In vitro Behavior of Blood Derived Macrophages Against Killed *M. leprae*[1, 2]

R. C. B. Pisani, B. Beiguelman and D. V. A. Opromolla[3]

Studies on the *in vitro* ability of blood derived macrophages to lyse phagocytized leprosy bacilli ([4, 5, 11, 13, 15, 16, 21]) have been motivated by two main purposes. The first is the possibility of obtaining a more sensitive test than the late lepromin reaction (Mitsuda reaction) for evaluating the resistance to infection by leprosy bacilli. Since the Mitsuda reaction is a consequence of events that follow the phagocytosis of killed leprosy bacilli by the histiocytes of the skin, it was presumed that the *in vitro* reaction of blood derived macrophages might be an advance over the Mitsuda test, since it is a response at the cellular level, free from other *in vivo* influences. It is thought that leprosy bacilli are intracellular parasites which are able to multiply within the macrophages after phagocytosis. The second purpose is the possibility of applying this *in vitro* test to an evaluation of host hereditary factors relating to the tissue response to *M. leprae*. Family and twin-pair studies of the Mitsuda reaction ([8, 9, 12, 14]) have led to the conclusion that tissue resistance to *M. leprae* as evaluated by the Mitsuda reaction is a familial trait. Nevertheless, there are several reservations against acceptance of the monogenic explanation of familial association of that trait ([10]).

Except for Treo and Silva ([21]) who studied *in vitro* blood macrophage reactons to *M. leprae* in two dimorphous (intermediate) and seven indeterminate cases, all other concerned investigators have concentrated their attention only on the polar forms of leprosy ([4, 5, 11, 13, 16]). Data relating to healthy individuals were reported by Barbieri and Correa ([4, 5]) and Delville ([15]). The present paper describes a technic that makes easier the assessment of the *in vitro* ability of the blood derived macrophages to lyse killed leprosy bacilli; records the results of the application of this improved technic to patients having varying clinical forms of leprosy, as well as to healthy individuals as represented by both contacts and noncontacts of leprosy cases; examines the association between the results of the *in vitro* and the Mitsuda tests in both leprosy and healthy individuals, in order to explore the possibilities of the *in vitro* test for genetic studies; presents a comparison of the results obtained by applying the improved technic to those reported in pertinent literature.

MATERIALS AND METHODS

Blood samples (20 ml each) from 111 individuals (54 leprosy patients and 57 healthy individuals) were collected by means of sterile heparinized syringes. There were 10 lepromatous, 10 tuberculoid, 10 dimorphous, 17 indeterminate and 7 cases of uncertain leprosy classification. The healthy individuals, all of whom submitted to clinical and complete hematological examinations, consisted of 40 persons having had no known leprosy contact (students, and researchers at the State University of Campinas, SP, Brazil) and of 17 nonconsanguineous spouses of leprosy patients registered at the Department of Sanitary Dermatology of Campinas.

The Mitsuda test was given to all examined individuals before the collection of blood samples by injecting intradermally 0.1 ml of standard Mitsuda type lepromin. Except for six indeterminate cases, whose Mitsuda reaction could not be read because they moved from the Sanatório Aimorés, the results in all other individuals were read at 28 to 30 days after the lepromin injection. The criteria used for classifying the Mitsuda reaction were those recom-

1 Received for publication 5 July 1972.
2 This work was supported by grants of the World Health Organization.
3 R. C. B. Pisani, Assistant, and B. Beiguelman, Chairman, Department of Medical Genetics, State University of Campinas, C.P. 1170, Campinas, SP, Brazil; D. V. A. Opromolla, Clinical Director of the "Sanatorio Aimorés," Bauru, SP, Brazil.

mended by the VIth International Leprosy Congress, Madrid, 1953.

The *in vitro* reactions were carried out under sterile conditions in Leighton tubes provided with coverslips to which leprosy bacilli were adherent. The leprosy bacilli were extracted with chloroform from integral lepromin diluted 20 times in 0.9% NaCl solution using a 1:1 mixture which was vigorously shaken in a test tube for five minutes. The chloroform was aspirated with a Pasteur pipette and transferred to a test tube kept in an ice bath. The final suspension was used within 20 minutes to avoid possible damage of the bacilli by chloroform. A volume of this suspension, sufficient to fill the capillary end of a Pasteur pipette was placed on each coverslip. Each volume was estimated to contain an average of 7.5×10^4 bacilli.

A sample of heparinized venous blood from each individual was divided into each of four sterile tubes which were then left for 30 to 40 minutes at 37°C at an angle of 45°. In cases of slow hemosedimentation rate the tubes were centrifuged at a low speed (300 rpm). The plasma of the four tubes, containing a large number of leukocytes, was aspirated with a Pasteur pipette and transferred to a conical graduated tube. Hanks balanced salt solution (BSS), supplemented with 0.5% lactalbumin hydrolysate and antibiotics (100 I.U. of penicillin and 100 μg of stereptomycin per ml), was added to the plasma in the proportion of 6:4 v/v.

