IMMUNOLOGICAL REGULATION OF EXPERIMENTAL CUTANEOUS LEISHMANIASIS

III. Nature and Significance of Specific Suppression of Cell-mediated Immunity in Mice Highly Susceptible to *Leishmania tropica*

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The development of diffuse cutaneous leishmaniasis has been associated with impairment of cell-mediated immunity (CMI)[1] in patients infected with *Leishmania tropica* (1). The relative susceptibility of certain inbred mouse strains and the persistence of lesions in resistant mice given an overwhelming challenge have also shown some rough correlation with an analogous diminution of delayed-type hypersensitivity (DTH) (2, 3). Although this was originally attributed to a blocking effect of humoral antibody (4), the development of higher anti-leishmanial titers does not appear to determine either mouse strain susceptibility to *L. tropica* (5) or inhibition of DTH during the infection.[2]

An important role for CMI in the control of leishmanial infections has also been inferred from experimental approaches. Widespread cutaneous dissemination developed in *L. enriettii* infected guinea pigs when they were immunosuppressed with antilymphocyte serum (6) or when the primary lesion was in a skin site with interrupted lymphatic drainage (7). T cell deprivation was found to retard healing and diminish DTH with *L. tropica* infections in thymectomized, irradiated, bone marrow reconstituted CBA mice (8). The present paper establishes not only that the converse situation is obtained in the highly susceptible BALB/c strain - prior thymectomy slows progression of the disease - but that the suppression of DTH reactivity which develops in these mice is both leishmania-specific and accompanied by the emergence of a T cell population which, when transferred, impairs its induction. The data collectively imply that the characteristic failure of the BALB/c strain to control *L. tropica* infection, largely attributable to one major regulatory gene,[3] involves

[1] *Abbreviations used in this paper:* ATxXBM, adult thymectomy, x-irradiation, and bone marrow-reconstitution; CMI, cell-mediated immunity; CY, cyclophosphamide, DNFB, 2,4-dinitrofluorobenzene; DTH, delayed-type hypersensitivity; GPBS, glucose phosphate-buffered saline; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; PSA, protein-soluble antigen derived from *L. tropica*; SRBC, sheep erythrocytes; XBM, x-irradiated, bone marrow-reconstituted.

[2] Hale, C., and J. G. Howard. Immunological regulation of experimental cutaneous leishmaniasis. II. Studies with Biozzi high and low responder lines of mice. *Parasite Immunol.* In press.

[3] Howard, J. G., C. Hale, and W. L. Chan-Liew. Immunological regulation of experimental cutaneous leishmaniasis I. Immunogenetic aspects of susceptibility to *Leishmania tropica* in mice. *Parasite Immunol.* In press.

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cancellation of the normal cell-mediated immune response by potent suppressor T cell generation.

Materials and Methods

Mice. BALB/c, CBA/T6T6, C57BL/6, and (BALB/c × C57BL/6)F_1 mice were obtained from our own inbred colonies. In all experiments, animals were of the same sex and age 10–12 wk.

Leishmania Parasites. The strain of *Leishmania tropica major* used in this study was kindly provided by Dr. R. A. Neal, Wellcome Research Laboratories, Kent, England (9). The organisms were cultivated by passaging a piece of nonulcerated lesion into conventional Nicolle's modification of Novy and McNeal's medium blood agar slopes overlaid with 1% glucose phosphate-buffered saline (GPBS), pH 7.2. After incubating at 26°C for 4 d, promastigotes were collected, sedimented by centrifugation, and resuspended in 1% GPBS. The number of organisms was estimated microscopically using a hemocytometer and by diluting the sample in 10% Lugol's iodine. Mice were infected by injecting subcutaneously 0.1 ml of the desired concentration of promastigotes in shaved rumps. The lesions developed were measured with a direct reading vernier calliper gauge (GMH-390-T, Gallenkamp, London) in two perpendicular diameters. The average diameter (in millimeters) was recorded and corrected for the thickness of skin at the same site of an infected mouse.

Antigens and Chemicals. Protein-soluble antigen (PSA) fraction from *L. tropica* used for the elicitation of DTH, was prepared following the method of Bryceson et al. (10) in which 4-d-old cultures of promastigotes were collected, washed three times in phosphate-buffered saline (PBS), and then sonicated for 6 min in an MSE sonicator (MSE ultrasonic power unit, Sussex, England). When sonication was complete, as judged microscopically, urea was added to a final concentration of 8 M. The resulting suspension was incubated for 2 h at 37°C and then dialyzed extensively against 0.1 M ammonium carbonate: acetic acid buffer, pH 7.4. The dialysate was centrifuged at 33,000 rpm for 1 h in a Beckman zonal ultracentrifuge (Beckman Instruments, Palo Alto, Calif.) to remove any debris. The supernate was quantified according to protein content by Lowry's method and stored at −20°C in aliquots until use. Sheep erythrocytes (SRBC) were stored in Alsever's solution at 4°C and washed three times in saline before use. 2,4-Dinitrofluorobenzene (DNFB) was obtained from BDH (British Drug House Chemicals Ltd., Poole, Great Britain). Cyclophosphamide monohydrate was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks, Great Britain, and Paromomycin sulphate from Parke, Davis and Co., Usk Road, Pontypool, Gwent, Great Britain.

