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Suppressor T-Cells in BCG-Infected Mice

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Specific pathogen-free B6D2 hybrid mice were infected with high (10^8 cells, intravenous), moderate (10^6 cells, intravenous), and low (10^3 cells, aerogenic) doses of viable BCG Pasteur. The growth of the BCG in the lungs and spleens of the three groups was followed over a 90-day period and correlated with the level of tuberculin hypersensitivity. Spleen cells were harvested from the three groups of mice at increasing time intervals and filtered through nylon wool to remove adherent cells, and the level of blast transformation after exposure to phytohemagglutinin and purified protein derivative was determined. Early in the BCG infection both the high- and the intermediate-dose groups showed enhanced thymidine incorporation by the spleen cell cultures, followed by a profound depression late in the infection. At this time, both groups of mice were anergic to purified protein derivative injected into footpads. Cell mixing studies demonstrated the presence of a population of suppressor cells in the spleens of the anergic animals. The suppressive abilities of these cells would be ablated by treatment with anti-Thy-1 antiserum and complement. The aerogenically infected mice were unresponsive to purified protein derivative but showed no evidence of suppressor T-cells. The lack of tuberculin sensitivity in these mice seemed to be due to a lack of sensitized T-cells in the spleen rather than to active immunosuppression.

BCG vaccination has been widely employed in the prophylaxis of human pulmonary tuberculosis (4) and more recently has been tested for its protective value against leprosy (12). Because of its ability to stimulate the normal mononuclear defenses, BCG has been used extensively as a nonspecific adjuvant for a number of immunological responses (18). Mackaness et al. (16) showed that different substrains of BCG varied in their ability to potentiate cellular proliferation within the draining lymph nodes in footpad-inoculated mice. The extent of the cellular proliferative response could be correlated directly with the subsequent delayed footpad response to sheep erythrocytes introduced into stimulated nodes (17). An analogous immunomodulation has been reported in BCG-stimulated individuals suffering from both experimental and human tumors (1, 2). Recently, several investigators have shown that large doses of living BCG, far from potentiating cellular responsiveness, may be toxic (21) and can suppress many cellular activities, including both graft versus host and mixed lymphocyte culture responses (10, 11). Orbach-Arbouys and Poupon (20) reported that spleen cells from heavily infected mice exhibit reduced responsiveness to the nonspecific T-cell mitogen phytohemagglutinin (PHA) and that such suppression appeared to be mediated by a subpopulation of lymphocytes generated within BCG-infected spleens.

Mice and guinea pigs infected with large doses of mycobacteria have long been known to quickly become anergic to tuberculin (3, 4, 5, 9). Reduction of this infecting inoculum by as little as 10-fold is associated with significant levels of tuberculin hypersensitivity, again indicating that the suppression of the cellular response in a BCG-vaccinated host is dose dependent. The actual mechanism mediating this suppressive effect has yet to be determined fully (3, 14). The present study shows that mice infected systemically with large numbers of BCG quickly develop T-cell populations within their spleens which are capable of suppressing both nonspecific mitogenic and specific antigenic proliferative responses in vitro about the time when the host becomes anergic to tuberculin injections in vivo.

MATERIALS AND METHODS

Animals. Specific pathogen-free B6D2 hybrid mice were obtained from the Trudeau Animal Breeding Facility. They were maintained as described earlier (5) and given sterile food and acidified drinking water ab libitum (6).

Bacteria. *Mycobacterium bovis* (BCG Pasteur TMC 1011) was grown in gently stirred, modified Sauton synthetic medium as described elsewhere (8). The logarithmic-phase culture was stored in 1-ml ampoules at −70°C until required (6). An ampoule was thawed, homogenized in an equal volume of modified

Sauton synthetic medium to break up any clumps, and diluted suitably in 0.05% Tween-saline just before inoculation into mice. The viability of the inoculum was checked by plating 0.05% Tween-saline dilutions on Middlebrook 7H10 agar and counting the resulting colonies after 4 weeks of incubation at 37°C in sealed plastic bags (5).

Delayed hypersensitivity measurements. Vaccinated and control mice were injected in the right hind footpad with 2.5 μg of purified protein derivative (PPD; Connaught Laboratories, Toronto, Canada) in 0.03 ml of 0.05% Tween-saline (5). Diluent alone was injected into the opposite footpad. Increases in foot thickness were measured with dial gauge calipers after 3, 6, 24, and 48 h (5). An increase of 0.18 mm (1.8 Schnelltaster units) over control values was significant at the 1% level (7).