The amount of leukocyte suspension in the final medium was sufficient for distribution to at least five Leighton tubes which were incubated at 37°C. The tissue culture medium was renewed twice a week with a solution of Hanks BSS containing lactalbumin hydrolysate and antibiotics (60%) and fetal bovine serum (40%).

The phagocytic and lysing ability of the macrophages which developed from monocytes and perhaps from some lymphocytes as well, were followed during 25 days by removing a coverslip from each set of Leighton tubes, at five day incubation intervals. The coverslips were fixed for five minutes in absolute methanol and dried at room temperature. Bacilli were stained with carbolfuchsin at room temperature for 20 minutes. After rinsing with 1% hydrochloric acid alcohol, the coverslips were washed three times in tap water, one minute each. The cellular elements were stained for a few seconds in an aqueous 0.5% methylene blue solution and washed in running tap water. The coverslips were dried at room temperature, cleared in xylol and mounted in balsam.

Three bacterioscopic scores are proposed for quantifying the *in vitro* response of the macrophages to killed *M. leprae*. The criteria are chiefly based on the decrease in bacillary concentration in the inoculum as an indirect evaluation of the rate of phagocytic and lysing activity of the macrophages. They are as follows:

Score O. Free acid-fast bacilli are absent or very scarce. Macrophages are represented by histiocyte-like cells as well as by giant cells, but the frequency of the latter may decrease considerably after a long incubation period. Cells considered as epithelioid, because of either a homogeneous or a thinly granulated, sharply outlined, cytoplasm are frequently found. Macrophages containing undigested bacilli may be absent, rare or frequent, while signs of lysis may be present or have disappeared. Necrotic masses containing undigested bacilli are usually absent.

Score 1. The bacillary concentration in the inoculum is reduced to a variable degree. Macrophages are represented by histiocyte-like cells as well as by giant cells, but the frequency of the latter may decrease considerably after a long incubation interval. Epithelioid cells are frequently found. Macrophages with undigested bacilli may be frequent or rare, but signs of lysis are always clearly observed within the macrophages. Necrotic masses containing undigested bacilli may or may not be present.

Score 2. The bacillary concentration in the inoculum is practically unchanged. Macrophages, usually with low affinity for leprosy bacilli, are represented by histiocyte-like cells, giant cells being absent or sparse. Epithelioid cells are absent or sparse. Macrophages surrounding the inoculum are either full of or free of undigested

TABLE. 1. *Distribution of leprosy patients according to various characteristics.*

Case	Clinical form[a]	Racial stock[b]	Sex	Age	Years under observation	Mitsuda reaction	Combined Bacterioscopic Score
1. N.F.	LL	C	M	51	1	—	2-2
2. E.P.F.	LL	C	M	18	1	—	2-2
3. F.F.S.	LL	C	M	45	15	—	2-2
4. A.T.	LL	C	M	52	1	—	2-2
5. F.M.F.	LL	N	M	59	4	—	2-2
6. M.M.	LL	C	M	29	1	—[c]	2-2
7. A.M.C.	LL	C	M	51	1	—	2-2
8. J.G.	LL	C	M	27	1	—[c]	2-2
9. J.A.A.	LL	C	M	34	1	—	2-2
10. A.P.S.	LL	C	M	26	<1	—	2-2
11. A.L.B.	TT	C	F	62	7	++	0-0
12. B.S.	TT	N	M	54	7	+[c]	0-0
13. A.C.	TT	C	M	64	30	+++	0-0
14. J.R.O.	TT	C	M	41	8	+++	0-0
15. A.A.F.	TT	C	F	48	13	+++[c]	0-0
16. E.L.	TT	C	M	15	<1	++[c]	0-0
17. L.C.R.	TT	C	M	16	<1	+++[c]	0-0
18. J.N.S.	TT	N	M	15	<1	++[c]	0-0
19. V.A.S.	TT	N	F	32	3	+[c]	0-0
20. G.B.	TT	C	M	76	20	+++	0-0
21. J.D.R.	BB	C	M	49	6	−	1-0
22. V.L.R.	BB	C	F	18	1	−	1-0
23. F.M.	BB	C	M	58	3	−	1-0
24. M.Y.	BB	M	M	45	15	+[c]	1-0
25. J.O.C.	BB	C	M	39	10	+[c]	1-0
26. R.C.	BB	C	M	73	2	−	1-0
27. J.F.	BB	C	M	41	<1	−	0-0
28. J.M.	BB	C	M	53	2	+[c]	1-0
29. D.V.L.F.	BB	C	M	51	1	+[c]	1-0
30. M.F.G.	BB	N	F	60	3	++[c]	1-0
31. B.J.	II	N	M	50	<1	?	0-0
32. F.F.	II	N	M	51	1	++	0-0
33. J.C.B.	II	C	M	60	10	++	0-0
34. A.E.L.	II	C	M	52	5	?	2-2
35. E.G.S.	II	C	F	43	17	++	0-0
36. A.C.I.	II	C	M	27	4	−	1-0
37. A.M.	II	C	M	49	<1	?	0-0
38. H.R.	II	C	M	57	<1	+	1-1
39. F.D.B.	II	C	M	73	<1	?	1-1
40. M.G.	II	C	M	70	<1	++	1-0
41. E.A.	II	C	F	37	8	++[c]	0-0
42. A.C.	II	C	M	64	4	−	1-0
43. J.B.P.	II	C	M	67	23	+++	1-0
44. F.L.	II	C	M	52	9	?	1-0
45. A.G.	II	C	M	57	20	−	0-0
46. G.S.	II	N	M	50	7	?	0-0
47. A.C.B.	II	C	F	79	35	−	1-1
48. J.G.	LL or TT?	N	M	45	1	+[c]	0-0
49. A.P.D.	TT or BB?	C	F	27	2	+++	1-0
50. S.E.A.	TT or BB?	N	M	22	2	+[c]	0-0
51. E.F.	TT or BB?	N	M	40	5	+[c]	1-0
52. M.J.R.	TT or BB?	N	M	32	8	+[c]	0-0