Induction and Measurement of DTH. In some cases mice were injected intraperitoneally with 150 mg/kg of cyclophosphamide 2 d before DTH induction. For DTH to SRBC, mice were injected subcutaneously with 1×10^8 SRBC. 5 d later they were tested for DTH by injecting 1×10^8 SRBC (50 μl volume) into the right hind footpad. The increase in footpad thickness was measured 24 h after the injection, using a dial Caliper (Pocotest, reverse Spring-loaded caliper, Carobronze, Great Britain). DTH was expressed as percentage increase at 24 h. For contact sensitivity, DNFB was dissolved in acetone/olive oil (1:1) at a concentration of 10 mg/ml. Mice were primed by painting the shaved regions of the abdomen and the back with a total of 0.5 ml of the DNFB preparation. 5 d after priming, 10 μl of the DNFB solvent mixture was painted on the right pinna. Ear thickness increases were measured at 24 h with a dial caliper (model 7309, Mitutoyo Co. Japan). Contact sensitivity was expressed as percentage increase of ear thickness. ((right-left/left × 100). For DTH to *L. tropica* antigen, mice were injected in the right hind footpad with 50 μl of *L. tropica* antigen preparation (2 mg/ml) and footpad thickness increase measured 3, 24, and 48 h later with a dial-caliper (Pocotest). DTH was again expressed as percentage increase of footpad thickness. In each experiment a group of unimmunized mice was injected in the footpad with the test antigen. The increase of footpad thickness at 24 h in these mice was taken as the background footpad swelling caused by the eliciting antigen alone. All DTH data presented in this paper are corrected for the background readings.

In preliminary experiments it was established that the DTH reaction to *L. tropica* has classical characteristics: it peaks between 24 and 48 h after antigen elicitation and is transferable by immune spleen and lymph node cells but not by immune serum. Histological examination shows marked infiltration of mononuclear cells at the site of antigen elicitation.

Thymectomy, Irradiation, and Bone Marrow Reconstitution. Mice were thymectomized at 4 wk of age using a 1 in 50 dilution of avertin (Winthrop, Surrey, Great Britain) in 10% alcohol for anesthetic. 3 wk later the mice were irradiated in a caesium source (^{137}C) at a rate of 50.7 rads/min (800 rads for BALB/c and 900 rads for CBA/T6T6 and F_1 mice) and reconstituted with 5×10^6 syngeneic bone marrow cells injected intravenously. The mice were infected with either 2×10^5, 2×10^6, or 2×10^7 promastigotes 8–10 wk later.

Anti-θ Treatment of Cells. Bone marrow and spleen cells were collected in Eagle's minimum essential medium (MEM) and sedimented by centrifugation. 1×10^8 aliquots of cells were resuspended in 1 ml of 1:20,000 dilution of an anti-Thy-1.2 serum (F7D5 monoclonal IgM antibody, Olac Ltd., Oxon, Great Britain). After a 30-min incubation at room temperature, the cells were washed once and resuspended in 2 ml of a 1:10 dilution in MEM of heat-inactivated, spleen cell-absorbed rabbit complement, and incubated for a further 45 min. The cells were then washed twice in MEM and their percentage viability estimated by trypan blue dye exclusion assay.

Anti-IgG Column Fractionation of Spleen Cells. T cell-enriched fractions were prepared according to the method described by Shand (11). Ammonium sulphate-precipitated mouse immunoglobulin was coupled at pH 7.4 to plastic diakon beads (MG 101, Imperial Chemical Industries, Ltd, Herts, Great Britain) by incubating for 1 h at 45°C followed by overnight incubation at 4°C. The coated beads were washed three times in PBS and loaded into a column (1.5 × 30 cm). A 1:3 dilution of rabbit anti-mouse IgG serum was added to the column and allowed to react for 1½ h at room temperature. The column was extensively washed with PBS, and a spleen cell suspension (5×10^7 cells/ml) then passed through at a rate of 4 ml/min at room temperature. The extent of lymphocyte depletion in the eluted cells, assessed by an indirect immunofluorescence method, usually indicated a reduction from 40 to 50% to <0.5%.

Statistical Analysis. Standard errors of the mean were calculated and the statistical significances of the results analyzed by Student's *t* test.