Enumeration of in vivo bacterial populations. The lungs, livers, and spleens from five randomly selected mice from each group of infected mice were homogenized in saline and plated on 7H10 agar as described previously (5). The counting error was similar to that observed in earlier papers (6).

T-lymphocyte enrichment. Single-cell suspensions from spleens were fractionated on nylon wool (Leuko-Pak leukocyte filter; Fenwal Labs, Deerfield, Ill.) which had been soaked in saline for 2 h at 37°C, then rinsed three times in double-distilled water over a period of 3 days, and dried at 37°C (13). A 60-ml disposable syringe barrel was packed with 6 g of nylon wool and gas sterilized. The columns were used 3 to 4 days after sterilization. They were equilibrated by rinsing with 20 ml of RPMI 1640 medium containing 5% fetal calf serum, drained, and placed in a 37°C incubator for 1 h before they were loaded with 10^9 spleen cells in 20 ml of medium. The column was incubated at 37°C for 45 min before the nonadherent cells were eluted at a rate of 1 ml/min. The T-cell-enriched suspension was concentrated by centrifugation at 250 × *g* for 15 min and counted with a hemacytometer. Viability was checked by dye exclusion and was normally greater than 80%.Cells passed through nylon wool were between 94 and 97% theta positive.

Lymphocyte transformation. Spleens were removed aseptically from mice infected at increasing time intervals with 10^6 or 10^8 viable BCG and expressed through a stainless steel grid into cold RPMI 1640 medium containing 5% heat-inactivated fetal calf serum. Samples (200 μl) of spleen cell suspension (2 × 10^6 cells per ml) were dispensed in quadruplicate into Linbro flat-bottomed microtest plates (Flow Laboratories, Irvine, Scotland) and cultured in the presence of 0.01, 0.1, 1, and 10 μg of PHA (Difco Laboratories, Detroit, Mich.), lipopolysaccharide (*Escherichia coli* 055:BS; Difco), or PPD (Connaught Laboratories, Toronto, Canada). The plates were incubated at 37°C in humidified 5% CO_2-enriched air for 48 h before each well received 0.1 μCi of tritiated thymidine (specific activity, 5 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom). The plates were reincubated for 16 h, the cultures were harvested with a Mash II sampler (Microbiological Associates, Bethesda, Md.), and the radioactivity was measured with a Beckman liquid scintillation counter (19, 24). The results presented in this paper are the responses to 0.1 μg of mitogen in all cases.

Anti-theta serum treatment. Nylon wool-passaged spleen cells were mixed with a 1:80 dilution of anti-Thy-1 antiserum (Cedarlane Labs, Hicksville, N.Y.) and a 1:10 dilution of Low-Tox rabbit complement (19).

Mitomycin C treatment. Nylon wool-passaged spleen cells at a concentration of 10^7 cells per ml were incubated for 25 min at 37°C in the presence of 20 μg of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) per ml. The cells were washed three times in fresh medium, and the cell concentration was adjusted for use in a transformation assay (24).

RESULTS

Growth of BCG in B6D2 mice. Groups of mice were infected intravenously with 10^6 or 10^8 BCG or aerogenically with 10^3 BCG. The growth curves for the three groups of animals are shown in Fig. 1. Mice infected intravenously with 10^6 viable BCG showed an initial 100-fold increase in lung and spleen counts over a 14-day period, followed by a sharp decline observed throughout the remainder of the experiment. When the inoculum was increased 100-fold, the bacterial

