# Suppressor Cells in Experimental Murine Leprosy <sup>1,2</sup>

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The lepromatous form of human leprosy is characterized by a marked impairment in cell-mediated immunity which is not apparrent in antibody mediated immunity (5. <sup>17</sup>). Similarly, Mycobacterium lepraemurium (MLM) infection in susceptible strains of mice causes a marked suppression in cellmediated immune responses, as shown by delayed rejection of skin homografts and depression or absence of contact sensitivity to chemicals (11). According to some workers (1,11), infected mice show no depression of their humoral immunity, whereas others (12) observed a depression but at an advanced stage of the disease. The mechanism(s) underlying the inhibition of immune responses in both human and murine leprosy has not been elucidated.

In the present work, we have observed an impairment in the *in vitro* proliferative responses to T and B cell mitogens of spleen and lymph node cells from mice infected for various periods of time with MLM. These results suggest that suppressor cells are involved in the immunologic depression of leprous mice since infected spleen cells mixed with normal cells have the ability to suppress their proliferative response.

## MATERIALS AND METHODS

Animals. Female C3H/St mice were obtained from Canadian Breeding Farm and Laboratories, Ltd., Laprairie, Quebec. They weighed 16-18 gm at the beginning of the experiments. They were maintained under standard laboratory conditions and fed Purina chow and water *ad libitum*.

Infection and cell preparation. The Hawaii strain of *Mycobacterium lepraemurium* (MLM), obtained from Dr. O. K. Skinsnes, Honolulu, Hawaii was maintained by successive passages in female C3H/St mice. At the time of infection, fresh bacilli were isolated from the spleen of infected mice and counted according to the method of Shepard and McRae (15). Forty mice were injected intraperitoneally (i.p.) with 1 × 107 bacilli suspended in 0.5 ml phosphate buffer (0.15 M, pH 7.4), or with phosphate buffer alone as controls (20 mice). Under these conditions, the survival time of mice varied from 201 to 271 days with a mean of 230.3 days. At two, five and eight months after infection, groups of two infected and three control mice, chosen at random, were sacrificed by cervical dislocation and the lymphoid organs of each group were pooled separately. The spleen and peripheral lymph nodes (pools from inguinal, popliteal and axillary nodes) were removed under sterile conditions and the lymphoid cells isolated according to a method already described (16).

Lymphocyte transformation. The responses of spleen and lymph node cells to the T cell mitogens, phytohemagglutinin (PHA) and concanavalin A (Con A), and to the B-cell mitogens lipopolysaccharide (LPS) and dextran sulfate (DS) were determined by measuring the incorporation of tritium-labeled thymidine (3H-TdR) into DNA. Viable lymphoid cells  $(5 \times 10^5)$  were cultured with optimal concentrations of mitogens in 0.1 ml RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco) and antibiotics (penicillin: 100 units/ml, streptomycin: 100  $\mu$ g/ml). PHA was used at a final concentration of 50  $\mu$ l/ ml, Con A at 2  $\mu$ g/ml, LPS and DS at 50  $\mu g/ml$ . All cultures were set up in triplicate in flat-bottomed tissue culture plates (Linbro) and incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cultures were pulsed with  $1.0 \mu$  Ci <sup>3</sup>H·TdR (Amersham Searle Corp., Oakville, Ontario; specific activity 2.0 Ci/mmole) 18 hours before the end of the incubation period and harvested on glass fiber filters with a MASH multiple automated sample harvester (Microbiological Associates, Bethesda, MD). The radioactivity was counted in a Beckman DPM-100 scintillation spectrome-

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FIG. 1. <sup>3</sup>H-thymidine incorporation in response to PHA, Con A, LPS and DS in the spleen and lymph node cells of normal and MLM-infected mice, as a function of time after infection.

ter. Results are expressed as net cpm (i.e., mean cpm of stimulated triplicate cultures minus mean cpm of nonstimulated triplicate cultures).

# RESULTS

Intraperitoneal infection of C3H mice with  $1 \times 10^7$  MLM caused an enlargement of the spleen that began about two months after infection and progressed thereafter. At death, the weight of the spleen was six to seven times that of normal spleens. Enlargement of peripheral lymph nodes, more marked for the inguinal ones, also occurred but only around the fifth month after i.p. infection.