Results

The Converse Effects of Thymectomy on the Outcome of L. tropica Infection in CBA, BALB/c, and (BALB/c × C57BL/6)F_1 Mice. It has been shown previously that the ability of the 'resistant' CBA strain to control and heal cutaneous *L. tropica* lesions is impaired or weakened after adult thymectomy, x-irradiation, bone marow-reconstitution (ATxXBM) treatment (8). Our own experience is entirely consistent with this. Whereas XBM CBA mice did not differ from normal CBA in the maximum size attained (5–7 mm) and time for complete regression (90 d) of lesions induced by 2×10^7 *L. tropica*, ATxXBM animals showed retardation of healing such that only 4 out of 12 had complete regression by day 160 (Fig. 1). The effect was even more dramatic in a second experiment, for 9 out of 10 ATxXBM animals died from generalized

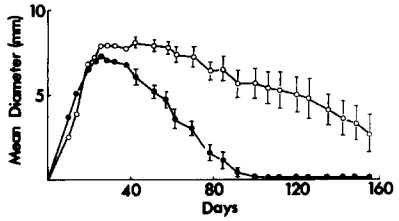

FIG. 1. *L. tropica* in relatively T-depleted CBA mice. Adult mice were either thymectomized (○) or sham thymectomized (●). 3 wk later, they were lethally irradiated with 900 rads whole body irradiation and reconstituted with 5×10^6 syngeneic bone marrow cells. The mice were infected subcutaneously with 2×10^7 *L. tropica* promastigotes in the rump 8 wk after irradiation and reconstitution. The lesion at the site of infection was read at weekly intervals. Each point represents the mean lesion size of 12 mice. Vertical bars show standard errors of the mean.

infection during days 90–170, whereas lesions in the x-irradiated, bone marrow-reconstituted (XBM) controls had regressed by day 90 as in Fig. 1.

A striking contrary trend was found in the 'susceptible' BALB/c strain. In a cumulative experience of >600 BALB/c mice infected with 2×10^7 *L. tropica*, all without exception have shown inexorable growth of the lesion, without detectable immune retardation, leading to 100% mortality from visceralization of the infection. Whereas XBM animals reacted indistinguishably from these, a pronounced slowing in lesion growth was apparent in the ATxXBM group from day 25 onwards (Fig. 2). All the XBM mice died in <100 d, whereas 5 out of 12 ATxXBM animals were surviving at 155 d, 2 of them with completely healed lesions. The same effect was found in (BALB/c × C57BL/6)F_1 mice which are somewhat less susceptible to *L. tropica* than the BALB/c parents (Fig. 3). The steady progression of lesion growth in the XBM group was completely arrested from day 30 onwards in the ATxXBM group. In this instance 7 out of 17 animals (42%) had achieved total healing by day 165, in comparison with 0 out of 23 controls. Parallel converse effects were found in the specific DTH reactivity of these mice (Fig. 4). Whereas prior thymectomy weakened the 24/48-h footpad response in infected XBM CBA mice, it had the opposite effect in BALB/c and (BALB/c × C57BL/6)F_1 mice. While the ATxXBM

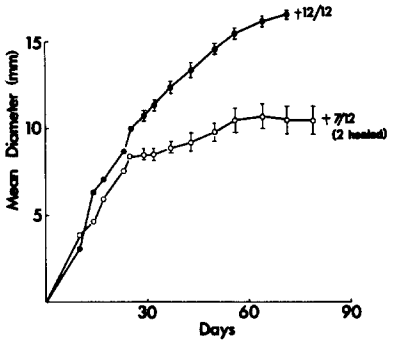

FIG. 2. *L. tropica* in relatively T-depleted BALB/c mice. Adult BALB/c mice were either thymectomized (○) or sham thymectomized (●). 3 wk later they were irradiated (800 rads) and reconstituted with 5×10^6 syngeneic bone marrow cells. They were infected with 2×10^7 *L. tropica* 8 wk later and lesions read weekly. The cross and figure at the end of each line denotes the cumulative mortality of each group at the time shown after infection with *L. tropica*.

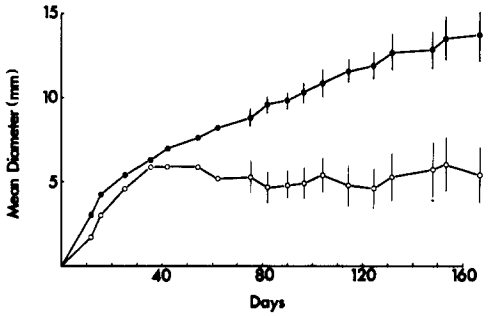

FIG. 3. *L. tropica* in relatively T-depleted (BALB/c × C57BL/6)F_1 mice. For details see legend to Fig. 1. There were 17 mice in the ATxXBM group (○) and 23 mice in the XBM group (●).

