IMMUNOLOGICAL REGULATION OF EXPERIMENTAL CUTANEOUS LEISHMANIASIS

IV. Prophylactic Effect of Sublethal Irradiation as a Result of Abrogation of Suppressor T Cell Generation in Mice Genetically Susceptible to *Leishmania tropica*

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The BALB/c mouse strain is characteristically unable to control *Leishmania tropica* infection (1–4) even when a primary lesion is induced with minimal numbers of protozoa (4). This local disease progresses without restraint, leading to widespread cutaneous dissemination and fatal visceralization. In contrast, other strains examined contain the disease such that spontaneous healing or non-fatal chronicity are the outcome (3–5). The hypersusceptibility of BALB/c mice is largely determined by a single autosomal non-H-2-linked gene (4), seemingly dissociable from the Lsh gene, which regulates innate susceptibility to systemic *Leishmania donovani* infection (6, 7). Evidence has been presented in a previous article that implied that a major component in the overwhelming susceptibility of the BALB/c strain involves cancellation of a potentially curative cell-mediated immune (CMI)[1] response by the generation of a specific suppressor T cell population (8). The main observations were: (*a*) anti-leishmanial delayed type hypersensitivity response (DTH) reactivity is detectable in BALB/c mice only early in *L. tropica* infection and then becomes subject to profound antigen-specific suppression, (*b*) only the T cell-enriched fraction from the spleens of such suppressed mice impairs the induction of leishmania-specific DTH in normal syngeneic mice, and (*c*) adult thymectomized, x-irradiated, and bone marrow-reconstitution (ATxXBM) BALB/c mice show retardation of lesion growth and even some cures in parallel with expression of DTH reactivity, whereas the converse effects are found in normally resistant CBA mice subjected to the same treatment (8, 9).

Our paper is concerned with an analogous abrogation of BALB/c susceptibility to *L. tropica* infection by sublethal whole-body irradiation, which similarly parallels impairment of suppressor T (T_S) cell generation. Decisive experiments with this system have now shown that whereas T cell-enriched fractions from healed BALB/c mice transfer both DTH reactivity and protective immunity, similar fractions from nonhealed animals inhibit both the expression of leishmania-specific DTH reactivity and the prophylactic effect of irradiation. A determining role for T_S cells in BALB/c

[1] *Abbreviations used in this paper:* ATxXBM, adult thymectomized, x-irradiated, bone marrow-reconstituted; CMI, cell-mediated immunity; DTH, delayed-type hypersensitivity; PSA, protein-soluble antigen derived from *L. tropica*; T_D, T cells mediating DTH; T_S, T suppressor cells.

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Volume 153 March 1981 557–568

strain susceptibility to *L. tropica* infection, previously argued from correlative data, has thereby been demonstrated directly in vivo.

Materials and Methods

Mice. Members of inbred BALB/c and CBA strains and Biozzi Ab/H and Ab/L Selection I lines were obtained from our own colonies (Wellcome Research Laboratories Beckenham, Kent, England). BALB/B congenic mice were obtained from Olac Ltd., Bicester, Oxon, England. Female mice 10–12 wk of age were used throughout.

Leishmania Parasites and Antigen. The strain of *Leishmania tropica major* used in this study was kindly provided by Dr. R. A. Neal, Department of Parasitology, Wellcome Research Laboratories.

Maintenance, cultivation, and isolation of the parasites have been described in detail previously (8, 10). Mice were infected by injecting subcutaneously 0.1 ml of the appropriate concentration of promastigotes into their shaved rumps. The lesions which developed were measured with a direct reading vernier calliper gauge (GMH-390-T; Gallenkamp, London) in two perpendicular diameters. The average diameter (mm) was recorded and corrected for the thickness of skin at the same site of an uninfected mouse.

Protein-soluble antigen (PSA) fraction from *L. tropica* used for the elicitation of DTH was prepared following the method of Bryceson et al. (11) as described in detail elsewhere (8).

Measurement of DTH. DTH to *L. tropica* antigen was estimated by the footpad swelling method. Mice were injected in the right hind footpad with 50 μl of PSA (2 mg/ml) and footpad thickness increase measured 3, 24, and 48 h later with a dial-calliper (Pocotest; reverse spring-loaded calliper; Carobronze, England). DTH at 24 h was expressed as absolute footpad thickness increase in 10^{-2} mm and also as a percentage increase as described previously (8). In each experiment a group of unprimed mice was injected similarly with the test antigen. The increase in footpad thickness at 24 h in these mice was taken as the background footpad swelling due to the eliciting antigen alone.