TABLE. 1. *Distribution of leprosy patients according to various characteristics. —Cont.*

Case	Clinical form[a]	Racial stock[b]	Sex	Age	Years under observation	Mitsuda reaction	Combined Bacterioscopic Score
53. A.G.O.	TT or BB?	N	M	51	9	+[c]	0-0
54. W.R.	TT or NL?	N	M	32	2	+++[c]	0-0

[a] LL=lepromatous; TT=tuberculoid; BB=borderline (dimorphous); II=indeterminate; NL=nonleprous individuals.
[b] C=Caucasoid; N=Negroid; M=Mongoloid.
[c] Mitsuda reaction histologically confirmed by Professor Dr. José Lopes de Faria, Chairman, Department of Pathology, State University of Campinas, Campinas, SP, Brazil.

TABLE 2. *Distribution of healthy Caucasoid noncontacts of leprosy cases according to various characteristics.*

Noncontact	Sex	Age	Mitsuda reaction	Combined Bacterioscopic Score
1. A.C.S.	M	25	+++	1-1
2. A.B.N.	M	23	±	1-1
3. C.M.	M	22	+	2-2
4. C.M.M.	F	21	+	2-1
5. C.L.C.V.	M	22	+++	1-1
6. D.J.S.	M	26	+++	2-2
7. D.A.	F	25	+++	1-0
8. E.F.	M	24	−	2-2
9. F.C.A.	M	24	+	1-1
10. F.S.F.	M	25	+	1-1
11. G.M.W.	M	23	+	2-2
12. H.C.S.	M	25	+++	1-0
13. H.O.S.	M	21	+	1-0
14. I.S.	F	24	±	2-2
15. J.B.L.	M	25	++	1-1
16. J.P.M.	M	22	+	1-1
17. N.S.	M	24	+	2-2
18. J.M.S.	F	22	±	1-1
19. L.G.F.	M	23	+++	2-2
20. M.A.B.P.	F	23	±	2-2
21. M.A.F.M.	F	23	−	1-1
22. J.A.F.M.	M	27	++	2-2
23. M.B.V.P.	F	21	±	1-1
24. P.M.H.	M	23	+++	2-2
25. P.C.T.	M	21	−	1-1
26. S.B.	F	22	+	1-1
27. S.P.	M	21	+	1-1
28. W.R.P.	M	20	−	2-2
29. R.P.	F	37	+++	1-1
30. A.M.C.	M	25	+++	1-1
31. R.O.	M	29	+++	1-1
32. A.M.C.	M	31	++	1-1
33. P.C.C.	M	29	++	2-2
34. W.P.J.	M	27	+++	1-0
35. P.L.G.	M	24	+++	1-0
36. A.C.C.	M	20	++	1-1
37. F.A.	M	30	++	2-1
38. B.B.	M	39	+++	1-0
39. E.M.C.	F	20	±	1-1
40. C.A.M.	M	25	+	1-1

bacilli, while signs of lysis are usually absent or insignificant. Necrotic masses containing undigested bacilli are usually found after 15 days incubation.

RESULTS

The bacterioscopic scores determined at the two last incubation intervals (20th and 25th day) for each examined individual are expressed as a Combined Bacterioscopic Score (CBS) and presented in Tables 1-3. The CBS has been adopted to describe the *in vitro* reaction of macrophages against killed leprosy bacilli because it was observed that the scores of the two last incubation intervals are a sensitive indicator of the trend that the *in vitro* reactions have had from the beginning with respect to the phagocytic and lysing activities of the macrophages.

As illustrated in Table 4, the samples scoring "zero" at the 20th day, that is having a 0-0 CBS, were those presenting macrophages with the highest phagocytic and lysing activities in all incubation intervals. A lesser degree of those activities was observed in samples scoring "one" at the 20th day and "zero" at the 25th day of incubation (1-0 CBS). The poorest phagocytic and lysing activities were presented by the samples exhibiting 1-1, 2-1 and 2-2 CBS.