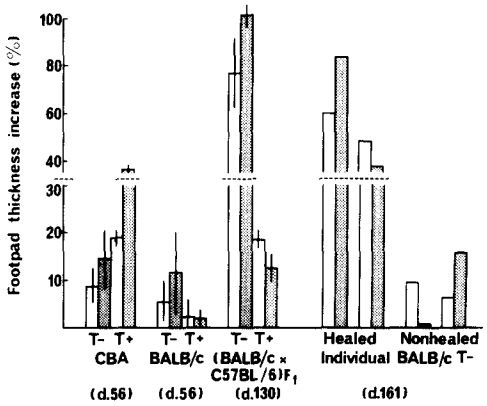

FIG. 4. DTH to *L. tropica* in T-deprived mice. The ATxXBM mice (T−) and XBM mice (T+) described in Figs. 1–3 were tested for DTH to *L. tropica*. They were injected in the footpad with 100 μg PSA at various times after infection as indicated. DTH was expressed as percentage of specific footpad thickness increase at 24 (open columns) and 48 h (hatched columns). Vertical bars represent standard errors of the mean.

animals of either CBA or BALB/c strain showed equivalent relatively weak DTH reactivity (in the latter case a gain over the complete suppression of the XBM group), the corresponding augmentation in the F_1 situation was striking. Similar large responses were elicited at day 161 in the two ATxXBM BALB/c mice of Fig. 2 which had healed their lesions in comparison with two other survivors of the group which had not.

These results implied that a potent suppressive influence operates in *L. tropica*-infected BALB/c and (BALB/c × C57BL/6)F_1 mice such that even the weak residual CMI reactivity in ATxXBM animals is more effective than that which can function normally.

DTH Responses to L. tropica in Mice. Several strains of inbred and F_1 hybrid mice were infected with 2×10^7 parasites and their DTH reactivity to *L. tropica* antigen (PSA) compared at various times subsequently. BALB/c mice developed variable levels of DTH early on during the infection, but showed little or no detectable DTH by 30 d (Fig. 5a). In contrast, CBA, C57BL/6, and (BALB/c × C57BL/6)F_1 hybrid mice developed uniformly high levels of DTH which persisted long after infection (Fig. 5b–d). In an earlier report[2] it was shown that BALB/c mice are uniquely susceptible to even minimal infecting doses of *L. tropica*, whereas CBA, C57BL/6, and (BALB/c × C57BL/6)F_1 mice are able to resolve or contain infections induced by high doses of the parasite. Data presented in Fig. 5 therefore demonstrate a positive correlation between the DTH responses and the ability to exert some control over *L. tropica* infection.

Effect of Cyclophosphamide (CY) on the DTH Response to L. tropica. Administration of CY before antigen priming significantly enhances DTH reaction. The effect of CY on the DTH response to *L. tropica* was investigated in BALB/c mice which generally express lower levels of DTH to this parasite than other strains of mice so far tested. BALB/c and CBA mice were injected with 2×10^7 live or killed *L. tropica* 2 d after receiving 150 mg/kg of CY. Control mice were not pretreated with CY. 10 d after immunization, both BALB/c and CBA mice pretreated with CY developed signifi-

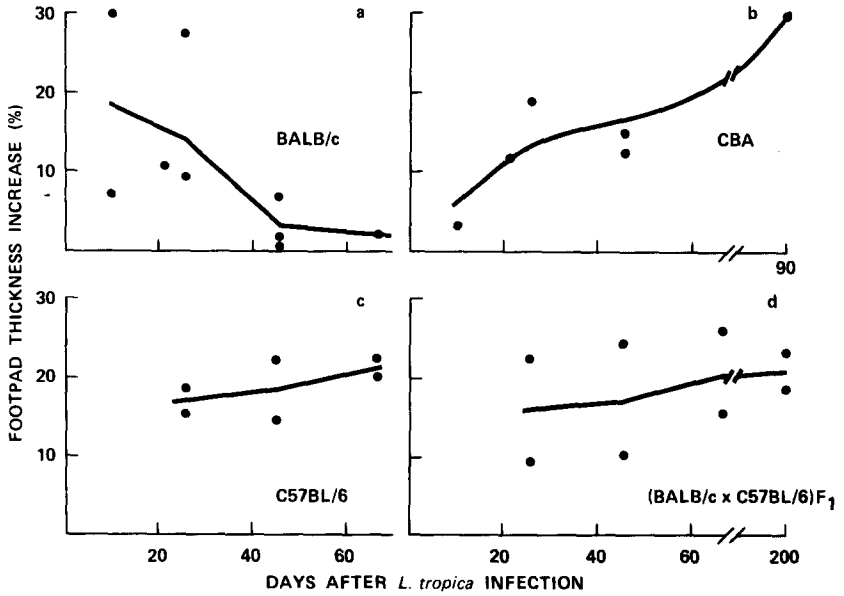

FIG. 5. Time-course of DTH responsiveness to *L. tropica* in mice. BALB/c (a), CBA (b), C57BL/6 (c) and (BALB/c × C57BL/6)F_1 (d) mice were infected subcutaneously with 2×10^7 promastigotes in the rump. DTH was elicited with 100 μg PSA at various times after infection, each mouse being tested once only. This figure shows the cumulative data of seven independent experiments. DTH was expressed as specific 24-h footpad thickness increase. Each point represents the mean DTH of 5–12 mice.

