

## Inhibition of Antibody-dependent, Cell-mediated Cytotoxicity by Serum from Lepromatous Leprosy Patients<sup>1</sup>

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Persistent infection of man with *Mycobacterium leprae* may lead to two polar forms of leprosy: tuberculoid leprosy (TT) and lepromatous leprosy (LL) (13). While patients with TT develop high resistance to the infectious agent, those with the baciliferous form (LL) have impaired cell-mediated immunity (CMI) (5). It is not known whether the alterations of CMI in LL are a consequence of a regulatory defect or if LL is genetically determined. Moreover, it has not been established if the CMI alterations are restricted to the response to *M. leprae* or are part of a generalized defect.

Patients with LL may present acute inflammatory episodes: erythema nodosum leprosum (ENL) (15). ENL is characterized by acute vasculitis and subcutaneous nodules. In many cases it is associated with the onset of antileprosy treatment in baciliferous patients and has many histological features in common with the Arthus reaction (16). Circulating immune complexes (CIC) and deposits of immunoglobulin and complement (C) at the ENL lesions have been demonstrated in LL patients during or near the time of ENL episodes. The persistence of CIC is thought to play a role in the regulation of CMI since many of the cellular functions that depend on surface receptors

for Fc (FcR) can be abrogated or stimulated by immune complexes (4).

The purpose of this study was to examine the impact of the CIC in LL patients on antibody-dependent, cell-mediated cytotoxicity (ADCC) and complement (C) activity, since activation of both mechanisms can be triggered by CIC both *in vitro* and *in vivo*. It will be shown that while CIC present in LL sera can activate C and block ADCC *in vitro*, both activities are preserved *in vivo*. We propose that the complement system plays a role in the maintenance of these functions in LL patients.

### MATERIALS AND METHODS

**Patients.** Seventeen patients (7 women and 10 men) with lepromatous leprosy (LL) classified according to Ridley and Jopling (13) were studied. The patients did not reside in endemic areas and their ages ranged from 16–68 years. Venous blood samples were obtained from five patients before treatment and from 12 patients who had undergone specific treatment with diamino-diphenyl sulfone (dapsone, DDS), rifampin, and clofazimine. Four of these 12 were studied during ENL episodes (ENL-tr-LL); the remaining eight had no recent ENL episodes (tr-LL). During ENL, thalidomide was added to the therapeutic protocol. Fourteen healthy controls were studied simultaneously.

**Mononuclear cells.** Mononuclear leukocytes (PBL) were isolated by centrifugation of defibrinated blood on Ficoll-Hypaque (2). Cells were collected from the interphase and contained 85–95% lymphocytes. PBL were suspended in RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, New York, U.S.A.) containing antibiotics and 10% heat-inactivated fetal calf serum (FCS) (GIBCO).

<sup>1</sup> Received for publication on 8 August 1984; accepted for publication on 5 November 1984.

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**Serum.** Serum samples were obtained from patients and stored in aliquots at  $-80^{\circ}\text{C}$ . Heat-inactivated sera (HI-sera) were prepared by incubating serum aliquots for 30 min at  $56^{\circ}\text{C}$ .

**Antibody-dependent cell-mediated cytotoxicity (ADCC).** ADCC was assayed as described previously (<sup>11</sup>), using <sup>51</sup>Cr-labeled chicken red blood cells (CRBC) sensitized with rabbit anti-CRBC. Briefly, duplicate tubes containing  $1 \times 10^6$  PBL and  $4 \times 10^5$  <sup>51</sup>Cr-CRBC-anti-CRBC were incubated in 0.2 ml RPMI-FCS for 18 hr at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  humidified atmosphere. After centrifugation (10 min at  $400 \times g$ ), 0.1 ml of the supernatant was collected with an automatic pipette, and the radioactivity of the pellet and the supernatant was measured in a Tecnar Trias-300 gamma counter. ADCC was calculated as follows:

$$\text{ADCC \%} = \frac{\text{counts in the supernatant} \times 2}{\text{counts in supernatant} + \text{pellet}} \times 100$$

Nonspecific cytotoxicity in the absence of anti-CRBC was subtracted. It did not exceed 5%.