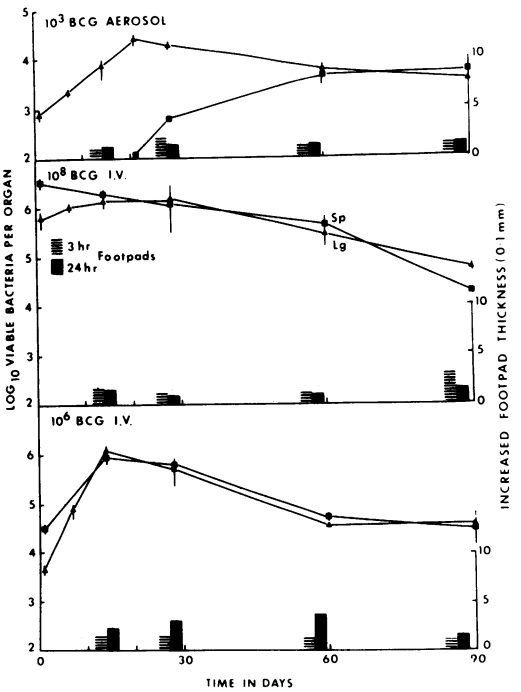

FIG. 1. *Growth curves for BCG Pasteur after administration of high (10^8 cells intravenous), moderate (10^6 cells, intravenous), or low (10^3 cells, aerogenic) doses into SPF B6D2 mice. The histograms represent immediate (3-h) and delayed (24-h) hypersensitivity after 2.5 μg of PPD was injected into the right hind footpad. Swelling due to diluent alone injected into the left footpad was subtracted from the test results. SP, Spleen; LG, lung.*

counts for both lungs and spleens declined, with little or no sign of early multiplication in vivo. On the other hand, when small numbers of viable BCG were introduced directly into the lungs, substantial growth occurred within the lungs and later in the spleens (Fig. 1). The 10^6 BCG-vaccinated mice developed positive delayed-type hypersensitivity reactions to PPD within 7 days, whereas the group vaccinated with 10^8 BCG failed to develop detectable levels of tuberculin hypersensitivity. The aerogenically infected mice never became more than marginally hypersensitive to the tuberculin.

Lymphocyte transformation. At increasing time intervals after BCG inoculation, spleen cells were harvested from all three groups of mice, and the T-cell-enriched suspensions were tested for responsiveness to PHA (Fig. 2) or PPD (Fig. 3). The actual degree of the PHA and PPD responses varied depending upon the dose and the route of inoculation, and the uptake also changed dramatically as the infections progressed. On the other hand, the response of the lipopolysaccharide-stimulated cells, which was determined by using unfractionated spleen cells, did not differ significantly from that of normal spleen cells, regardless of the mode of infection used.

Mice vaccinated with 10^8 BCG (high intravenous dose) showed an early enhanced response

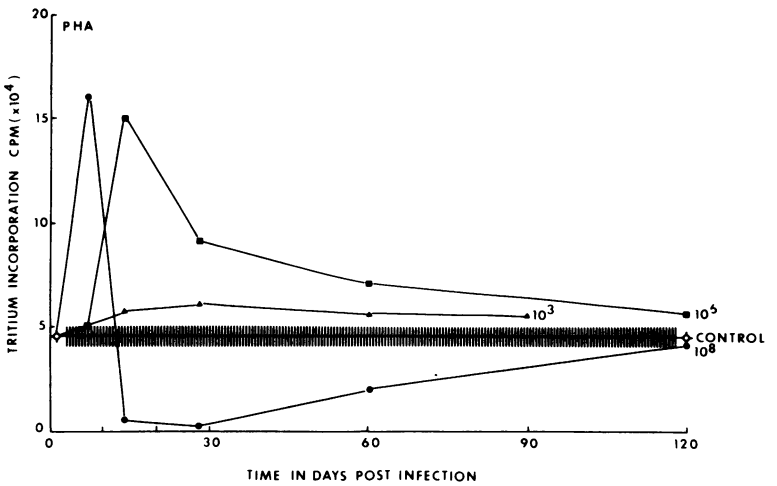

FIG. 2. *Tritiated thymidine incorporation by nylon wool-filtered spleen cells exposed in vitro to 0.1 μg of PHA at increasing time intervals after administration of high (●), moderate (■), or low (▲) doses of viable BCG Pasteur. Normal incorporation levels ± standard error of the mean are represented by the hatched area.*