TABLE I

*Effect of CY on DTH to L. tropica**

Immunization with 2×10^7 *L. tropica*	Mouse strain	Specific DTH(%)‡			
		d10		d45	
		+CY	−CY	+CY	−CY
Live	BALB/c	28.7 ± 6.7§	7.2 ± 3.0	0	1.8 ± 1.4
	CBA	16.8 ± 5.4	3.3 ± 1.5	8.4 ± 6.4	15.0 ± 2.3
Killed	BALB/c	21.7 ± 4.9	6.1 ± 1.9	2.2 ± 2.4	0
	CBA	16.3 ± 3.7	9.9 ± 3.3	2.8 ± 1.6	4.7 ± 1.9

* Groups of five mice were injected intraperitoneally with CY or remained untreated. 2 d later they were injected subcutaneously with 2×10^7 live or killed *L. tropica*. 10 or 45 d after immunization DTH was elicited in the footpads with PSA as detailed in Materials and Methods.

‡ For each mouse strain, a group of five unimmunized controls was injected with the eliciting antigen which provided the background footpad swelling (varying from 0 to 5%). Specific DTH denotes values after subtracting the background.

§ Values represent means ± SE.

cantly higher levels of DTH to PSA compared to respective untreated controls (Table I). Furthermore, the DTH responses in CY-treated BALB/c mice were significantly higher than those of CBA mice. However, 45 d after immunization there was little or no detectable level of DTH in BALB/c mice, whether or not they had been treated with CY. Although the effect of CY on CBA mice had also waned by day 45, substantial DTH reactivity was still detectable. Thus it appears that CY enhanced

the DTH reaction soon after antigenic stimulation, but failed to avert the onset of immunosuppression in BALB/c mice at the later stage.

Specificity of DTH Suppression in BALB/c Mice Infected with L. tropica. BALB/c mice infected with *L. tropica* 35 d previously developed significantly lower levels of DTH to PSA compared to uninfected controls. However the ability of these mice to mount DTH responses to SRBC or DNFB was unaffected (Table II). Thus suppression of CMI in *L. tropica*-infected BALB/c mice appears to be specific to the parasite and is not a generalized phenomenon.

Effect of Passive Transfer of Spleen Cells from Infected Mice on DTH to L. tropica in Recipients. Data presented so far are consistent with the notion that the susceptibility of BALB/c mice to *L. tropica* infection may involve failure to develop a sustained level of CMI and that antigen-specific suppression of CMI in these mice is mediated by suppressor T cells. Experiments were therefore designed to investigate this further. BALB/c and CBA mice were infected with 2×10^7 *L. tropica*, and 49 d later spleen cells from these mice were harvested and transferred intravenously into normal syngeneic recipients which were then injected with 2×10^7 promastigotes. The recipients were tested for DTH to *L. tropica* 10 d after being infected. BALB/c mice receiving spleen cells from infected donors developed significantly lower levels of DTH compared to mice receiving uninfected donor spleen cells (Table III). In contrast, CBA mice receiving spleen cells from infected or uninfected donors showed

TABLE II

*Specificity of Suppression of DTH in BALB/c Mice Infected with L. tropica**

Mice infected with *L. tropica*	CY	Immunization	Elicitation	Specific DTH‡	Suppression
				%	%
+	+	Live *L. tropica*	PSA	23.9 ± 3.7	65.4§
−	+			69.1 ± 2.7	
+	−			14.9 ± 3.4	45.8
−	−			27.5 ± 4.3	
+	+	DNFB	DNFB	60.4 ± 14.4	13.0
−	+			69.0 ± 10.8	
+	−			39.0 ± 4.7	−19.7
−	−			31.3 ± 7.5	
+	+	SRBC	SRBC	86.4 ± 3.8	−8.3
−	+			79.2 ± 3.0	
+	−			82.3 ± 3.7	−5.9
−	−			77.4 ± 3.5	

* BALB/c mice were infected with 2×10^7 *L. tropica* or left uninjected as controls. 33 d later they were either treated with CY or left untreated as indicated. On day 35, mice were immunized with 2×10^7 live *L. tropica*, 500 μg of DNFB, or 1×10^8 SRBC as described in Materials and Methods. DTH was elicited 5 d after immunization with the corresponding immunizing antigens.

‡ For each antigen, a group of five uninfected and unimmunized control mice were injected with the eliciting antigen. This provided the background footpad swelling caused by the eliciting antigen alone. Specific DTH denotes values after subtracting this background.