Table 5 summarizes the distribution of leprosy and healthy samples according to CBS scores.

DISCUSSION

Lepromatous and tuberculoid cases. The data concerning leprosy patients having the polar types of this disease (Tables 1 and 3) are consistent with the physiopathology of these forms. Thus, the blood derived macrophages of the lepromatous cases, who usually have no effective cellular resistance to *M. leprae*, behaved homogeneously in their inability to lyse leprosy bacilli, since they scored 2 at all incubation intervals. In contrast, all tuberculoid patients, who are the leprosy cases with the highest effective cellular resistance to the presence and growth of leprosy bacilli, yielded a 0-0 CBS.

The *in vitro* behavior of the blood derived macrophages of both lepromatous and tuberculoid cases was also consistent with what is known about the behavior of the skin macrophages of these patients against the leprosy bacilli contained in lepromin.

As a matter of fact, among 30 biopsies of

TABLE 3. *Distribution of healthy Caucasoid contacts of leprosy cases according to various characteristics.*

Contact	Sex	Age	Spouse: leprosy type[a]	Years of cohabitation after leprosy onset	Mitsuda reaction	Combined Bacterioscopic Score
1. M.S.	F	56	LL	11	+++	1-0
2. C.G.B.	F	51	LL	9	++	1-1
3. M.A.S.	F	34	LL	7	+++	1-0
4. A.F.	M	49	LL	17	++	1-1
5. B.J.	F	73	LL	18	++	2-2
6. J.L.A.	M	46	LL	8	++	2-1
7. O.M.	F	33	LL	12	++	1-0
8. A.J.	M	65	LL	5	+	2-2
9. A.L.	F	72	LL	15	+++	1-0
10. B.L.B.	F	35	LL	5	+++	1-1
11. C.B.C.	F	24	LL	4	+++	1-1
12. F.P.N.	M	59	TR	11	++	1-0
13. B.B.C.	F	63	LL	24	++	1-0
14. M.A.G.B.	F	26	TR	5	++	1-1
15. J.C.B.	M	27	TR	3	++	1-0
16. J.B.A.D.	M	32	LL	7	+++	1-1
17. M.P.A.	F	29	LL	2	++	2-2

[a] LL=lepromatous; TR=tuberculoid in reaction.

TABLE 4. *Distribution of the average bacterioscopic scores during the 25 days of incubation, according to the combined score presented in the last two incubation intervals (CBS).*

Combined Bacterioscopic Score	No. of cases	Incubation intervals days)				
		5	10	15	20	25
0-0	24	1.42	0.91	0.60	0	0
1-0	29	1.69	1.41	1.24	1	0
1-1	29	1.97	1.62	1.28	1	1
2-1	3	2	2	2	2	1
2-2	26	2	2	2	2	2

TABLE 5. *Distribution of the leprosy and healthy samples according to the Combined Bacterioscopic Score (CBS).*

Sample	No. patients	0-0	1-0	1-1	2-1	2-2
Lepromatous	10	—	—	—	—	10
Tuberculoid	10	10	—	—	—	—
Borderline	10	1	9	—	—	—
Indeterminate	17	8	5	3	—	1
Uncertain classification	7	5	2	—	—	—
Healthy noncontacts	40	—	6	20	2	12
Healthy contacts	17	—	7	6	1	3
Total	111	24	29	29	3	26

Mitsuda negative reactions exhibited by lepromatous cases, 27 studied by Bechelli *et al* (7) and three by Anzulay *et al* (3); and Andrade (2), all but one (96.7%) disclosed a histologically negative reaction which is represented by an infiltrate with histiocytes full of undigested phagocytized leprosy bacilli. Among 59 biopsies of strongly positive Mitsuda reactors (38 being 2+ and 21 being 3+) exhibiting the tuberculoid type of leprosy, 55 analyzed by Bechelli *et al*, and 4 by Azulay *et al* and Andrade; only 2 were found to be histologically negative (both were 2+ reactors). The remainder were classified as histologically positive, i.e., they consisted of either a granulomatous infiltrate composed chiefly of epithelioid cells assuming a tuberculoid or a tuberculoid-like structure where the epithelioid cells are scattered and sparcely grouped with few or no giant cells. In both situations the acid-fast bacilli were absent or sparse.

The frequency of histologically positive reactions among the tuberculoid cases with a clinically weakly positive Mitsuda reaction is lower and may be considered as 64.7% on the basis of the data of Bechelli *et al* (7), on 30 cases of Azulay *et al* (3), and on 4 cases of Andrade (2). If those proportions are used as estimates of the probability of histologic correspondence of the clinically read Mitsuda reaction among the lepromatous and tuberculoid cases, then in a sample of 10 Mitsuda negative lepromatous cases it would be expected that 9.67 would have macrophages unable to digest killed *M. leprae*. Among 10 tuberculoid cases showing Mitsuda reactions distributed as in the present sample (Table 1), the theoretical number of individuals presenting macrophages capable of phagocytosing and

disposing of killed *M. leprae* would be 9.02. In the samples analyzed these theoretical numbers are higher. Thus, the expected number of histiologically-negative late lepromin reactions among the lepromatous cases is 9.74 since two of the ten cases have been histologically examined (cases 6 and 8). Concerning the tuberculoid cases, the expected number of histologically positive reactors would be 10 because such type of reaction has been actually observed in the biopsies of the weak reactors (cases 11 and 19), 2+ reactors (cases 16 and 18), as well as in two 3+ reactors (cases 16 and 17), the four remaining, not histologically examined cases, being 3+ reactors (Table 1).