Measurement of Antibody. The agglutination assay of Allain and Kagan (12) was used as described in detail elsewhere (13).

Adoptive Cell Transfer. Spleen and lymph node (inguinal, brachial, axillary, and mesenteric) cells from donor mice were collected in Eagle's minimum essential medium and sedimented by centrifugation. In some experiments, these cells were fractionated into T or B cell-enriched populations by treatment with anti-Thy-1.2 serum and complement or by anti-IgG column fractionation as described previously (8). Various numbers of viable cells were injected intravenously into intact or sublethally irradiated syngeneic recipients which were infected with 2×10^7 promastigotes 24 h later.

Irradiation. Mice were irradiated in a Cesium source (^{137}Cs) at a rate of 50 rad/min.

Statistical Analysis. Standard errors and standard deviations of the mean were calculated and the statistical significance of the results analyzed by Student's t test. $P < 0.05$ is considered statistically significant.

Results

Effect of Sublethal Irradiation on the Course of L. tropica Infection. BALB/c mice were exposed to 350- or 550-rad doses of whole-body irradiation 4 h before infection with 2×10^7 *L. tropica* promastigotes (Fig. 1). The development of lesions was indistinguishable from that in nonirradiated controls until day 30, at which time progression was arrested in both irradiated groups. Lesion growth commenced again in the 350-rad group from day 70 at the same rate as in the controls, leading to a uniformly fatal termination in both (the mean survival time in the irradiated group increased from 110 to 152 d). Control of the disease persisted in the 550-rad group, however, with three out of six animals becoming completely cured within 140 d and the remainder surviving with contained chronic lesions. The cumulative results of total healing

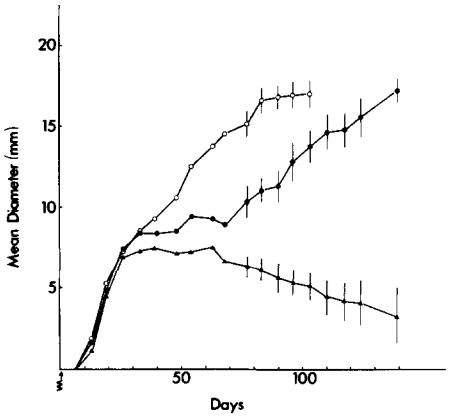

FIG. 1. Ability of BALB/c mice to control *L. tropica* infection induced by sublethal irradiation 4 h before injection with 2×10^7 promastigotes. ▲, 550 rad; ●, 350 rad; ○, nonirradiated ($n = 6$; means ± SE).

TABLE I

Effect of Prior Sublethal Irradiation on the Outcome of Infection with L. tropica in BALB/c and BALB/K Strain Mice

Infecting dose	Strain	Irradiation‡	No. of mice	Fate of infected mice*		
				Healed	Chronic contained disease	Fatal progression
		rad			%	
2×10^7	BALB/c§	550	53	60	21	19
		350	31	6.5	6.5	87
		0	54	0	0	100
2×10^5	BALB/K	550	8	100	0	0
		0	8	0	25	75

* Assessment 120–150 d postinfection.
‡ 4 h before infection.
§ Cumulative data from seven experiments.

found in seven consecutive prophylactic radiation experiments were: 550 rad: 60%, 350 rads: 6.5%, and controls: 0% (Table I). Fatally progressive disease developed in only 19% of 53 mice pretreated with 550 rad. A similar effect of 550 rad was also obtained in relatively susceptible congenic BALB/K (Table I) and Biozzi Ab/H (selection I) mice (data not shown). In contrast, this pretreatment did not modify the extent or duration of self-healing disease in resistant CBA or Biozzi Ab/L (selection I) mice, which has been described elsewhere (4, 13).