§ Comparing infected mice with the uninfected controls, figures underlined are statistically significant (at least $P < 0.05$), $n = 5$.

TABLE III

*Effect of Passive Transfer of Spleen Cells from Infected Mice on the Induction of DTH to L. tropica in Normal Recipients**

Mice	Donor spleen cells	Specific DTH	Suppression
		%	%
BALB/c	Infected BALB/c	8.3 ± 1.5	41.7‡
BALB/c	Normal BALB/c	14.2 ± 2.7	
CBA	Infected CBA	19.1 ± 3.3	1.2
CBA	Normal CBA	19.3 ± 4.8	

* Donor mice were infected subcutaneously with 2×10^7 *L. tropica* promastigotes on day −49. They were injected daily with 100 μg of Paromomycin sulphate subcutaneously around the infected lesion on day −6 to −3. On day 0, 1×10^8 viable spleen cells from infected or normal donors were transferred intravenously into normal syngeneic recipients. Immediately after cell transfer, the recipients were infected subcutaneously with 2×10^7 live *L. tropica* promastigotes. DTH was elicited in the footpad on day 10 with 100 μg PSA.

‡ $P < 0.01$, $n = 5$.

TABLE IV

*Effect of Passive Transfer of Spleen Cells from Infected Mice on the Expression of DTH to L. tropica in Sensitized Recipients**

Mice	Donor spleen cells	Days between cell transfer and DTH elicitation	Specific DTH	Suppression
			%	%
BALB/c	Infected BALB/c	0	8.7 ± 2.7	−4.8
BALB/c	Normal BALB/c		8.3 ± 1.7	
CBA	Infected CBA	0	17.9 ± 2.8	−23.4
CBA	Normal CBA		14.5 ± 2.4	
BALB/c	Infected BALB/c	4	11.6 ± 1.6	10.8
BALB/c	Normal BALB/c		13.0 ± 1.8	
CBA	Infected CBA	4	22.4 ± 1.9	−194.6‡
CBA	Normal CBA		7.5 ± 0.7	

* The donor spleen cells were the same as those for Table III. The cells were transferred intravenously into recipients which were sensitized subcutaneously 14 d before cell transfer with 2×10^7 promastigotes. DTH was elicited in the recipients either immediately after cell transfer or 4 d later with 100 μg PSA.

‡ Figures underlined are statistically significant ($P < 0.05$). Negative values denote enhancement, $n = 5$.

similar degrees of DTH reactivity to *L. tropica* antigen. These results demonstrate clearly that spleen cells from infected BALB/c mice, but not CBA mice, contain elements which are capable of suppressing the induction of DTH to *L. tropica*. Attempts to demonstrate suppression of the expression of DTH in BALB/c mice primed to *L. tropica* before transferring spleen cells from infected BALB/c mice were unsuccessful (Table IV). This was so whether DTH was elicited on the same day or

4 d after the cell transfer. Indeed, transfer of spleen cells from infected CBA mice significantly enhanced the DTH to *L. tropica* in sensitized recipients. This suggests that DTH effector cells, rather than suppressor cells, were passively transferred. The fact that similar enhancement was not evident in BALB/c mice confirms an earlier finding (Table I) that these mice failed to show DTH reactivity 45 d after infection with *L. tropica*.

Characterization of Suppressor Cells. The cell type responsible for the suppression of DTH in *L. tropica*-infected BALB/c mice was investigated. Spleen cells from *L. tropica*-infected mice were treated in vitro before cell transfer with anti-θ serum plus complement or filtered through an anti-Ig column to enrich the T cell population. Table V shows that unfractionated spleen cells or the Ig^- spleen cells from infected donors significantly suppressed the induction of DTH to *L. tropica* in normal syngeneic recipients. This suppressive activity was completely abrogated by treatment with anti-θ serum and complement. Thus, the antigen-specific suppression of DTH response to *L. tropica* appears to be mediated by T cells.

Discussion

Spontaneous control and healing of experimental and clinical cutaneous leishmaniasis has been associated with the development of cell-mediated immunity. The striking inability of BALB/c mice to mount any effective resistance to *L. tropica* infection is paralleled by the emergence of profound antigen-specific suppression of DTH reactivity shortly after its initial brief phase of detectablity (Table I and Fig. 5). An analogous association was also found in cases of disseminated cutaneous leishmaniasis by Bryceson (1) who suggested that it might represent a form of immunological tolerance. Developing this notion Preston et al. (3) and Arredondo and Perez (12)

TABLE V

*Fractionation of the Spleen Cells Suppressing the Induction of DTH to L. tropica**

Donor spleen cells (1×10^8)	Specific DTH	Suppression
	%	%
Infected donors		
Unfractionated	10.6 ± 2.1	57.2‡
Anti-θ + C′	23.4 ± 2.4	5.6
Filtrate through anti-Ig column	7.9 ± 2.2	68.1‡
Normal donors	26.5 ± 3.1	−7.0
	24.8 ± 2.1	0

* BALB/c mice were infected subcutaneously with 2×10^7 *L. tropica* 42 d before cell transfer. They were treated with paromomycin sulphate as described in legend to Table III. Aliquots of spleen cells from these mice were treated with anti-θ serum plus complement or filtered through anti-Ig column as described in Materials and Methods. After washing, the fractionated or unfractionated cells were transferred intravenously into normal syngeneic recipients. Control groups were either injected with normal spleen cells or not. All groups were infected subcutaneously with 2×10^7 *L. tropica* immediately after cell transfer. DTH was elicited with 100 μg PSA 10 d after immunization.