Inhibition of ADCC of normal PBL by patient's serum or preformed immune complexes (IC) was done by incubating the pellet of  $2 \times 10^6$  PBL for 2 hr at  $37^{\circ}\text{C}$  with: 20  $\mu\text{l}$  RPMI-FCS, 20  $\mu\text{l}$  of fresh patient's serum; HI-patient's serum or preformed IC with or without 50  $\mu\text{l}$  normal human serum (NHS) as a source of C. After 4 washes with RPMI, the effector cells were used in the ADCC assays. Inhibition was calculated as follows:

Inhibition % =

$$\left( 1 - \frac{\text{ADCC (PBL + patient's serum or IC)}}{\text{ADCC PBL}} \right) \times 100$$

**Complement determination.** Total hemolytic activity was assayed by the method of Kent and Fife (<sup>8</sup>), and the alternative pathway activity was measured as described by Platts-Mills (<sup>12</sup>). Complement fixation by HI-serum or preformed IC was performed by incubating equal volumes of control NHS with HI-patient's serum or HI-NHS containing 5, 10, or 20  $\mu\text{l}$  of soluble preformed

IC for 2 hr at  $37^{\circ}\text{C}$ . The remaining hemolytic activity was assayed, and the fixation of C calculated:

% C fixation =

$$\left( 1 - \frac{\text{CH50 U/ml (NHS + HI-patient's serum or IC)}}{\text{CH50 U/ml NHS}} \right) \times 100$$

**Soluble preformed immune complexes (IC).** Rabbit anti-ovalbumin (anti-Ova) purified by affinity chromatography was reacted with ovalbumin (grade V, Sigma, U.S.A.) at a molar ratio of 1:2 for 1 hr at  $37^{\circ}\text{C}$ . After centrifugation at  $1500 \times g$  for 20 min, the supernatant was collected and used in inhibition assays.

**Immune complex assays.** Circulating immune complexes were assayed by polyethylene glycol (PEG) precipitation (<sup>3</sup>) or by the <sup>125</sup>I-C1q binding assay of Zubler (<sup>17</sup>). The levels of CIC by PEG are expressed as  $\text{OD}_{280}$  units/ml and <sup>125</sup>I-C1q binding as a percent of <sup>125</sup>I-C1q precipitation.

## RESULTS

**ADCC, complement (C) hemolytic activity and circulating immune complexes.** As shown in Table 1, ADCC and alternative C activity were normal in LL patients with or without specific treatment. Total C activity was variable in the three groups (162–327 U/ml). It is interesting to note that ADCC was higher in patients whose alternative C activity was great (Table 1).

PEG precipitable CIC and C1q fixing complexes were significantly above normal values in LL patients (Table 1). In agreement with previous results of this laboratory (data not shown), <sup>125</sup>I-C1q binding activity was higher in LL and tr-LL than in ENL-tr-LL patients.

**Inhibition of ADCC or normal PBL by incubation with LL sera.** Since ADCC activity was normal in LL patients with high levels of CIC, we studied the ability of these CIC to block the Fc receptors of PBL. The results shown in Table 2 indicate that incubation of normal PBL with either LL or tr-LL serum (LL-S or tr-LL-S) does not affect ADCC. However, ADCC inhibition is revealed upon elimination of C activity from

TABLE 1. ADCC, total and alternative complement hemolytic activity and circulating immune complexes in serum from lepromatous leprosy patients.<sup>a</sup>

Patients	No.	ADCC % ± S.D.	Total comple- ment activity CH50 U/ml ± S.D.	Alternative pathway activity APCH50 U/ml ± S.D.	Immune complexes	
					<sup>125</sup> I-C1q binding % ± S.E.M.	PEG precipitation OD <sub>280</sub> U/ml ± S.E.M.
LL	5	58.4 ± 16.0	234 ± 55	120 ± 10	32.9 ± 5.6 <sup>b</sup>	0.46 ± 0.12 <sup>b</sup>
ENL-tr-LL	4	51.8 ± 10.9	281 ± 40	112 ± 5	12.6 ± 6.1 <sup>c</sup>	ND <sup>d</sup>
tr-LL	8	53.2 ± 9.0	214 ± 63	129 ± 11	29.9 ± 8.5 <sup>b</sup>	0.51 ± 0.04 <sup>b</sup>
Normal controls	14	55.4 ± 6.0	230 ± 20	106 ± 12	6.6 ± 0.5	0.09 ± 0.01

<sup>a</sup> ADCC, total complement and alternative pathway hemolytic activity and immune complexes were assayed in untreated lepromatous leprosy patients (LL), LL patients who received specific treatment (tr-LL), tr-LL patients with erythema nodosum leprosum (ENL-tr-LL) and normal subjects. The correlation between ADCC and alternative pathway activity was statistically significant in the lepromatous leprosy patients studied ( $n = 9$ ,  $r = 0.68$ ;  $p < 0.05$ ).