The titration of incremental dosages of 100 rad was assessed from 150 rad upwards (Fig. 2). Although a progressively more potent retarding influence on the disease was detectable from 250 rad, the maximum tolerated dose of 550 rad was found necessary to obtain a substantial proportion of cures. The time of administering this optimal dosage was also found to be critical (Fig. 3). Irradiation with 550 rad 10 or 20 d postinfection or 20 d preinfection was without effect, whereas administration 10 d previously was much less potent than administration at 4 h.

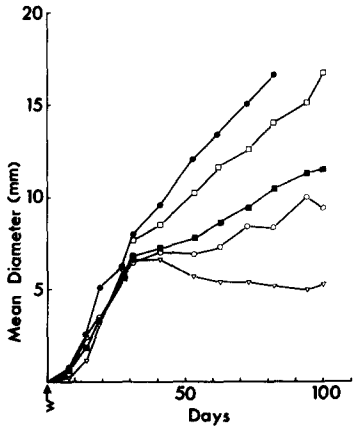

FIG. 2. Relative effect of different doses of sub-lethal irradiation immediately before infection of BALB/c mice with 2×10^7 *L. tropica* promastigotes. Rads: 250 (□); 350 (■); 450 (○); 550 (▽). Nonirradiated (●) ($n = 5$). Mice given 150 rad were indistinguishable from nonirradiated group.

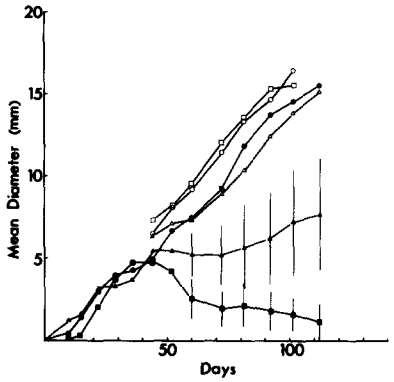

FIG. 3. Effect of varying the time of administering 550 rad irradiation to BALB/c mice in relation to their response to infection with 2×10^7 *L. tropica* promastigotes. Before infection: −20 d (□); −10 d (▲); −4 h (■). After infection: +10 d (△); +20 d (○). No irradiation (●) ($n = 5$; means ± SE). Data for mice irradiated day −20, day +10, and day +20 are omitted earlier than 44 d postinfection for clarity of representation. They did not differ significantly from corresponding nonirradiated controls.

DTH Reactivity and Resistance to Reinfection of Convalescent, Irradiated BALB/c Mice. Mice that had spontaneously healed *L. tropica* lesions as a consequence of prior exposure to 550 rad possessed strong anti-leishmanial DTH reactivity when tested during the convalescent phase (Table II). In contrast, irradiated mice with persisting lesions, which remained nonhealed at day 174, gave minimal or insignificant DTH responses, as had been found previously in nonirradiated BALB/c mice with uncontrolled disease (8). A group of nine mice that had completely resolved their rump lesions were rechallenged with promastigotes subcutaneously in the interscapular region 110–140 d after initiating the primary infection. All of four convalescent mice were totally resistant to reinfection with 2×10^5 parasites, whereas a high level of resistance was also expressed to 2×10^7 by two out of five animals in which only minute, transient lesions appeared.

The peak antibody titers attained in 550-rad pretreated mice by 68 d postinfection

TABLE II

Comparison of DTH Reactivity in 550 rad-irradiated BALB/c Mice According to Healing or Nonhealing of Lesions Induced by 2 × 10^7 L. tropica

Mice	No.	24-h footpad thickness increase*	
		%	× 10^{-2} mm
Healed lesions (day 118)‡	6	45.3 ± 3.3	65.0 ± 5.0
Nonhealed lesions (day 174)‡	3	19.8 ± 8.4	28.3 ± 11.7
Noninfected	5	12.8 ± 1.6	19.0 ± 2.4

* Response to PSA.
‡ Days postinfection.