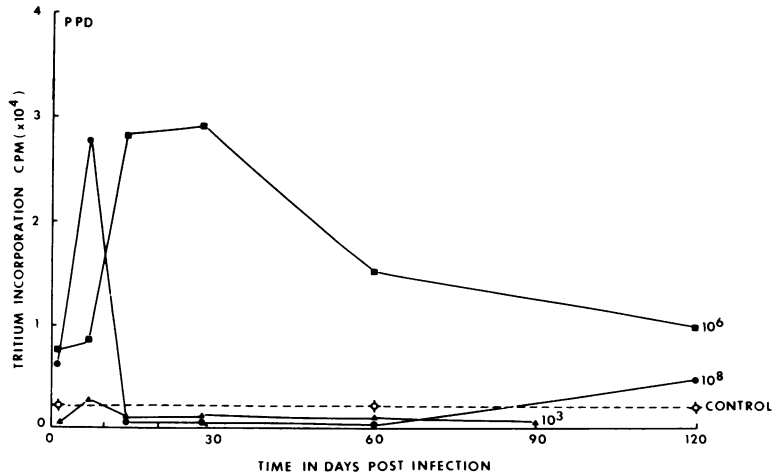

FIG. 3. *Tritiated thymidine incorporation by filtered splenic lymphocytes exposed in vitro to 0.1 μg of PPD at increasing time intervals after BCG vaccination. See legend to Fig. 2 for details.*

to both PHA and PPD stimulation, but this was quickly replaced by a level of responsiveness approaching that of background. Subsequently, the tritium incorporation rates returned slowly to near normal levels (Fig. 2). The response by T-cell-enriched spleen cells from the 10^6 BCG group also showed a sharp early increase, which reached a peak between days 14 and 21 after BCG infection and was followed by a slow return to near normal levels at 4 months. Finally, spleen cells taken from mice infected aerogenically with the low dose of BCG showed incorporation rates after exposure to PHA and PPD which were little different from those seen with normal cells (Fig. 2 and 3). To determine possible reasons for the lack of lymphocyte transformation shown by cells from the 10^8 BCG group and the group infected aerogenically with BCG, filtered spleen cells were prepared 28 days into the infections (2×10^5 cells per 100 μl) and mixed with an equal number of cells taken from day-14 10^6 BCG-infected mice (positive footpad and maximum incorporation response to PPD). The effects of PHA and PPD stimulation on the mixed cell cultures are shown in Table 1. The thymidine uptake in response to both PHA and PPD stimulation in the day-14 10^6 BCG spleen cells was markedly inhibited by the addition of the 10^8 BCG cell suspension. On the other hand, the day-28 aerosol cells showed no suppressive effect on the day-14 10^6 BCG cell suspensions exposed to either mitogen (Table 1). When T-cell-enriched suspensions were prepared from day-120 10^8 BCG donors and mixed with day-14 10^6 BCG spleen cells, no suppression of PHA or PPD incorporation rates occurred (Table 1). Mitomycin C treatment of the day-28 10^8 BCG cells before mixing with day-14 10^6 BCG cells (Table 2) showed none of the suppression seen in untreated controls exposed to PHA or PPD.

In a final experiment, the day-28 10^8 cell suspension was exposed to anti-Thy-1 antiserum in the presence of complement and then mixed with the day-14 10^6 BCG cells. As Table 3 shows, the ability of the day-28 T cell-enriched cell suspension to ablate the PHA and PPD responses by day-14 10^6 BCG cells was prevented almost completely (Table 3). The control values for these tests were all within expected limits.

TABLE 1. *Cell mixing experiments*

Cell source	Response (cpm) with:	
	PHA	PPD
Normal	39,439 ± 1,956[a]	476 ± 83
BCG (10^6, day 14)	134,955 ± 4,501	30,469 ± 1,496
BCG (10^8, day 28)	3,466 ± 985	348 ± 61
BCG (10^8, day 120)	32,125 ± 2,426	4,569 ± 304
BCG (10^3, day 28)	42,994 ± 2,061	589 ± 43
BCG (10^3, day 90)	46,003 ± 1,874	384 ± 69
Cell mixing		
BCG (10^6, day 14) + BCG (10^8, day 28)	34,466 ± 1,876	776 ± 99
BCG (10^6, day 14) + BCG (10^8, day 120)	73,005 ± 3,118	13,099 ± 2,143
BCG (10^6, day 14) + BCG (10^3, day 28)	69,441 ± 2,003	16,451 ± 1,395
BCG (10^6, day 14) + BCG (10^3, day 90)	65,436 ± 2,809	15,684 ± 2,063
Half number BCG (10^6, day 14)[b]	89,996 ± 3,459	12,129 ± 991

[a] Mean ± standard error.
[b] Cells (2×10^5) mixed with medium to halve the number of cells exposed to the mitogen.