<sup>b</sup>  $p < 0.0005$ , Student's  $t$  test, compared with normal controls.

<sup>c</sup>  $p < 0.005$  Student's  $t$  test, compared with normal controls.

<sup>d</sup> ND = Not done.

LL-S (HI-LL and HI-tr-LL). The degree of inhibition was not correlated to the level of CIC as measured by the <sup>125</sup>I-C1q binding test or PEG precipitation (data not shown). Incubation of PBL with HI-NHS had virtually no effect on ADCC.

Inhibition of ADCC did not occur if HI-LL-S were supplemented with fresh NHS (Fig. 1). These results emphasize the role of heat labile factors in preventing the blockade of ADCC by immune complexes. Pre-

formed soluble IC that do not bear any relationship with leprosy inhibit ADCC of PBL and addition of fresh serum also abolished the inhibitory reaction (Fig. 2). Heat labile serum factors (C?) could act by re-organizing the IC in a way that renders them inactive (<sup>9</sup>), or by the recovery of the blocked FcR at the cell surface (<sup>7</sup>). To answer this question, we performed the inhibition assay in two steps: in the inhibitory phase we allowed contact of PBL with HI-LL sera in the absence of C for 2 hr, and we added fresh normal serum to allow recovery thereafter. The results shown in Table 3 indicate that the action of C was exerted both by preventing the blockade of FcR and by rendering blocked FcR available for the cytotoxic reaction. The degree of inhibition of ADCC achieved in the absence of C was not correlated with the absolute amount of CIC measured by <sup>125</sup>I-C1q binding or PEG precipitation.

**Complement fixing ability of heat-inactivated LL-S and NHS.** Because it has been shown that the C system, via the alternative pathway, may promote the functional recovery of blocked Fc receptors on lymphocytes (<sup>6</sup>), we determined if CIC present in LL-S were able to activate the alternative C pathway. The results shown in Table 4 demonstrate that this is the case. While HI-NHS is relatively ineffective to consume total and alternative pathway activity, both HI-LL-S

TABLE 2. Inhibition of ADCC of normal PBL by LL serum and NHS.<sup>a</sup>

PBL reacted with	ADCC % ± S.E.M.	Inhibition % ± S.E.M.
RPMI-FCS	62.1 ± 3.0	—
NHS	62.0 ± 3.6	0
HI-NHS	59.4 ± 3.1	4.6 ± 1.2
LL-S	59.5 ± 7.3	4.3 ± 1.2
HI-LL-S	40.5 ± 2.6	41.0 ± 8.3
tr-LL-S	62.8 ± 3.7	0
HI-tr-LL-S	31.4 ± 5.3	53.6 ± 9.4

<sup>a</sup> Pellets of  $2 \times 10^6$  PBL resuspended in 20  $\mu$ l RPMI-FCS were incubated at 37°C for 2 hr with 20  $\mu$ l of sera from lepromatous leprosy patients (LL-S) ( $n = 5$ ) or sera from LL patients who received specific treatment (tr-LL-S) ( $n = 5$ ). The reaction was also performed with heat inactivated sera (HI-NHS, HI-LL-S, HI-tr-LL-S). After the incubation, PBL were washed 4 times with RPMI-FCS, resuspended to  $2 \times 10^6$  PBL in 20  $\mu$ l and the reaction was carried out as described in Materials and Methods.