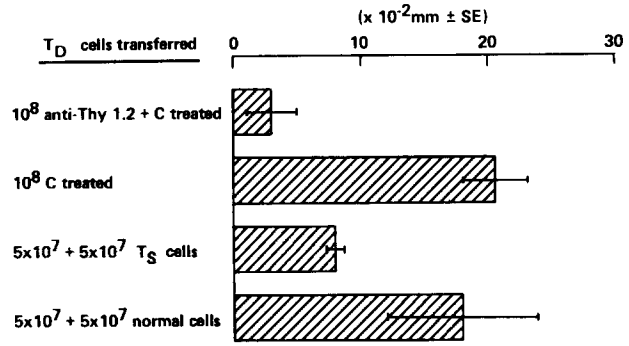

FIG. 4. Transfer of specific DTH reactivity and suppression of its expression by spleen and lymph node cells from *L. tropica*-infected BALB/c mice. 24-h footpad thickness increase in response to PSA injection. Cells injected into normal BALB/c mice: 'T_D', spleen + lymph node cells from 550-rad-irradiated mice 118 d postinfection (healed). 'T_S', spleen + lymph node cells from 550-rad-irradiated mice 174 d postinfection (nonhealed).

(mean $\log_2$: 7.8 in BALB/c and 11.0 in Ab/H) did not differ significantly from control-infected animals (8.7 and 10.8, respectively). Furthermore, no dissociation was found on monitoring individual antibody levels in irradiated, infected BALB/c mice according to differing disease outcome.

Transfer of DTH Reactivity and Suppression of Its Expression. The aforegoing results strongly suggested that the disease outcome in *L. tropica*-infected BALB/c mice after irradiation depends largely on a delicate balance between curative CMI and its suppression. Direct evidence for an interaction between T cells mediating DTH (T_D) and suppressor cells was sought in an adoptive cell transfer system after their incubation in vitro. Spleen and lymph node cells from healed, *L. tropica*-infected, preirradiated BALB/c mice which showed high levels of DTH to PSA (in Table II), were capable of transferring a significant degree of DTH reactivity into normal syngeneic recipients. The cells responsible for the transference were sensitive to anti-Thy-1.2 and complement treatment (Fig. 4) and therefore appear to be T_D effector cells. When such cells were incubated in vitro with spleen and lymph node cells from non-healing preirradiated BALB/c mice, the ability to transfer DTH was greatly impaired ($P < 0.01$) as compared with incubation with normal cells (Fig. 4).

Effect of Reconstituting Spleen Cell Fractions on the Prophylactic Effect of 550 rad Against L. tropica Infection

NORMAL DONORS. Immediately after exposure to 550 rad, BALB/c mice were injected with 2×10^7 splenic T cells (anti-Ig column passaged) or 5×10^7 splenic B cells (anti-Thy-1.2 + C treated) or remained uninjected. The next day, together with normal controls, they were infected with 2×10^7 *L. tropica* promastigotes (Fig. 5). Lesion development was similar in all groups until day 30. Thereafter arrest occurred in all irradiated groups. Whereas this led to regression in the 550-rad-alone and 550-rad + B cell groups (which did not differ significantly), progression again commenced uniformly from day 60 in the 550 rad + T cell group.

INFECTED DONORS OF T CELLS. BALB/c recipients were reconstituted immediately after 550 rad with the following splenic T cells: 5×10^7 from normal donors, 3.5×10^7 from donors with progressive *L. tropica* infection (44 d after injection of 2×10^7 promastigotes), and 2×10^7 from donors with healed lesions because of prior 550-rad irradiation (90–120 d postinfection). Together with unreconstituted irradiated and normal controls they were infected with 2×10^7 promastigotes (Fig. 6). As in Fig. 5, the prophylactic effect of irradiation was overcome by normal T cell replacement, with a shorter phase of arrest. Even more strikingly, 550-rad-irradiated mice injected with T cells from DTH-suppressed, infected donors were indistinguishable in their susceptibility from normal, nonirradiated BALB/c mice. Conversely, recipients of T cells from healed donors showed even greater resistance than irradiated controls, with minimal lesion development and early resolution.

DTH activity measured in these same mice on days 39, 62, and 82 showed striking parallelism with their disease status throughout (Fig. 7). Normal mice and 550-rad-irradiated mice injected with T cells from suppressed donors showed similar weak reactivity initially which was extinguished by day 62. Irradiated mice with or without normal T cell injection showed equivalent stronger reactivity at day 39. Although,