‡ Figures underlined are significantly different ($P < 0.05$) from controls (line 5), $n = 5$–6.

speculated that suppressor T cells might explain some DTH impairment found respectively in CBA mice with large lesions resulting from massive *L. tropica* infection and in BALB/c mice infected with *L. mexicana*. The present data collectively provide cogent direct evidence for the involvement of suppressor T cell-mediated abrogation of DTH in the unique susceptibility of the BALB/c strain.

The potential DTH responsiveness of BALB/c mice is high — mice given CY 2 d before infection showed greater reactivity after 10 d than did similarly treated CBA (resistant) animals. On the other hand, DTH reactivity persisted 5 wk later in the latter, but not in BALB/c mice whether or not they had been pretreated with CY. A clear case has been made for the DTH-augmenting effect of CY being caused by selective impairment of suppressor T cell induction rather than inhibitory antibody formation (13–15). In particular, DTH amplification is obtained by nonantibody suppressive dosages of CY (16) and not elicited in adult thymectomized mice or those >6 mo old (17). Virgin suppressor T cells are known to be relatively short-lived, such that within 4 wk of adult thymectomy their depletion leads to augmentation of both DTH reactivity (18) and antibody responses to certain T-independent antigens (19). This selective elimination of suppressor T cells affords a likely explanation for the opposing effects of thymectomy in the CBA and BALB/c strains. ATxXMB CBA mice show great impairment in healing of *L. tropica* lesions (originally demonstrated by Preston et al. [8]) and confirmed by us) together with weakened specific DTH reactivity. In contrast, similarly pretreated susceptible BALB/c and (BALB/c × C57BL/6)F_1 mice acquire the ability to restrain lesion growth during the immune phase to an extent that complete healing results in some cases. This effect is paralleled by greater DTH in thymectomized mice (especially those posthealing) than in intact controls. The paradox of stronger DTH in thymectomized than intact BALB/c or F_1 mice must be attributable to residual effector T cells freed from suppressor T cell restraint, for BALB/c nu/nu mice are no different from euthymic BALB/c in their susceptibility to *L. tropica* (5). It has been known for a long time that thymectomized, irradiated and bone marrow reconstituted mice retain weak cell-mediated immune reactivity (20) which coincides with the persistence of some virgin radioresistant T cells (21). The fact that ATxXBM BALB/c mice acquire greater resistance to *L. tropica* infection than XBM or untreated controls, despite inevitably weak DTH potential, stresses the potency of suppression which must be involved during the normal course of infection in this strain.

A comparable change of modality towards immune resolution of *L. tropica* lesions has recently been induced by subjecting BALB/c mice to sublethal irradiation (550 rad or less) immediately before infection. (Howard et al. Manuscript in preparation.) This effect is similarly associated with DTH amplification, but does not correlate with any diminution of antibody formation, which, overall, has not been causally implicated. We are currently investigating the capacity of cells from ATxXBM and sublethally irradiated BALB/c mice which have recovered from *L. tropica* infection to transfer CMI adoptively.

The aforegoing observations imply that a potent suppressor T cell population is generated in *L. tropica* infected BALB/c mice which (*a*) inhibits DTH and not antibody responses, and (*b*) is antigen specific in that induction of DTH to SRBC and contact sensitivity to DNFB is unimpaired. Direct demonstration of this was obtained by cell transfer studies. The induction, although not the expression, of DTH to *L.*

tropica was suppressed by adoptively transferred spleen cells from infected BALB/c mice. This property was present in the T cell-enriched (anti-IgG column eluate) fraction, but absent from the T cell-depleted (anti-θ + C′ treated) fraction. Inhibition of induction of DTH is easier to detect than impairment of its expression by cell transfer studies (22, 23), so that failure to detect the latter need not imply that the suppressor T cell activity involved is restricted to the inductive phase during the natural development of the infection. On the other hand, the distinction between the various suppressor T cells regulating humoral and cell-mediated immunities has been documented (24) and has been discussed in detail elsewhere (25).