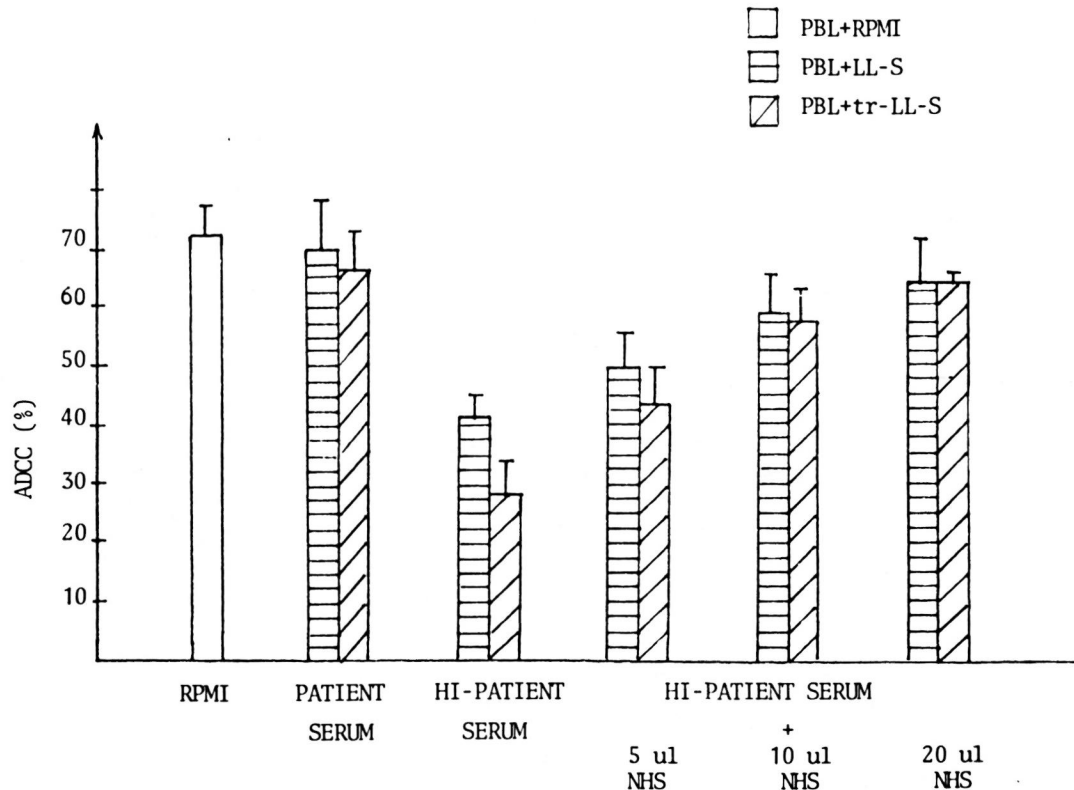


FIG. 1. Prevention of inhibition of ADCC of normal PBL incubated with LL patients' sera by heat labile serum factors.  $2 \times 10^6$  PBL were incubated at  $37^\circ\text{C}$  for 2 hr with  $20 \mu\text{l}$  RPMI-FCS, freshly frozen patient serum, heat inactivated (HI) patient serum, or HI-patient serum plus 5, 10, or  $20 \mu\text{l}$  of NHS. ADCC was determined as described in Materials and Methods. LL-S = sera from lepromatous leprosy patients; tr-LL-S = sera from lepromatous leprosy patients who received special treatment.

and HI-tr-LL-S are able to activate and fix 74–80% of total activity and 40–50% of the alternative pathway.

#### DISCUSSION

In the present study we show that normal ADCC and C activities coexist in LL patients with extremely elevated CIC levels assayed both by an assay based on C component binding (<sup>17</sup>) and by an assay that detects high molecular weight aggregates with or without C binding ability (<sup>3</sup>). Normal C activity and high CIC levels have been well documented previously (<sup>2, 14</sup>). Normal ADCC and C levels could be the consequence of failure of the CIC present in LL patients to block cellular FcR-dependent functions or to consume C activity. Alternatively, compensatory mechanisms could operate in LL to prevent the inhibi-

tory action of CIC. Data shown in Table 2 indicate that the whole sera are unable to block ADCC of normal PBL. However, elimination of C activity (or of other heat labile serum factors) allows expression of the inhibitory activity of CIC present in LL sera. These results are similar to those obtained when adding preformed soluble complexes to a standard ADCC reaction (Fig. 1) and, as in that case, the inhibition can be prevented if C is introduced in the reaction.