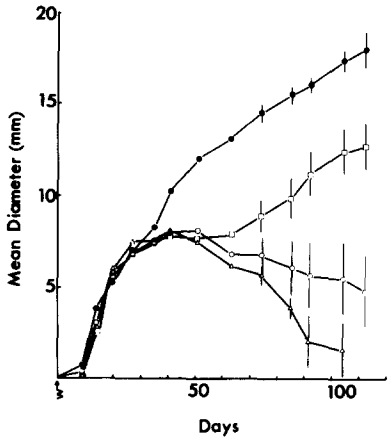

FIG. 5. Effect of replacement syngeneic spleen cell fractions on the protective effect of 550 rad against *L. tropica* infection in BALB/c mice. Pretreated mice: (*a*) 550 rad only (○); (*b*) 550 rad + 2×10^7 T cells (anti-Ig fractionated) (□), (*c*) 550 rad + 5×10^7 B cells (anti-Thy-1.2 + C treated) (△), (*d*) normal mice (●). Mice were infected with 2×10^7 promastigotes 1 d after irradiation and cell reconstitution (means ± SE; $n = 6$). (*b*):(*a*)—highly significant ($P < 0.01$), (*c*):(*a*)—not significant ($P > 0.05$).

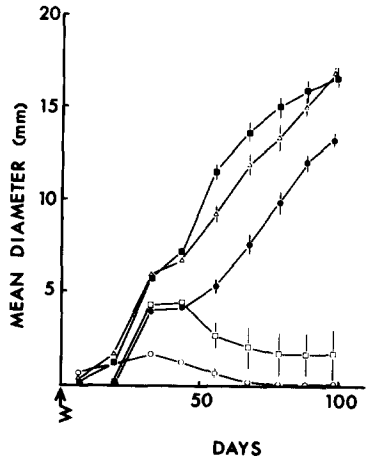

FIG. 6. Comparative effect of replacement with syngeneic splenic T cell fractions from healed, nonhealed, and normal BALB/c mice on the protective effect of 550 rad against *L. tropica* infection. Pretreated mice: 550 rad only (□); 550 rad + 5 × 10^7 T cells from normal donors (●), 550 rad + 3.5 × 10^7 T cells from nonhealed donors (day 44 postinfection) (△); 550 rad + 2 × 10^7 T cells from healed preirradiated donors (○), and normal mice (■). Mice were infected with 2 × 10^7 promastigotes 1 d after irradiation and cell reconstitution (means ± SE; $n = 6$).

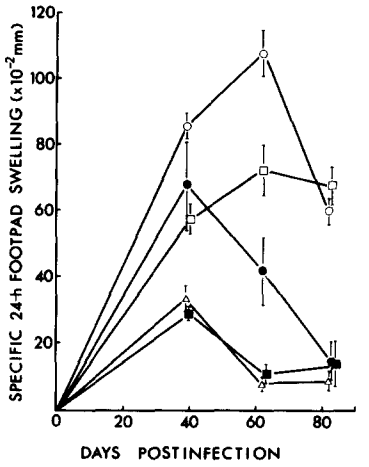

FIG. 7. DTH reactivity in BALB/c mice infected with *L. tropica* after irradiation and reconstitution with various syngeneic T cell populations. The mice were the same as those described in Fig. 6. DTH was elicited with PSA at various times after infection as indicated. BALB/c mice were irradiated with 550 rad and reconstituted with T cells from nonhealing (△), healed (○), or normal (●) donors (see Fig. 6). Control mice were either irradiated (□) or not (■) and were not given cells ($n = 6$; means ± SE).

this was subsequently sustained in the nonrepopulated group, it was progressively reduced to extinction in the T cell reconstituted group (in parallel with resumed progression of the disease). The 550-rad-irradiated mice injected with T cells from healed mice developed the strongest DTH reactivity of all which fell to the level of the irradiated controls subsequent to total lesion regression.

Discussion

The overwhelming susceptibility of BALB/c mice to infection with *L. tropica* was shown, in a preceding paper (8), to involve failure of a potentially curative CMI response which correlated with leishmania-specific suppression of DTH reactivity. The greater resistance of ATxXBM BALB/c and (BALB/c × C57BL/6)F_1 mice together with the results of cell transfer studies implicated an antigen-specific suppressor T cell population which inhibits DTH induction. The objective of demonstrating a direct causal role for this T_S cell in the pathogenesis of uncontrolled leishmaniasis in the BALB/c strain has now been attained by studying the effects of sub-lethal irradiation.