The results of a typical experiment on the mitogen-induced lymphoblastic transformation in normal and MLM-infected mice are illustrated in Figure 1. In normal mice (time 0), the proliferative response of spleen cells to PHA was low (and very often absent) while that of lymph node cells was quite high. Similar findings, although less marked, were observed with Con A. Inversely, the reactivity of B cells to LPS and DS was relatively high in normal spleens and very low in the lymph nodes. These results might be explained either by differences in the ratio of T and B cells between the two lymphoid organs (<sup>10</sup>), or by different numbers or the differential activity of regulatory cells.

In the spleen of MLM-infected mice, the reactivity of T cells was completely absent two months after infection, while the B cell response although lower than that of the controls was still detectable (upper part of Fig. 1). However, five and eight months after infection, the response to LPS and DS was respectively suppressed. These results differed markedly from those obtained with lymph node cells from infected mice (lower part of Fig. 1). First, the PHA response was maintained at high levels till the fifth month after infection and a relatively high response was still present at eight months; second, the Con A response decreased early and steadily to reach a very low value at eight months; and third, both LPS and DS responses increased to some extent around the fifth month and decreased thereafter. These findings indicate that at different times after MLM infection, the spleen and lymph node cells become refractory to stimulation first by the T and later by the B cell mitogens.

The inhibition of the lectin-induced transformation in the spleen of MLM-infected mice can be due either to the presence of suppressor cells or to a relative lack of T and B cells owing to their replacement by parasitized macrophages or histiocytes. To test these possibilities, spleen cells from leprous mice, or from normal mice as controls, were added to normal cells just prior to their stimulation with the mitogens. As seen in Table 1, the addition of  $0.5 \times 10^5$  infected cells inhibited to a significant extent the <sup>3</sup>H-TdR incorporation by the normal spleen cells when stimulated with each of the four mitogens. With the addition of  $2.5 \times 10^5$  infected cells, the response to PHA and Con A was totally suppressed, that to LPS was significantly depressed while, for unknown reasons, the response to DS was enhanced. Infected spleen cells were also found to inhibit the <sup>3</sup>H-TdR incorporation by normal lymph node cells (data not shown). The addition of normal spleen cells was without any significant effect on the proliferative response. Since the addition of infected cells to normal spleen or lymph node cells caused an inhibition of their mitogen-induced proliferation, it appears that the decreased response of spleen cells from MLM-infected mice is due to an active suppression rather than to the absence or the refractoriness of mitogen reactive cells.

In an attempt to characterize the type of cells involved in the suppression of the mitogen-induced blastogenesis, a T lymphocyte enriched population was prepared from the spleen cells of MLM-infected mice by passage through a nylon wool column (7) and assayed for their PHA responsiveness in vitro. The results, although still preliminary, would indicate that at an advanced stage of the disease (e.g., seven to eight months) suppressor cells were present among the T cell population since this population failed to respond to PHA stimulation. However, it has to be noted that earlier during the course of MLM infection (e.g., two to three months), a significant restoration of the PHA responsiveness was observed in the T cell population when compared to that found in the corresponding unfractionated population.

In another series of experiments, phagocytic cells were removed from the spleen cells by the carbonyl iron-magnet method (<sup>13</sup>) prior to the stimulation of the remaining nonphagocytic cells with T and B cell mitogens. Following this treatment the recovery of the nonphagocytic cells was essentially the same for both MLM-infected and noninfected mice. The results obtained in three independent experiments performed with mice infected for five to eight months indicated that the removal of phagocytic cells led to a significant restoration of the B cell responsiveness whereas the T cell responsiveness remained profoundly depressed.

## DISCUSSION

The present study has shown that following intraperitoneal infection of C3H/St mice with M. lepraemurium, a marked depression of the mitogen-induced blastogenesis occurred first in the spleen and later during the course of infection in the peripheral lymph nodes. The fact that the suppression of blastogenesis occurred earlier in the spleen than in the nodes might be due to the route of inoculation used. Another interpretation would be that suppressor cells originate in the spleen and then some of these cells leave the spleen to reach the peripheral lymph nodes as shown recently in mice rendered tolerant to chemical agents (9). At the spleen level, the depression of the proliferative response to the T cell mitogens was found to precede that of the B cell mitogens. This finding suggests either a difference in the susceptibility of T and B cells to the action of suppressor cells, the T cells being more susceptible; or the presence of two types of suppressor cells, one affecting the T lymphocytes, the other which is activated later affecting the B lymphocytes. On the other hand, in the peripheral lymph nodes, although the T cell proliferative response was significantly depressed at an advanced stage of the disease (e.g., eight months), no significant depression of the B cell response was noted throughout all of the investigation period. On the contrary, an enhancement of the B cell response was temporarily observed near the fifth month after the infec-

 TABLE 1. Effects of adding spleen cells from MLM-infected mice on the in vitro proliferative response of normal spleen cells to T and B cell mitogens.

