin reactivity. Cell walls or whole bacillary antigens should also be tested.

#### SUMMARY

Two hundred forty-four children between six months and 59 months of age from nursery schools and playgrounds in Ribeirão Preto, São Paulo, Brazil, were randomly allocated into five groups. These received: 1) intradermal BCG; 2) placebo; 3) antigen *F*, containing soluble cytoplasmic components of the mycobacteria *Mycobac*- terium tuberculosis, M. aquae, and M. lepraemurium; 4) antigen G, containing soluble cytoplasmic components of M. piscium, and M. balnei, and 5) antigen H, containing soluble cytoplasmic components of M. leprae.

BCG preinjected children had the highest proportion of positive late lepromin reactions. The injection of antigens F, G, or Hhad no effect on the late lepromin reactions. These findings contrasted with those observed in our earlier trail in which the injection of antigens X (containing soluble cytoplasmic components of the mycobacteria M. avium and M. gallinarum), Y (M. simiae, M. gallinarum, and M. avium), and Z (M. leprae, M. simiae, and M. borstelense) had caused significantly lower positive late lepromin reactions.

By comparing the data of the present investigation with the findings of the first trial, it appears that the antigens obtained from *M. borstelense* and from either *M. avium* or *M. gallinarum* could have been instrumental in the impairment of lepromin activity observed in the first trial.

#### RESUMEN

Doscientos cuarenta y cuatro niños, entre 6 y 59 meses de edad, de casas cuna y guarderías en Ribeirão Preto, São Paulo, Brasil, fueron agrupados al azar en 5 grupos. Estos recibieron (1) BCG intradérmico, (2) placebo, (3) antígeno F, conteniendo componentes citoplásmicos solubles de las micobacterias M. tuberculosis, M. aquae y M. lepraemurium, (4) antígeno G, conteniendo componentes citoplásmicos solubles de M. piscium y M. balnei, y (5) antígeno H, conteniendo componentes citoplásmicos solubles de M. leprae.

Los niños preinyectados con BCG tuvieron la mayor proporción de reacciones positivas tardías a la lepromina. La preinyección de antígenos F,  $G \circ H$ , no tuvieron efecto sobre las reacciones tardías a la lepromina. Estos hallazgos contrastaron con aquellos observados en nuestro trabajo inicial donde la inyección de antígeno X conteniendo componentes citoplásmicos solubles de M. avium y M. gallinarum, de antígeno Y (M. simiae, M. gallinarum y M. avium), y de antígeno Z (M. leprae, M. simiae y M. borstelense), causó un número significativamente más bajo de reacciones tardías positivas a la lepromina.

Comparando los datos del presente estudio, con los hallazgos del estudio anterior, parecería que los antígenos obtenidos del *M. borstelense* y del *M. avium* o del *M. gallinarum*, pudieron haber sido instrumentales en el abatimiento de la reactividad a la lepromina observada en el primer estudio.

# RÉSUMÉ

On a procédé à une répartition au hasard, en 5 groupes, de 244 enfants âgés de 6 à 59 mois, et fréquentant les crêches et les jardins d'enfants de Ribeirao Preto, Sao Paulo, Brésil. Ces enfants ont reçu respectivement: (1) du BCG intradermique; (2) un placebo; (3) l'antigène F, contenant des constituants cytoplasmiques solides des mycobactéries suivantes, M. tuberculosis, M. aquae, et M. lepraemurium; (4) l'antigène G, contenant des constituants cytoplasmiques solubles de M. piscium et de M. balnei; (5) l'antigène H, contenant des composants cytoplasmiques solubles de M. leprae.

Les enfants qui avaient reçu auparavant une injection de BCG ont présenté la proportion la plus élevée de réactions tardives positives à la lépromine. L'injection des antigènes F, G, ou H, n'avait pas d'effet sur les réactions tardives de la lépromine. Ces observations sont à l'opposé de celles faites lors des essais antérieurs, où l'on avait eu recours à l'injection de l'antigène X, contenant les constituants cytoplasmiques solubles des mycobactéries suivantes: M. *avium*, M. gallinarum, de l'antigène Y (M. simiae, M. gallinarum, et M. avium), et de l'antigène Z (M. leprae, M. simiae, et M. borstelense), qui tous entraînaient une proportion significativement plus faible de réactions tardives à la lépromine.

Quand on compare les données de l'étude publiée ici, avec les observations faites au cours des premiers essais, il apparaît que les antigènes obtenus à partir de *M. borstelense* d'une part, et soit de *M. avium* ou de *M. gallinarum* d'autre part, pourraient avoir joué un rôle important dans l'altération de la réactivité à la lépromine observée au cours de la première étude. Acknowledgments. The authors express thanks to Dr. Lygia M. C. Andrade who provided human lepromin for the study; to the assistants (Mr. Oswaldo Compos Borelli and Luci Aparecida dos Santos) who helped in the field trail; to the municipal health authorities; and to the directors and staffs of children's institutions of Ribeirão Preto.

#### REFERENCES

- BECHELLI, L. M., HADDAD, N., KWAPINSKI, J. B. G. and SIMÃO, E. T. Action inhibitrice de certains antigenes mycobacteriens, sur la reactivite leprominique tardive. Acta Leprol. 66–67 (1977) 217–222.
- HANKS, J. H., ABE, M., NAKAYAMA, T., TUMA, M. and MARTINEZ, D. V. Studies towards the standardization of lepromin. Bull. WHO 42 (1970) 703-709.
- KWAPINSKI, J. B. G. and ALCASID, A. Immunological specificity and relationships of cytoplasmic antigens of non-chromogenic mycobacteria. Can. J. Microbiol. 18 (1972) 445–448.
- KWAPINSKI, J. B. G., ALCASID, A., KWAPINSKI, E. H. and NAIRN, V. The immunology of cytoplasmic antigens of mycobacteria. Can. J. Microbiol. 18 (1972) 1201–1211.
- KWAPINSKI, J. B. G., ALMEIDA, J. O. and KWA-PINSKI, E. H. Immunological determination of *Mycobacterium leprae* by means of cytoplasmic antigens. Bull. WHO 46 (1972) 509-513.
- KWAPINSKI, J. B. G., BECHELLI, L. M., HADDAD, N. and SIMÃO, E. T. Impairment of reactivity to lepromin by mycobacterial antigens related to, or identical with, *Mycobacterium leprae*. Can. J. Microbiol. 21 (1975) 896–901.