At first sight the prophylactic effect against *L. tropica* infection of sublethal irradiation within the range 350–550 rad appears heterodox in that similar treatment has for long been known to increase susceptibility to many bacterial species by impairing the microbicidal function of phagocytic cells (14–16). A macrophage basis for the present converse effect seems unlikely, however, on two counts. First, the normal resistance of CBA and Biozzi Ab/L mice to *L. tropica* shows no diminution after even 550 rad irradiation. Second, the development of *L. tropica* cutaneous lesions in irradiated BALB/c mice is unchanged during the first 30 d or so, i.e., throughout the innate susceptibility (macrophage) stage. The subsequent arrested progression of the disease coincides in onset temporally with that found in normally resistant strains (4) and in ATxXBM BALB/c mice (8). This prophylactic effect of irradiation seems not to involve initial impairment of antibody synthesis, for 350 rad (which is known to ablate antibody responsiveness down to 1% [17]) had only a minimal effect against *L. tropica* infection, whereas only the largest tolerated dose (550 rad) led to a high cure rate. Furthermore, similar antibody titers were observed in nonhealing and radiation-induced healing BALB/c and Ab/H mice alike. The critical time and dosage requirement for irradiation before infection is consistent with deletion of the precursors of T_S cells, whose radiosensitivity has been established in a number of different systems (18–20). Direct evidence for a key role of these cells in BALB/c susceptibility was sought using 550 rad rather than the analogous effect of ATxXBM pretreatment, in view of the greater amplitude of the prophylactic effect obtained, shorter duration of the experiments, and quantitative benefits in cell transfer studies.

Unequivocal evidence was obtained by syngeneic cell replacement studies that reversal by irradiation of the outcome of *L. tropica* infection is determined at the level of T cell regulation. When 550-rad-irradiated BALB/c mice were reconstituted with only 2×10^7 normal T cells (anti-Ig column-passaged spleen cells), inexorable progression of the disease again occurred after a transient phase of arrest between days 30–50. No such temporary respite was found when a corresponding number of T cells from DTH-suppressed mice with progressive disease were injected instead. In this case the behavior pattern was indistinguishable from that of nonirradiated normal BALB/c mice. In contrast, no significant change in the prophylactic effect of irradiation resulted from transfer of B cell (anti-Thy-1 + complement treated) fractions. The close correlation between the disease profile in these different groups and changes in their DTH reactivity was striking (Figs. 6 and 7). Both normal mice and those irradiated and injected with T cells from suppressed donors showed absence of significant DTH reactivity after a small initial rise. Irradiated mice possessed strong DTH reactivity at day 40 which was sustained or lost in parallel with disease

regression or progression depending on whether or not they had been injected with normal T cells. Furthermore, the minority of 550-rad-irradiated mice which did not acquire disease regression showed little or no DTH reactivity, in contrast with cured members of the same group. The ability to abrogate the prophylactic effect of irradiation with a normal T cell fraction or, *a fortiori*, T cells from nonhealing (DTH inhibited) mice provides direct evidence of a causal role for suppressor T cell lineage in the BALB/c strain susceptibility to *L. tropica* infection.

We reported previously (8) that the T_S cell in BALB/c mice with progressive *L. tropica* infection could, on transfer, suppress the induction of DTH reactivity to *L. tropica*, but not its expression in a presensitized animal. This latter activity has, however, now been demonstrated successfully in a mixed cell transfer system involving T_S cells (from nonhealed) and T_D cells (from irradiation-induced healed) mice. Although such rapid suppression must involve preformed effector T_S cells, the prolonged impairment of both DTH reactivity and protective CMI in *L. tropica*-infected BALB/c mice probably involves memory T_S cell generation (21). It should be stressed that the T_S lineage involved in this system is leishmania specific (8) and restricted to CMI in its effects, because antibody formation to *L. tropica* infection is not diminished in the BALB/c strain (3, 22).

The close correlation between DTH reactivity and outcome of the disease, which has been a consistent feature of this investigation, suggests that they are causally related. Direct evidence of this is provided by the transfer of both strong DTH reactivity (Fig. 4) and protective immunity (Fig. 7) by T cell fractions from healed (preirradiated) BALB/c mice. Irradiated recipients of such cells showed minimal lesion development during the initial phase (up to day 30) accompanied by even stronger DTH than in mice irradiated only. The use of the irradiated BALB/c recipient provides a sensitive in vivo assay for the protective (T_D) or inhibitory (T_S) potential of total T cell fractions. That either may ultimately predominate in *L. tropica*-infected mice previously exposed to 550-rad irradiation emphasizes the delicate homeostatic cellular balance on which the outcome of this infection depends.