Origin and no. of spleen cells in cultures		Thymidine incorporation after stimulation with:			
Normal cells	Infected cells <sup>a</sup>	РНА	Con A	LPS	DS
5.0 × 10 <sup>5</sup> +	0	12,556b	89,446	72,022	11,780
5.0 × 10 <sup>5</sup> +	0.5 × 10 <sup>5</sup>	0	25,023	52,708	3,132
2.5 × 10 <sup>5</sup> +	2.5 × 10 <sup>5</sup>	0	0	21,066	18,117
2.5 × 10 <sup>5</sup> +	0	1,862	23,316	126,569	2,850

<sup>a</sup> Infected cells taken from a mouse infected with MLM six months previously.

<sup>b</sup> This data represents the net cpm, i.e., cpm of stimulated minus cpm of nonstimulated cultures. Experiments were done in triplicate, the range was less than 10% and is not included in this table.

tion. These *in vitro* observations might have some relevance to the *in vivo* observations of the early suppression of cell-mediated immunity and the persistence for long periods of time of antibody-mediated immunity (<sup>11,</sup> <sup>12</sup>).

The addition of infected spleen cells to normal spleen or lymph node cells caused an inhibition of their mitogen-induced proliferation. This finding strongly suggests the presence of suppressor cells (or factors) in the spleen of MLM-infected mice which actively inhibit the lymphoproliferative response, thereby eliminating in part the possibility that an absence of mitogen reactive cells in the infected spleens was responsible for the suppression observed. This interpretation finds support from the fact that following the carbonyl iron-magnet treatment of spleen cells, the recovery of viable spleen cells was essentially the same for both infected and uninfected mice. Nevertheless, additional studies are needed to find out the relative proportion of T and B lymphocytes in the spleens of MLM-infected mice and to study the effects of MLM-parasitized macrophages and MLM alone in relation to the suppression of the lymphoproliferative responses. In addition, it must be determined whether the *in vitro* depression of the blastogenic response of lymphocytes is related to the humoral and cell-mediated immune responses of mice infected with MLM for various periods of time.

It should be noted that suppressor cells, as found in this study, have been demonstrated in the spleen of mice infected with unrelated mycobacteria (4,16), Corynebacterium parvum (14), parasitic agents (3.6) and in mice bearing a variety of tumors (8). The exact nature of these cells is still a matter of controversy; indeed, they have been identified either as T lymphocytes (18), B lymphocytes  $(^{2})$  or as monocyte macrophages  $(^{4,8})$ . The present results do not allow conclusions about the nature of suppressor cells involved in murine leprosy. However, the fact that they cannot be totally eliminated either by adherence to nylon wool or by the carbonyl iron-magnet technic might suggest that they differ from the macrophage-like suppressor cells found recently in the spleen of BCG-infected mice (4,16). Obviously, it remains to be determined whether the adherent and phagocytic properties are preserved

tion. These in vitro observations might have in a cell overloaded with M. lepraemurium.

The observation that spleen suppressor cells are induced following MLM infection in mice might be of primary importance in understanding the mechanism responsible for the impairment of cellular immunity in murine and human leprosy.

### SUMMARY

Female C3H/St mice were infected intraperitoneally with  $10^7$  *M. lepraemurium* (MLM), and the *in vitro* proliferative responses to the T cell mitogens PHA and Con A, and to the B cell mitogens LPS and dextran sulfate were determined in the spleen and peripheral lymph node cells at two, five and eight months after infection.