It has been clearly shown (<sup>9</sup>) that the C system can alter the physical properties of immune complexes and that such changes would render IC unable to produce many of their biological effects. Recent work by Isturiz, *et al.* (<sup>7</sup>) has emphasized the role of the C system in the recovery of FcR activity blocked by exposure to IC. With the experimental design presented in Table 3, we were

TABLE 3. Recovery of ADCC of normal PBL inhibited by LL patients' sera upon incubation with NHS.<sup>a</sup>

Serum	CIC		Inhibition of ADCC (%)			
	<sup>125</sup> I-C1q binding (%)	PEG-precipitation OD <sub>280</sub> U/ml	PBL + HI-S 2 hr at 37°C	(PBL + HI-S) 2 hr at 37°C then	NHS 18 hr at 37°C	(PBL + HI-S + NHS) 20 hr at 37°C
Patient 1						
LL	26.0	0.31	70		34	20
Patient 2						
tr-LL	8.5	0.61	72		38	28
Patient 3						
tr-LL	65.0	0.42	59		21	1
Controls	7.1	0.19	10		5	1

<sup>a</sup>  $2 \times 10^6$  PBL resuspended in 20  $\mu$ l RPMI-FCS were incubated for 2 hr at 37°C with 20  $\mu$ l heat-inactivated serum (HI-S) from LL patients or a normal donor; alternatively PBL were incubated first for 2 hr at 37°C with 20  $\mu$ l HI-S and then for 18 hr at 37°C with 100  $\mu$ l NHS or for 20 hr at 37°C with a mixture of 20  $\mu$ l HI-S and 100  $\mu$ l NHS. Treated PBL were washed 3 times with RPMI-FCS and ADCC was assayed as described in Materials and Methods. Control ADCC of PBL incubated 18 hr at 37°C with RPMI-FCS was 61% and of PBL exposed to 100  $\mu$ l NHS for 18 hr at 37°C was 60%. The degree of inhibition of ADCC was calculated taking those values as controls.

able to determine that C acted by preventing CIC blockade of ADCC and by recovering FcR activity of blocked effector cells.

Because of the role of the alternative pathway of C, both in the redissolution of

CIC<sup>(10)</sup> and in the recovery of blocked function<sup>(6)</sup>, it was important to determine if CIC present in LL patients were able to activate this pathway. The results shown in Table 4 demonstrate that when endogenous C activity is eliminated, CIC present in LL sera are able to consume both the alternative and total C activity.

The possible relevance of the alternative pathway of C in preserving the ADCC activity of effector cells from lepromatous leprosy patients is further supported by the fact that in these patients ADCC correlates positively with the hemolytic activity of the alternative C pathway.

It could be postulated that, while immune complexes that are potentially able to trigger inflammatory responses by C activation or blocking cellular Fc receptors exist in LL patients, compensatory mechanisms such as those demonstrated *in vitro* may contribute to maintain intact ADCC capability *in vivo*.

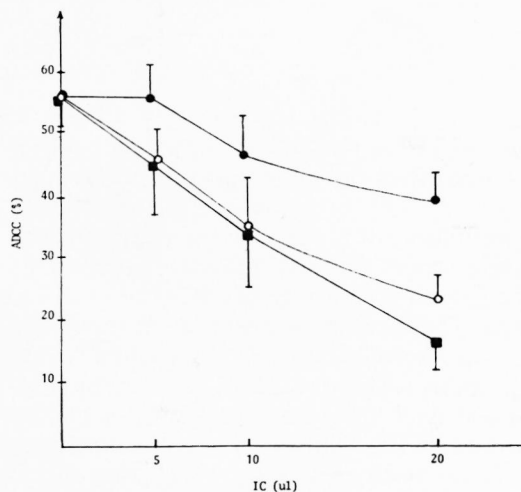


FIG. 2. Inhibition of ADCC of normal PBL by soluble preformed immune complexes.  $2 \times 10^6$  PBL were suspended in 20  $\mu$ l RPMI containing different amounts of soluble preformed immune complexes (IC) and incubated for 2 hr at 37°C in the presence of 50  $\mu$ l RPMI-FCS (■—■); HI-NHS (○—○); or fresh frozen NHS (●—●). ADCC was determined as described in Materials and Methods.