One caveat, albeit unlikely, could be that the results obtained with T cell replacement of irradiated BALB/c mice involve helper and not suppressor T cells i.e., that impairment of CMI is because of humoral antibody. Against this are (*a*) the failure of healing/nonhealing discrimination to correlate with antibody titers, (*b*) the requirement for relatively high irradiation dosage (c.f. antibody suppression), (*c*) the very rapid suppression of DTH in mixed T cell transfers (Fig. 4), and (*d*) the consistent failure of transferred anti-leishmanial sera to modify the outcome (S. Nicklin, C. Hale, F. Y. Liew, and J. G. Howard, manuscript in preparation). We are currently delineating the Lyt and I-region product phenotype of the T_S cell under study as well as its sensitivity to cyclophosphamide metabolites in vitro (23) which may reveal distinctive features from T_H cells.

In conclusion, BALB/c mice are perfectly capable of producing protective CMI (like members of other strains) and this, once acquired, is transferrable with their T cells. Because (*a*) this property resides in the Lyt-1^+,2^- subset ([24]; and F. Y. Liew, C. Hale, S. Nicklin, and J. G. Howard, manuscript in preparation), and (*b*) macrophages develop intensified anti-leishmanial activity upon activation by concanavalin A or mixed lymphocyte reaction products (25, 26), it is implicit that anti-leishmanial CMI involves specific T cell-macrophage interaction and not cytotoxic T

cell generation. Despite their potentiality to develop this immune mechanism, BALB/c mice do not normally do so as a result of its total suppression by parallel induction of potent T_S cells. Our data identify a causal role for these cells in abrogating the development of protective immunity and in suppressing the induction and expression of leishmania-specific DTH reactivity. There are grounds for suspecting that the primary genetic defect in BALB/c mice is expressed in the innate susceptibility of their macrophages to *L. tropica* (6, 27, 28), which could lead to T_S cell generation via rapid accumulation of relatively high antigen dosage (29, 30). Nevertheless, because the immune phase can be enabled to develop by experimental maneuver leading to a curative outcome, it is implicit that BALB/c macrophages must be inherently capable of being specifically activated to destroy their parasite content.

Summary

The overwhelming susceptibility of BALB/c mice to infection with *Leishmania tropica* can be substantially reversed by immediately prior sub-lethal irradiation. This is related to radiation dosage, and at 550 rad, causes 60% complete cures and only 19% (instead of 100%) incidence of progressive disease. Irradiation 10 d before infection is only weakly prophylactic, whereas 10 d after is without effect. Control of lesion development is only apparent after the first 30 d, coincident with the analogous onset previously found in resistant strains and adult thymectomized, x-irradiated, bone marrow-reconstituted BALB/c mice. Instead of the specific suppression of DTH characteristic of *L. tropica* infection in the BALB/c strain, healed irradiated mice express strong anti-leishmanial DTH reactivity and resistance to reinfection. T cells from these mice transfer DTH reactivity which is suppressed by admixture with cells from nonhealed, nonreactive donors.

Irradiated BALB/c mice again develop inexorable disease progression, after its transient arrest, when they are reconstituted with normal T cells. When the T cells are derived from uncontrollably-infected donors, the susceptibility regained is indistinguishable from that of normal mice. B cells do not modify the prophylactic effect of 550 rad, whereas T cells from healed mice confer strong protective immunity throughout the initial phase. Regression or progression of disease correlates completely with DTH reactivity in all these groups.

Although BALB/c mice express an extreme level of genetic susceptibility to *L. tropica* infection, they are nevertheless capable of mounting a curative cell mediated immune response. That this is ineffective during pathogenesis of the disease was previously associated correlatively with potent specific suppressor T cell generation, which is now shown to be preventable by prior irradiation. Most important, however, a causal role for these cells in vivo has been demonstrated directly by reconstitution.

We thank our colleague Dr. Stephen Nicklin for preparation of the anti-Ig coated columns.

Received for publication 1 October 1980.

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