In view of the amplifying effect of CY on anti-leishmanial DTH reactivity only 10 d after infection, the induction of this suppressor T cell generation must be a relatively early event. DTH suppression was first detected at 25 d in some instances, became a generality by 35 d and persisted until the death of the animals from 90 d onwards. Although evidence of some general depression of immune reactivity has been obtained in the late stage of heavily infected CBA mice (3), our BALB/c strain showed little or no diminution of antibody response to the T-independent antigen dextran B1355 or of DTH reactivity to SRBC throughout 60 d (unpublished data). We did not follow mice beyond this in view of the generalization of their disease, when nonspecific suppression is of less mechanistic interest. The specificity of the T cell-mediated suppression in the *L. tropica*/BALB/c model is notable in view of the highly nonspecific nature of immunosuppression found in other protozoal infections of mice, such as *Trypanosoma brucei* (26–28) and *Plasmodium* species (29, 30). On the other hand, evidence has recently been obtained for the analogous induction of specific suppressor T cells which prevent T cell-mediated regression of the Meth A fibrosarcoma in (BALB/c × C57BL/6)F_1 mice (31), and the induction of DTH to influenza virus (23).

To what extent can the leishmania-specific suppression of DTH by suppressor T cell generation be held accountable for the overwhelming susceptibility of the BALB/c strain which appears to be the expression of one regulatory gene? There are grounds for suspecting that although the resultant impairment of CMI is a major component, it could be a sequel to a primary innate deficiency of the macrophages. Bradley (32) demonstrated that genetic control of innate susceptibility or resistance of mouse strains to systemic *L. donovani* infection was expressed at the level of their macrophages. Suppressor T cells are characteristically generated by high doses of antigen (33, 34), which could be attained more readily by rapid expansion of *L. tropica* populations in genetically 'susceptible' macrophages. The role of antigen dosage is supported by the induction of more severe disease and some impairment of DTH by massive infection of normally resistant CBA mice (3). Thus suppressor T cell generation in cutaneous *L. tropica* infection could have a quantitative rather than a qualitative basis in the BALB/c strain.

The importance of the expression of acquired CMI for the resolution of cutaneous leishmaniasis is clear enough, but the means by which this is accomplished are not. The alternatives are essentially either (*a*) the classical specifically-induced T cell activation of macrophage function or (*b*) cytotoxic T cell killing of parasitized macrophages involving H-2 restriction, proposed by Handman et al. (5). The latter authors suggested that BALB/c susceptibility could reside in reduced H-2 expression on infected BALB/c macrophages. It is difficult, however, to reconcile this pathway with resolution of lesions induced by prior thymectomy or sublethal irradiation.

In summary, our proposal is that the major regulatory gene determining *L. tropica* susceptibility of BALB/c strain involves (*a*) a primary macrophage 'defect' leading to (*b*) rapid amastigote, and hence high antigen, accumulation with (*c*) consequent induction of DTH and suppressor T cells, but (*d*) profound impairment of the former by the latter, and (*e*) no consequent DTH-induced amplification of anti-leishmanial activity within infected macrophages.

Summary

BALB/c mice have an exceptional susceptibility to *Leishmania tropica* infection such that cutaneous lesions grow without restraint in all cases leading to fatal metastasis and visceralization in normal and x-irradiated, bone-marrow reconstitued (XBM) animals. Adult thymectomized, x-irradiated, bone marrow-reconstituted (ATxXBM) BALB/c mice, however, show pronounced retardation of lesion growth leading to some survival and even cures. A similar trend was also found in moderately susceptible (BALB/c × C57BL/6)F_1 mice, in contrast with the "resistant" CBA strain, in which, as previously known, ATxXBM animals showed impairment of normal, spontaneous self-healing. These converse effects are paralleled by respective leishmania-specific delayed-type hypersensitivity (DTH) reactivities, prior thymectomy leading to diminution in CBA and augmentation in BALB/c and (BALB/c × C57BL/6)F_1.

Anti-leishmanial DTH responses, amplifiable by cyclophosphamide pretreatment, can be detected in BALB/c mice within 10 d of infection with 2×10^7 promastigotes, but become near-totally suppressed by day 25–35. No such suppression is found in CBA, C57BL/6, or (BALB/c × C57BL/6)F_1 mice together with varying degrees of immune control of lesion development or regression. Suppression of DTH in BALB/c mice is leishmania specific and does not extend to 2,4-dinitrofluorobenzene (DNFB) or sheep erythrocytes specificities. Spleen cells from suppressed *L. tropica*-infected mice when transferred to normal BALB/c mice impaired the induction of DTH to leishmanial antigen. This property resided in the T cell-enriched fraction and not in the T cell-depleted fraction.

It is concluded that a major component of the striking inability of BALB/c mice to control *L. tropica* infection involves profound impairment of a potentially curative cell-mediated immune response by suppressor T cell generation. The possibility is discussed that this may be secondary to rapid amastigote (antigen) accumulation in macrophages expressing the primary genetic "defect."

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