## SUMMARY

Antibody-dependent cellular cytotoxicity (ADCC) and total and alternative pathway complement (C) activity were found to be normal in lepromatous leprosy (LL) patients in the presence of elevated circulating immune complexes (CIC) measured by the

TABLE 4. Complement fixing ability of heat LL serum and NHS.<sup>a</sup>

Control NHS reacted with	No.	Total C activity		Alternative pathway activity	
		CH50 (U/ml)	Fixation (%)	APCH50 (U/ml)	Fixation (%)
HI-NHS	2	168-172	11-13	108-110	11.5-13.5
HI-LL-S	3	38.0 ± 6.2	80.0 ± 3.5	68.0 ± 16.8	41.0 ± 8.5
HI-tr-LL-S	5	48.0 ± 8.5	74.0 ± 3.5	62.0 ± 12.6	48.0 ± 8.1

<sup>a</sup> Results are expressed as mean ± standard error of the mean.

<sup>125</sup>I-C1q binding assay. Heat inactivation (56°C, 30 min) uncovered the ADCC inhibitory activity of LL sera. The effect of C was exerted both by interfering with the blocking action of CIC and by recovering ADCC activity of CIC-blocked effector cells. Heat inactivation allowed the expression of total and alternative C pathway fixing ability of the LL sera. Thus, immune complexes potentially able to block Fc receptor-dependent functions or capable of fixing C can be detected in LL sera. We postulate that compensatory mechanisms such as those described *in vitro* may contribute to maintain intact ADCC activity *in vivo*.

### RESUMEN

Se encontró que tanto la citotoxicidad celular dependiente de anticuerpo (CCDA) como la actividad total de complemento, estuvieron dentro de límites normales en los pacientes lepromatosos (LL), aún en presencia de niveles elevados de complejos inmunes circulantes (CIC) medidos por el ensayo de enlazamiento de C1q-<sup>125</sup>I. La inactivación por calor (56°C, 30 min) permitió descubrir una actividad inhibitoria de la CCDA en los sueros LL. El efecto del C se ejerció tanto por interferencia de la acción bloqueadora de los CIC, como por recuperación de la actividad CCDA de las células efectoras bloqueadas por los CIC. La inactivación por calor permitió visualizar la capacidad fijadora del C de los sueros LL. Así, en los sueros LL se pueden demostrar CIC potencialmente capaces de bloquear las funciones dependientes del receptor para Fc, o capaces de fijar C. Postulamos que mecanismos compensatorios similares a aquellos descritos *in vitro* pueden contribuir al mantenimiento *in vivo* de la actividad CCDA.

### RÉSUMÉ

On a observé que la cytotoxicité cellulaire dépendant des anticorps (ADCC), de même que l'activation du complément (C) par la voie classique et par la voie alterne, étaient normales chez des malades atteints de lèpre lépromateuse (LL) en présence d'un taux élevé

de complexes immuns circulants (CIC) tel qu'il est déterminé par le test de fixation de C1q marqué à l'<sup>125</sup>I. L'inactivation par la chaleur pendant 30 minutes à 56°C a permis de révéler l'activité inhibitrice de l'ADCC exercée par le sérum de malades LL. L'activité de complément (CC) était à la fois le résultat d'une interférence avec l'action de blocage des complexes et de la récupération de l'activité ADCC des cellules effectrices bloquées par ces complexes immuns. L'inactivation par la chaleur a permis de démontrer la propriété qu'ont les sérums de lèpre lépromateuse de déclencher la voie classique et la voie alterne d'activation du complément. Dès lors, les complexes immuns sont le cas échéant capables de bloquer les activités biologiques dépendant du récepteur Fc. On peut dès lors supposer que des mécanismes compensatoires tels que ceux mis en évidence *in vitro* contribuent éventuellement à maintenir l'activité ADCC *in vivo*.

**Acknowledgments.** We thank Marta Felippo for expert technical assistance; Drs. Beatriz Ruibal Ares and Norma Riera for their critical advice; and Drs. L. M. Baliña, R. Valdez, J. Cardama, and J. C. Gatti for referring the patients.

We are grateful to Farmasa Farmacéutica Argentina for donating the Hypaque solution.

This work was done with the financial support of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Grant No. 9466a/83 and Grant No. 2755 ESILIM-CONICET.

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