Therefore, it may be said that the blood derived macrophages of patients with the polar forms of leprosy reproduce *in vitro* the reactions that the skin macrophages of those patients present *in vivo* against killed *M. leprae*. Stated in another way: among lepromatous and tuberculoid patients a complete agreement seems to exist between the *in vitro* and Mitsuda reactions either clinically or histologically analyzed.

The data on lepromatous and tuberculoid patients are in accordance with previous reports [4, 11, 13, 21] claiming that blood derived macrophages of lepromatous patients are unable to destroy leprosy bacilli *in vitro*, while under the same conditions those of tuberculoid patients are able to phagocytize and lyse *M. leprae*.

The findings disagree with those of Godal and Rees [16] who found no differences in the *in vitro* behavior of blood derived macrophages of five lepromatous, as compared with five tuberculoid patients, with respect to their phagocytic and lysing ability for killed *M. leprae*. Their hypothesis that the dissident results could be a consequence of racial variability does not seem to be very likely since samples of Brazilian lepromatous and tuberculoid patients have included individuals of different racial stocks. While it seems reasonable to suppose that technical variations may be responsible for the differing results of Godal and Rees [16], it is nevertheless a fact that other authors have reported findings similar to ours despite the technical variations that they adopted either in obtaining blood derived macrophages or in inoculating the bacilli. Thus, Treo and Silva [21] used total venous blood samples for their *in vitro* tests, while other authors used either fragments of buffy coat [4, 13] or leukocyte suspensions fairly free of erythrocytes [11].

Dimorphous cases. The macrophages of dimorphous patients demonstrated ability to lyse the killed *M. leprae* phagocytized *in vitro*, though in a lesser degree than those of tuberculoid patients. Except case 27 who exhibited a 0-0 CBS, the remaining nine presented a 1-0 CBS (Tables 1 and 5). The finding in case 27 may have been influenced by his reactional state and/or the corticoid therapy to which he was submitted. The slower phagocytic and lytic rate of the macrophages of the dimorphous as compared to tuberculoid cases finds a parallel in clinical data, since some dimorphous patients may be confused with tuberculoid-in-reaction cases.

Table 1 includes five negative, four weakly positive and one strongly positive reactors among the dimorphous cases. There was, therefore, no correlation between the clinical Mitsuda reaction and the *in vitro* macrophage behavior. The same conclusion cannot be extended to the possibility of association between *in vitro* reaction and histologic response to lepromin injection, since the biopsies of the five clinically positive reactors also proved to be histologically positive, i.e., to have skin macrophages with lysing ability against killed leprosy bacilli. Unfortunately, there is virtually no data on the histology of biopsies of clinically negative Mitsuda reactions in dimorphous cases in the literature.

Our observations on *in vitro* reactions of dimorphous cases are in disagreement with those of Treo and Silva [21] who reported that *in vitro* reaction of the two dimorphous cases they analyzed were similar to those of lepromatous patients. However, large variations may be observed among dimorphous cases in Mitsuda reaction. Thus, 7 of 39 dimorphous cases studied by Alonso [1] presented alternatively negative and strong

positive Mitsuda reactions in relatively short intervals.

Indeterminate cases. The *in vitro* reactions presented by blood derived macrophages from 17 indeterminate cases (Table 1 and 5) are in agreement with the well-known fact that the indeterminate group is heterogeneous because it is composed of individuals who may evolve to other leprosy forms. Thus, the macrophages of eight indeterminate cases behaved as those of tuberculoid patients by presenting a 0-0 CBS (cases 31, 32, 33, 35, 37, 41, 45, 46). Five behaved as those of most dimorphous cases, since they presented a 1-0 CBS (cases 38, 39, 47) or a 2-2 CBS (case 34).

If the patients exhibiting a 1-1 CBS are considered together with those who presented a 2-2 CBS, the present sample would be regarded as exhibiting three classes of reactions. However, Treo and Silva [21] were able to observe only two extreme classes by studying the *in vitro* responses disclosed by seven indeterminate cases, that is to say, the tuberculoid class, which is comparable to the 0-0 CBS, and the lepromatous class, which is comparable to the 2-2 or 1-1 CBS.

