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NOTES

Passive Transfer of Tuberculin Sensitivity from Anergic Mice

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Mice heavily infected with *Mycobacterium bovis* BCG rapidly became anergic to cutaneous injection with tuberculin. Evidence is presented suggesting that this anergy reflects an adaptive physiological change within the host in which antigen-reactive Thy-1.2$^+$ cells become sequestered in central lymphoid tissues, with a concomitant reduction in the circulating pool. No evidence could be provided to support the suggestion that anergy was a consequence of an acquired immunosuppressive mechanism.

Acquired cellular immunity to many facultative intracellular bacterial parasites is known to depend upon the generation of protective T lymphocytes which activate host macrophages to contain and control the growth of the infectious organism (9, 12, 15, 16). The generation of this protective mechanism is usually temporally associated with the emergency on the part of the host of an acquired capacity to express a delayed-type hypersensitivity (DTH) response to the specific bacterial antigens (25). There are some circumstances, however, in which the animal develops a state of hyporesponsiveness, or anergy, to cutaneous challenge with bacterial antigens; these include, for example, during advanced disseminated disease such as miliary tuberculosis (10, 24, 25), immunodeficiency or diminished immunocompetence (for instance, in neonates), or by desensitization, in which cutaneous responsiveness can be ablated by the systemic administration of antigen (20). Perhaps the best-known example, however, consists of experimental infection with *Mycobacterium bovis* BCG, or with *Mycobacterium tuberculosis*, in which cutaneous anergy invariably develops after the intravenous infection of animals with $>10^7$ viable mycobacteria (4, 5, 22).

The present study was designed to investigate the underlying mechanisms which predispose to cutaneous anergy after experimental intravenous infections of mice with large doses of viable *M. bovis* BCG. This study followed from the previous observation in this laboratory that mice heavily infected with *M. bovis* BCG rapidly became anergic to cutaneous injection with tuberculin (5) and thus, in keeping with present knowledge about the regulation of the DTH reaction (6, 11), it was hypothesized that the development of the anergic state might be due to immunoregulatory mechanisms, including control by suppressor T cells (3, 5). The present study has centered on this possibility and provides evidence that is not in keeping with this hypothesis. It shows, first, that attempts to inhibit or reduce the emergence of DTH to tuberculin in tuberculin-reactive recipients by the passive transfer of T cells harvested from anergic donors were unsuccessful. Moreover, during the course of these experiments it was noticed that despite the observed anergy in the heavily infected donors, spleen cells harvested from these donors possessed the capacity to passively transfer (both systemically and locally) statistically significant responses to tuberculin. In view of these findings, it is hypothesized that tuberculin anergy in mice heavily infected with *M. bovis* BCG reflects an adaptive physiological change in which antigen-reactive cells become sequestered in the central lymphoid tissues, with a consequent reduction in the circulating T lymphocyte pool.

Experiments were performed by using specific pathogen-free B6D2 (C57BL/6 × DBA/2) F_1 and AB/6 (A/Tru × C57BL/6) F_1 hybrids of either sex when they were 6 to 8 weeks of age. All animals were supplied by the Trudeau Animal Breeding Facility, Saranac Lake, N.Y. Mice were infected intravenously via a lateral tail vein with indicated doses of *M. bovis* BCG (BCG Pasteur, strain 1011, Trudeau Mycobacterial Culture Collection, Saranac Lake, N.Y.) suspended in 0.2 ml of phosphate-buffered saline. Growth, storage, and preparation of inocula of this organism are described elsewhere (17). The emergence of DTH to tuberculin in mice infected with various doses of *M. bovis* BCG was followed with time by injecting groups of mice ($n = 5$) in a hind footpad with 5 μg of purified protein derivative of tuberculin (PPD; Connaught Laboratories Ltd., Willowdale, Ontario, Canada) in 40 μl of pyrogen-free sterile saline. The contralateral footpad was injected with diluent alone. The development of DTH was measured by using dial gauge calipers (Schnelltaster, Hessen, West Germany) that are capable of measuring increments of 0.05 mm in thickness.