In the spleen, a complete suppression of the lymphocyte transformation to the T cell mitogens was observed two months after infection, while a complete suppression of the B cell proliferative response occurred at five to eight months. In the peripheral lymph nodes, the T cell responsiveness was maintained at a high level till the fifth month and decreased significantly thereafter, whereas the B cell proliferative response remained relatively high throughout the whole observation period. When spleen cells from MLMinfected mice were cocultured with normal spleen or lymph node cells, a strong depression of their reactivity to the mitogenic agents was observed.

These results strongly suggest the presence of suppressor cells in the spleens of MLM-infected mice that occur relatively soon after infection and that persist till the death of the animal. Since the suppressive activity in spleen cell suspensions was not totally abolished either by adherence to nylon wool or by the carbonyl iron-magnet technic, most of the suppressor cells would belong to a T cell subpopulation. The physiologic role of these cells in the impairment of cellular immunity in murine leprosy remains to be elucidated.

#### RESUMEN

Se infectaron ratones hembras C3H/St con  $10^7$ *M. lepraemurium* (MLM). A los 2, 5 y 8 meses después de la infección, se hicieron determinaciones *in vitro* de las respuestas proliferativas de las células del bazo y de los ganglios linfáticos periféricos hacia los mitógenos para células T, PHA y Con A, y hacia los mitogenos para celulas B, LPS y dextran-sulfato.

Dos meses después de la infección, se observó en el bazo una completa suppresión de la transformación de linfocitos por los mitógenos de las células T, mientras que una completa supresión de la respuesta proliferativa de las células B se presento entre 5 y 8 meses de infección. La capacidad de respuesta se mantuvo elevada en los ganglios linfáticos periféricos hasta el 5º mes y disminuyó significativamente después en tanto que la respuesta proliferativa de las células B permaneció relativamente elevada durante todo el periodo de observación. Cuando las celulas del bazo de los ratones infectados con MLM se cocultivaron con células normales de bazo o de ganglios linfáticos, se observó una fuerte depresión de la reactividad hacia los agentes mitogénicos.

Estos resultados sugieren fuertemente la presencia en el bazo de los ratones infectados con MLM, de células supresoras que aparecen relativamente pronto después de la infección y que persisten hasta la muerte de los animales. Puesto que la actividad supresora en las suspensiones de las células esplénicas no se eliminó totalmente por adherencia a fibra de nylon o por la técnica del "carbonilo férrico y magneto", la mayoría de las células supresoras deben pertenecer a una subpoblación de células T. El papel fisiológico de estas células en la alteración de la inmunidad celular en la lepra murina queda todavía por estudiarse.

## RÉSUMÉ

On a infecté par voie intra-péritonéale des souris femelles de souche C3H/St, avec  $10^7 M$ . *lepraemurium* (MLM); la prolifération observée *in vitro* en réponse à l'application de PHA mitogène des cellules T et Con A, et de mitogènes LPS des cellules B, ainsi que de dextran sulfate, a été déterminée dans la rate et dans les cellules des ganglions lymphatiques périphériques, deux, cinq et huit mois après l'infection.

Au niveau de la rate, on a observé une suppression complète de la transformation lymphocitaire à la suite d'application de mitogènes des cellules T, alors qu'une suppression complète de la prolifération des cellules B n'a été notée qu'après cinq à huit mois. Dans les ganglions lymphatiques périphériques, la réponse des cellules T s'est maintenue à un haut niveau jusqu'à la fin du cinquième mois, pour ensuite baisser significativement, alors que la réponse proliférative des cellules B est restée relativement élevée pendant toute la période d'observation. Lorsque les cellules spléniques, provenant de souris infectées par M. lepraemurium ont été cultivées en présence de cellules spléniques normales ou de cellules normales provenant des ganglions lymphatiques, on a observe une forte diminution de leur réactivité aux agents mitogéniques.

Ces résultats suggèrent fortement la présence au niveau de la rate de souris infectées par *M. lepraemurium*, de cellules suppressives, et ceci relativement tôt après l'infection; ces cellules persistent alors jusqu'à la mort de l'animal. L'activité suppressive notée dans les suspensions de cellules spléniques n'était pas entièrement abolie par l'adhérence à la laine de nylon, ou par la technique du "carbonyl iron-magnet"; on en conclut que la plupart des cellules suppressives doivent appartenir à une sous-population de cellules T. Le rôle physiologique de ces cellules dans la déterioration de l'immunité cellulaire dans la lèpre murine reste à élucider.

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