It seems premature to conclude, as did Treo and Silva [21] that the Mitsuda and the *in vitro* macrophage reactions are independent among indeterminate cases. Among the strong positive reactors no 2-2 or 1-1 CBS have been observed. Also, despite the strong association found between the clinical and histologic readings of the Mitsuda reaction among the indeterminate cases, the frequency of discordant results is not low. Pooling the data of Bechelli *et al* [7] on 82 patients and of Azulay *et al* [3] and Andrade [2] on 23 patients, it was found that the frequency of histologically positive reactions are 38.1% among the negative or doubtful reactors, 84.6% among the weak reactors and 84.4% among the strong positive reactors ($X^2 = 19.438$; 2 d.f.; $p < 0.001$).

Leprosy cases of uncertain classification. The seven leprosy cases of uncertain classification (cases 48-54 of Table 1) include one patient who was transferred to the "Sanatório Aimorés" as belonging to the lepromatous type (case 48), five patients who could be classified either in the dimorphous group or in the tuberculoid type (cases 50-53) and one patient exhibiting neurological disorders which suggested tuberculoid leprosy (case 54).

On admission to the "Sanatório Aimorés," case 48 exhibited signs of having manifested previously a lepromatous reaction, as well as a weak positive Mitsuda response. The 0-0 CBS observed in the *in vitro* reaction of his macrophages could perhaps be considered as favoring the hypothesis that such a patient might have developed the so-called "pseudoexacerbation" [18, 19, 20] or "reversal reaction" [17].

According to Souza-Lima and coworkers [18, 19, 20], the pseudo-exacerbation is an acute reaction which may occur among lepromatous patients under sulphone therapy, inducing the manifestation of tuberculoid-in-reaction-like lesions to a variable degree. Such lesions may coexist with leprosy of the lepromatous type and be either transient or of long duration.

Among the five cases who could be regarded as either tuberculoid or dimorphous, three exhibited a 0-0 CBS (cases 50, 52, 53) and two a 1-0 CBS (cases 49 and 51). If allowance is made for the exceptional case 27 found among the dimorphous patients, then the 1-0 CBS would support the attribution of a tuberculoid designation to cases 50, 52 and 53, and a dimorphous diagnosis for the remainder.

Finally, with regard to case 54, his 0-0 CBS might favor the attribution of tuberculoid leprosy to this patient since, as it will be seen later, no healthy individuals were found able to present a 0-0 CBS.

Healthy individuals. The data in Tables 2, 3 and 5 on healthy individuals indicate that there were no differences between contacts and noncontacts of leprosy cases with respect to their CBS. Thus, pooling together the 2-1 and 2-2 reactors in one class, an independence test will show $\chi^2 = 4.565$; 2 d.f.; $0.10 < P < 0.20$ when both samples are compared and $\chi^2 = 0.806$; 2 d.f.; $0.50 < P < 0.70$ when only the strong Mitsuda reactors are held for comparison. Therefore, the results of both samples may be pooled together and the 57 healthy individuals distributed as 13 (22.8%) with

1-0 CBS, 26 (45.6%) with 1-1 CBS, and 18 (31.6%) with either 2-1 or 2-2 CBS.

If the 57 healthy individuals are considered according to both the Mitsuda reaction and the CBS, it is seen that among the ten lepromin negative or doubtful reactors, six presented a 1-1 CBS and four a 2-2 CBS; among the twelve 1+ reactors, one showed a 1-0 CBS, six a 1-1 CBS and five either a 2-1 or 2-2 CBS; among the thirty-five 1+ or 3+ reactors, twelve disclosed a 1-0 CBS, fourteen a 1-1 CBS and nine a 2-1 or 2-2 CBS. After pooling together the 1-0 and 1-1 CBS, an independence test shows that no association can be attributed to the Mitsuda and the *in vitro* reactions since $\chi^2 = 2.609$; 2 d.f.; $0.20 < P < 0.30$.

Our data does not confirm the findings of Barbieri and Correa [4, 5] who claimed to have found complete association between the Mitsuda reaction and the *in vitro* test among healthy individuals by considering the lytic response of the blood macrophages as a positive reaction and the nonlytic response as a negative reaction. The results reported in Tables 2, 3 and 5 also show that the *in vitro* reactions disclosed by macrophages of healthy individuals are not comparable to the macrophages of leprosy patients. No 0-0 CBS determinations have been found among the former, while only 13 individuals were able to show a 1-0 CBS. Such results while agreeing with those of Delville [15], who used living *M. leprae*, are again in disagreement with those published by Barbieri and Correa [4, 5] who claimed to have found among healthy individuals *in vitro* reactions which reproduced those exhibited by lepromatous and tuberculoid patients.

The first study presented by Barbieri and Correa [4] on healthy individuals did not indicate the criteria they used for Mitsuda lepromin reaction classification and other data on their subjects was sparsely noted. Moreover, the concordance they claimed to have observed in both samples [4, 5] is surprisingly high, because the Mitsuda reaction among healthy contacts of leprosy cases does not always show a histologic correspondence [2, 3, 7].

If indeed the alleged correspondence betwen the Mitsuda and the *in vitro* tests were complete, then there is no sense in replacing the simplicity of the former with a sophisticated technic as intended by Barbieri and Correa [4] in advocating the latter as a routine procedure.