In passive transfer experiments, spleen cells from donor animals were enriched for T cells by the removal of adherent cells, followed by depletion of B cells by incubation of plastic petri dishes coated with anti-mouse immunoglobulin. Full details of these procedures are described elsewhere (17). Peritoneal exudate cells (PEC) were induced by injection of 3.5% casein and were harvested 48 h later into sterile phosphate-buffered saline plus 1% fetal calf serum supplemented with 10 U of heparin per ml. In some experiments, cells were treated before transfer with anti-Thy-1.2 antibody plus complement as described previously (17). After these preparatory procedures, 5×10^7 T-cell-enriched spleen cells were infused intravenously into age- and sex-matched syngeneic normal or previously infected (tuberculin-reactive) recipients; in systemic transfers involving PEC, recipients received 2 donor equivalents of cells. Unless stated otherwise, recipient animals were then immediately tested for their ability to mount a DTH response by injection in a hind footpad with PPD. In a separate series of experiments, the ability of spleen cells or PEC to mount a DTH response to

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TABLE 1. Development of tuberculin sensitivity in BCG-infected mice

Day of infection	Footpad swelling response (× 0.1 mm)[a] after initial infection with:			
	10^6 BCG		10^8 BCG	
	3 h	24 h	3 h	24 h
5	0.2 ± 0.2	0.4 ± 0.4	1.0 ± 0.5	0.8 ± 0.4
10	0.5 ± 0.2	1.3 ± 0.3	0.8 ± 0.3	1.9 ± 0.4
15	1.2 ± 0.3	3.5 ± 0.5	1.1 ± 0.2	0.4 ± 0.2
20	0.5 ± 0.3	3.2 ± 0.7	5.0 ± 1.2	0.5 ± 0.2
25	0.8 ± 0.4	2.8 ± 0.4	4.2 ± 0.8	0.4 ± 0.4
30	0.2 ± 0.2	3.8 ± 0.5	4.0 ± 1.0	0.2 ± 0.2

[a] Data are expressed as arithmetic mean value ± standard error of the mean. Responses above 1.8 were taken as positive reactions ($P < 0.01$). The response of uninfected mice to the batch of PPD tuberculin used in these experiments was 0.2 ± 0.2 (3 h) and 0.5 ± 0.3 (24 h).

tuberculin was also determined by the classical local passive cell transfer technique (14). Mice were inoculated in a hind footpad with an admixture of 3×10^6 cells plus 5 μg of PPD; control animals received cells alone.

The emergence of the capacity of the animal to mount a DTH response to tuberculin after infection with a moderate dose (10^6) or with a high dose (10^8) of *M. bovis* BCG was documented (Table 1). It was found, in accordance with earlier reports in the literature (5, 22), that whereas moderately infected mice gradually developed the capacity to express DTH reactions after injection in the footpad with tuberculin, heavily infected mice were for the most part unresponsive (anergic) to these antigens.

Two points should be made concerning the emergence of anergy in the groups of mice infected with 10^8 *M. bovis* BCG. First, a small but positive ($P < 0.01$) reaction to tuberculin was observed when animals were tested on day 10 of the infection; 5 days later, however, animals had become completely anergic to tuberculin injection. Second, from day 20 of the infection on, there was clear evidence for rapidly increasing immediate-type (Arthus) hypersensitivity in the heavily infected groups of mice. This resulted in large 3-h reactions, thus completely masking the emergence of any subsequent (delayed) responses. In contrast, mice infected with the lower dose (10^6) of *M. bovis* BCG showed no evidence of any immediate-type inflammatory reaction and mounted positive 24-h reactions to tuberculin from day 15 through to the end of the experiment.