The low degree of *in vitro* lysing activity of macrophages from healthy individuals against killed *M. leprae* seems to be reflected *in vivo*. Thus, an evaluation of the data of Bechelli *et al* [5] indicates that among the histologically positive lepromin reactors, the frequency of tuberculoid-like reactions is higher among healthy individuals than among leprosy cases. Pooling this data on tuberculoid and indeterminate cases it is found that 48 (36.1%) of 133 positive reactors exhibited a tuberculoid-like response, while among 48 healthy contacts who were lepromin positive reactors the proportion yielding that histological picture (30 or 62.5%) was significantly higher ($\chi^2 = 10.031$; 1 d.f.; $P < 0.001$). Furthermore, the observation that the peak of the Mitsuda reaction may be delayed among healthy noncontacts of leprosy cases [6] seems also to favor the assumption that the lysing ability of the macrophages of healthy individuals is expressed to a lesser degree than among leprosy patients whose macrophages are able to destroy killed *M. leprae*.

SUMMARY

A technic is described that renders easier the assessment of the *in vitro* phagocytic and lysing ability of blood derived macrophages against killed leprosy bacilli. Such *in vitro* reactions were followed and scored at 5 day intervals for 25 days. This technic was applied to 54 leprosy patients (10 LL, 10 TT, 10BB, 17 II and 7 of uncertain classification) and to 57 healthy individuals (40 noncontacts and 17 contacts of leprosy cases). Three classes of *in vitro* reaction were distinguished among the leprosy cases: lytic, weakly lytic and nonlytic reactions. The lepromatous cases consisted of nonlytic reactors, the tuberculoid patients of lytic reactors, the dimorphous of nine weakly lytic and one lytic reactors, and the indeterminate cases were represented by all three types of reactors.

The blood derived macrophages of the healthy individuals showed a low rate of *in*

vitro phagocytosis and lysis of *M. leprae* and none of them disclosed a lytic reaction similar to that observed among the tuberculoid cases. No association could be found between the intensity of the Mitsuda reaction and the *in vitro* lysing activity of blood derived macrophages of healthy individuals.

It is concluded that with present day technics the *in vitro* test cannot be considered as an advance over the Mitsuda reaction for practical purposes, nor does it yet give evidence as to whether or not the tissue resistance to *M. leprae* infection may be regarded as a genetic polymorphic trait.

RESUMEN

Se describe una técnica que facilita el estudio *in vitro* de la capacidad fagocítica y lítica de macrófagos derivados de sangre contra bacilos de lepra muertos. Estas reacciones *in vitro* fueron estudiadas y cuantificadas a intérvalos de 5 días durante 25 días. Esta técnica se aplicó a 54 pacientes con lepra (10 LL, 10 TT, 10 BB, 17 II y 7 de clasificación incierta) y a 57 individuos normales (40 no contactos y 17 contactos de casos de lepra). En los casos de lepra se observaron tres tipos de reacciones *in vitro*: reacciones líticas, líticas débiles y no líticas. Los casos lepromatosos fueron reactores no líticos, los pacientes tuberculoides fueron reactores líticos, de los casos dimorfos nueve fueron reactores líticos débiles y uno fué reactor no lítico y los casos indeterminados incluían todos los tipos de reactores.

Los macrófagos derivados de la sangre de individuos normales mostraron una tasa baja de fagocitosis y lisis del *M. leprae in vitro* y ninguno de estos individuos mostró una reacción lítica similar a la que se observó en los casos tuberculoides. No se encontró relación entre la intensidad de la reacción de Mitsuda y la actividad lítica *in vitro* de los macrófagos derivados de sangre de individuos normales.

Se concluye que, para fines practicos y con las técnicas de que se dispone actualmente, la prueba *in vitro* no puede considerarse como mejor que la reacción de Mitsuda y que, hasta el momento, esta prueba no ha proporcionado evidencia sobre si la resistencia tisular contra la infección por *M. leprae* puede ser considerada como una característica genética polimórfica.

RÉSUMÉ

On décrit ici une technique qui permet une évaluation plus facile de la capacité témoignée *in vitro* par des macrophages du sang à phagocyter et à détruire des bacilles de la lèpre tués. Ces réactions *in vitro* ont été suivies et cotées de 5 en 5 jours pendant 25 jours. La technique a été appliquée à 54 malades de la lèpre (10 lépromateux, 10 tuberculoïdes, 10 dimorphes, 17 indéterminés, et 7 de classification incertaine) et à 57 individus normaux (40 non contacts et 17 contacts de cas de lèpre). Trois groupes de réaction *in vitro* ont été distingués chez les cas de lèpre: une réaction lytique, une réaction faiblement lytique, et une réaction non lytique. Les cas lépromateux comprenaient des réacteurs non lytiques; les malades tuberculoïdes étaient du type lytique; on a relévé neuf lytiques faibles parmi les dimorphes et un réacteur lytique; les cas indéterminés comprenaient les trois types de réacteurs.