Experiments were then performed to examine the possibility that spleen or peritoneal exudate cells from heavily infected, tuberculin anergic mice contained cells capable of inhibiting the induction or expression of DTH in tuberculin-reactive mice (infected with 10^6 *M. bovis* BCG) and thus could be taken as evidence that anergy reflected an active acquired immunosuppressive mechanism (3, 5). To achieve this, 5×10^7 T-cell-enriched spleen cells or 2 donor equivalents of PEC were intravenously infused into tuberculin-reactive mice at various times before PPD injection in the footpad. The results of a representative experiment, however, demonstrate (Table 2) that neither population was capable of inhibiting either the induction or the expression phase of the DTH response. On the contrary, it was found that rather than proving inhibitory, this number of spleen cells from anergic donors were themselves capable of passively transferring tuberculin DTH when infused into normal recipients. Responses transferred by PEC from anergic donors were invariably negative ($P > 0.1$). Similar results were obtained with the alternative technical approach of local passive transfer (Table 2).

It was then formally determined whether the passive transfer of DTH from anergic donors reflected the activity of a T-cell-dependent mechanism. This was confirmed by the demonstration (Table 3) that prior treatment of T-cell-enriched spleen cells with monoclonal antibody to Thy-1.2 plus complement, but not complement alone, ablated the transfer of sensitivity.

This paper thus shows that the passive transfer of spleen cells from heavily infected anergic donors can confer upon normal recipients the capacity to mount a DTH response to injection in the footpad with tuberculin. We found, furthermore, that the passive transfer of sensitivity was mediated by a population of T lymphocytes, as evidenced by the demonstration that treatment of donor cells with anti-Thy-1.2 antibody plus complement before transfer ablated this capacity.

The finding in this study that spleen cells harvested from anergic animals provided a good source of antigen-reactive T cells and the peritoneal cavity did not, leads us to hypothesize that sequestration of such cells within the central lymphoid tissues may provide the underlying basis for the

TABLE 2. Failure to inhibit either induction or expression of DTH to tuberculin by the passive transfer of cells from anergic donors

Systemic transfer					Local passive transfer				
Donor infection (no. of BCG)	Donor cells	Recipient[a]	Day of transfer[b]	Footpad swelling response (× 0.1 mm)	Donor infection (no. of BCG)	Donor cells	PPD	Footpad swelling response (× 0.1 mm)	*P*
None	Spleen	PPD reactive	20	3.2 ± 0.5	10^8	Spleen	+	4.8 ± 0.8	<0.02
10^8	Spleen	PPD reactive	20	4.6 ± 1.0	10^6	Spleen	+	3.2 ± 0.2	<0.05
10^8	Spleen	PPD reactive	15	3.3 ± 0.4	10^8	PEC	+	1.4 ± 0.2	NS[d]
10^8	Spleen	PPD reactive	10	3.3 ± 0.6	10^6	PEC	+	4.0 ± 0.5	<0.01
10^8	Spleen	PPD reactive	5	3.2 ± 0.5	10^8	Spleen	−	2.0 ± 0.7	
10^8	PEC	PPD reactive	20	3.8 ± 0.6	10^8	PEC	−	0.2 ± 0.2	
10^8	PEC	PPD reactive	10	4.2 ± 0.7		None	+	0.2 ± 0.2	
10^8	Spleen	Normal		2.4 ± 0.4		None	−	0.2 ± 0.2	
10^8	PEC	Normal		1.2 ± 0.2[c]					

[a] PPD-reactive recipients were mice infected with 10^6 BCG 20 days earlier.
[b] Day of infection in recipient. All mice were tested with PPD on day 20.
[c] Not significant; all other values in this section represent a significant footpad swelling response ($P < 0.01$) and are means ± standard error of the mean ($n = 5$).
[d] NS, Not significant.