Les macrophages dérivés du sang d'individus normaux ont montré un faible pouvoir de phagocytose *in vitro*, de même qu'une lyse peu prononcée de *M. leprae;* aucun d'entre eux n'a témoigné d'une réaction lytique semblable à celle observée parmi les cas tuberculoïdes. Aucune association n'a pu être mise en évidence entre l'intensité de la réaction de Mitsuda, et l'activité lytique *in vitro* des macrophages dérivés du sang d'individus normaux.

On en conclut que, vu les techniques actuelles, l'épreuve *in vitro* ne peut pas être considérée comme un progrès par rapport à la réaction de Mitsuda en ce qui concerne la pratique. Elle ne permet pas davantage de supposer que la résistance tissulaire à l'infection par *M. leprae* peut être considérée comme un caractère génétique polymorphe.

REFERENCES

1. Alonso, A . M. *Lepra Dimorfa. Fundamentos de sua conceituacão.* Editora Livro S.A., Rio de Janeiro, 1966, pp 5-121.
2. Andrade, L. M. C. Comparação entre os aspectos microscópicos e macroscópicos do teste lepromínico. Bol. Serv. Nac. Lepra **21** (1962) 95-124.
3. Azulay, R. D., Andrade, L. M. C., Silva, C., Rabello-Neto, A. V., Azulay, J. D., Garrido-Neves, R. and Alonso, A. M. Comparison of the macroscopic readings and microscopic findings of the lepromin reaction. Internat. J. Leprosy **28** (1960) 38-43.
4. Barbieri, T. A. and Correa, W. M. Human macrophage culture. The leprosy prognostic test (LPT). Internat. J. Leprosy **35** (1967) 377-381.

5. Barbieri, T. A. and Correa, W. M. Delayed lepromin reaction and BCG application in first grade school children in S. Paulo, Brazil. Internat. J. Leprosy. **39** (1971) 750-755.
6. Bechelli, L. M., Garcia, G., Nakamura, S. and Quagliato, T. Determinação da data da leitura da reação de Mitsuda com lepromina integral em individous sãos, sem exposição prévia conhecida ao *M. leprae*. An. VIII Cong. Int. Lepr. (Rio de Janeiro) **3** (1963) 284-294.
7. Bechelli, L. M., Souza, P. R. and Quagliato, R. Correlação entre os resultados da leitura clínica e do exame histopatológico da reação de Mitsuda. Rev. Brasil. Leprol. **27** (1959) 172-182.
8. Beiguelman, B. Hereditariedade da reação de Mitsuda. Rev. Brasil. Leprol. **30** (1962) 153-172.
9. Beiguelman, B. The genetics of resistance to leprosy. Internat. J. Leprosy **33** (1965) 808-812.
10. Beiguelman, B. Leprosy and genetics. A review of past research with remarks concerning future investigations. Bull. WHO **37** (1967) 461-476.
11. Beiguelman, B. Some remarks on the genetics of leprosy resistance. Acta Genet. Med. Gemellol. (Rome) **17** (1968) 584-594.
12. Beiguelman, B. Lepromin reaction. Genetic studies including twin pair analysis. Acta Leprol. **44** (1971) 5-65.
13. Beiguelman, B. and Barbieri, T. A. Comportamento dos macrófagos nas formas polares de lepra. Ciência e Cultura **17** (1965) 304-305.
14. Beiguelman, B. and Quagliato, R. Nature and familial character of the lepromin reaction. Internat. J. Leprosy **33** (1965) 800-807.
15. Deville, J. *In vitro* behavior of macrophages from healthy persons against *M. leprae* and other mycobacteria. Internat. J. Leprosy **39** (1971) 329-339.
16. Godal, T. and Rees, R. J. W. Fate of *Mycobacterium leprae* in macrophages of patients with lepromatous or tuberculoid leprosy. Internat. J. Leprosy **38** (1970) 439-442.
17. Ridley, D. S. Reactions in leprosy. Leprosy Rev. **40** (1969) 77-81.
18. Souza-Lima, L. *Estado Atual da Terapêutica de Lepra*. Serv. Nac. Lepra (São Paulo, Brasil) 1953.
19. Souza-Lima, L. and Cerqueira, G. C. Tratamento experimental da lepra pelas diamino-difenilsulfonas. II Conf. Panamer. Lepra. Arq. Serv. Nac. Lepra (Rio de Janeiro) **4** (1946) 9-76.
20. Souza-Lima, L. .and Souza, P. R. de. Pseudoexacerbation of leprosy due to the diaminodiphenyl-sulfones. Internat. J. Leprosy **17** (1949) 19-21.
21. Treo, M. M. and Silva, C. O. Comportamento do *Mycobacterium leprae in vitro* em sangue total ou plasma de leprosos de diferentes formas clínicas. An. VIII Cong. Internac. Leprol. (Rio de Janeiro). Serv. Nac. Lepra **3** (1963) 484-494.