TABLE 3. Evidence that passive transfer of tuberculin sensitivity from anergic donor is mediated by T lymphocytes

Treatment of donor cells[a]		Footpad swelling response in normal recipients to PPD (× 0.1 mm)
Anti-Thy-1.2	Complement	
−	−	2.8 ± 0.5
+	+	0.5 ± 0.3
−	+	2.9 ± 0.6

[a]Spleen cells were harvested on day 15 of infection from mice that had been infected with 10^8 BCG.

observed cutaneous anergy. There is considerable evidence in the literature to support this hypothesis; for example, Schlossman et al. (20) demonstrated that guinea pigs that had been previously sensitized to hapten-conjugated peptides could be rendered anergic to subsequent challenge with antigen for up to 10 days by the administration of a single intravenous injection of specific antigen. During this time, circulating lymphocytes were completely unresponsive to in vitro antigenic stimulation; in contrast, cells harvested from lymph nodes were fully antigen reactive. A similar phenomenon of selective depletion of circulating lymphocytes by antigen stimulation was observed by Sprent et al. (21) and has been subsequently noted in a number of experimental systems (7, 13, 19). Furthermore, there is increasing evidence from guinea pig models that these adaptive changes are controlled by lymphokines (1), a finding which is supportive of the present study in that *M. bovis* BCG infection results in considerable lymphokine production.

In this regard, Bullock (2) has demonstrated that intravenous injection of rats with *Mycobacterium lepraemurium* results in a considerable disruption of normal patterns of lymphocyte trafficking in these animals, with widespread sequestration of radiolabeled lymphocytes in the spleen, liver, and lymph nodes. A similar demonstration of lymphocyte sequestration during mycobacterial infections was provided by Rook and his colleagues (18), who provided convincing clinical evidence for the presence of antigen-reactive cells within draining lymphoid tissue, in the absence of responsiveness by peripheral circulating cells. In the present study, we also found no evidence for antigen-reactive circulating cells, at least among the PEC population. However, it must be borne in mind that other cells with inhibitory properties may have been present (e.g., prostaglandin-secreting macrophages), and this point remains to be clarified.

The results of this study also support the general conclusion that the magnitude or intensity of the immune response predisposes to anergy. Mice infected with 10^6 *M. bovis* BCG cells do not become anergic, whereas in mice intravenously infected with $>10^7$ *M. bovis* BCG cells, anergy invariably occurs. It might be predicted, therefore, that reduction of the bacterial load in heavily infected animals would restore responsiveness, and indeed this prediction is borne out by the observation that responsiveness often returns in anergic tuberculosis patients after a course of appropriate chemotherapy (24). It is clear, however, that anergy may occur in a large number of circumstances such as miliary disease, moribundity, old age, in patients with viral infections, or with neoplasia (8), and it cannot therefore be assumed that mechanisms predisposing to anergy in mice heavily infected with mycobacteria also play a role in these other various anergic phenomena. However, with respect to the model discussed in the present study, it is proposed that at least three phases of cutaneous unresponsiveness appear to occur. The first or primary phase is apparently temporally related to the peak of the primary cellular response to intravenous infection with 10^8 *M. bovis* BCG (17) and may consist, as suggested here and elsewhere (2, 18, 20, 21), of a reduction in the number of circulating antigen-reactive cells due to central sequestration. A secondary phase then gradually develops in heavily infected mice which consists of increasing immediate-type (Arthus) hypersensitivity, resulting in blockage of or interference with the emergence of an interpretable 24-h reaction. A tertiary or "miliary" phase of anergy may subsequently occur in such animals as a result of senescence, immunosuppression, or overwhelming secondary infection; anergy under such circumstances may reflect a severe reduction in the blood monocyte pool as these cells are continually recruited into disseminating infectious lesions.

Finally, it is paradoxical that while DTH reactions are widely considered an important diagnostic procedure, the functional significance of the mechanisms underlying such reactions remains completely unexplained. The relevance of the presence or absence of such responses, and their relationship to the generation of acquired immunity to chronic or progressive intracellular bacterial and protozoal diseases, clearly remains an area of great importance for future study.

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