TABLE 2. *Cell mixing experiments after mitomycin C treatment*

Cell source	Response (cpm) with:	
	PHA	PPD
Before mitomycin C		
Normal	45,668 ± 2,196[a]	549 ± 65
BCG (10^6, day 14)	149,944 ± 3,014	32,944 ± 1,898
BCG (10^8, day 28)	4,009 ± 488	329 ± 20
After mitomycin C		
Normal	2,004 ± 459	84 ± 16
BCG (10^6, day 14)	4,667 ± 899	948 ± 49
BCG (10^8, day 28)	249 ± 43	90 ± 16
BCG (10^6, day 14, before mitomycin C) + BCG (10^6, day 14, after mitomycin C)	84,003 ± 4,198	18,006 ± 1,096
Half-number BCG (10^6, day 14)[b]	79,844 ± 3,446	18,499 ± 941

[a] Mean ± standard error.
[b] Cells (2×10^5) mixed with medium to halve the number of cells exposed to the mitogen.

TABLE 3. *Effect of anti-theta treatment on the suppressive phenomenon*

Cell source	Response (cpm) with:	
	PHA	PPD
Before anti-theta		
Normal	60,045 ± 2,948[a]	349 ± 24
BCG (10^6, day 14)	268,944 ± 4,046	40,866 ± 2,056
BCG (10^8, day 28)	6,498 ± 783	671 ± 98
After anti-theta		
Normal	2,498 ± 483	219 ± 43
BCG (10^6, day 14)	8,087 ± 1986	2,704 ± 339
BCG (10^8, day 28)	896 ± 84	380 ± 73
Cell mixing		
BCG (10^6, day 14, before anti-theta) + BCG (10^8, day 28, before anti-theta)	62,443 ± 2,985	3,945 ± 867
BCG (10^6, day 14, before anti-theta) + BCG (10^8, day 28, after anti-theta)	149,089 ± 2,189	23,477 ± 1,896
Half number BCG (10^6, day 14)[b]	150,049 ± 3,809	22,664 ± 3,098

[a] Mean ± standard error.
[b] Cells (2×10^5) mixed with medium to halve the number of cells exposed to the mitogen.

DISCUSSION

There is evidence in the literature that infectious anergy may be due to suppressor cells within the spleens of heavily infected animals (3, 14). The present study correlates the existence of tuberculin anergy seen in mice receiving 10^8 viable BCG with a reduced uptake of tritiated thymidine by splenic lymphocytes exposed to PPD in an in vitro assay. A population of suppressor T-cells could be demonstrated as early as 14 days into the BCG infection. By 4 months, this suppressor cell activity had largely disappeared again (Table 1), by which time the systemic BCG population had dropped to about 10^4 viable organisms within each spleen. It was not possible to demonstrate conclusively that these mice had undergone a restoration of tuberculin sensitivity at this time due to the large 3-h swelling responses observed in these animals at this time (Fig. 1).

Mitomycin C or anti-theta serum treatment ablated the suppressor T-cell activity in the day-28 10^8 BCG-infected spleens (Tables 2 and 3). Parallel studies carried out with whole spleen cell suspensions resulted in a prevention of suppressor cell activity essentially similar to that seen with the T-cell-enriched spleen cell suspensions. This is a strong argument against any contribution by adherent cells. Filtered spleen cells were used throughout the present study to avoid the complication of adding heavily infected macrophages to the cell mixtures in vitro. This could have resulted in the release of tuberculin or other mycobacterial antigens which might have functioned as mitogens within the test system. Viable counts routinely carried out on the filtered spleen suspensions indicated a contamination level in the cell mixing experiments of less than 0.01% BCG-infected cells. Such a low level of viable BCG recovery is a clear indication of the efficiency of the nylon wool filtration procedure and also makes it unlikely that endogenous tuberculin release occurred in any of the cell mixing experiments.

The present findings are compatible with those recently reported by Orbach-Arbouys and Poupons (20). These workers also noted an early stimulation phase in spleen cells taken from mice infected intravenously with 1 mg of BCG (estimated to represent 5×10^7 to 10×10^7 viable organisms), followed 1 week later by active suppression. Unfortunately, these authors did not examine the effect of the specific mitogen (PPD) on this response. They noted, however, the presence of an adherent suppressor cell population within the spleens of their mice. A similar finding has also been reported by Klimpel and Henney (15). Despite careful and repeated investigations, no evidence for such an adherent suppressor cell population could be found in the present study.

The responses by unfractionated normal and infected mouse spleen cells to lipopolysaccharide stimulation showed no evidence for an enhancement or depression of the thymidine incorporation levels. The response to lipopolysaccharide was totally ablated by passage of normal and infected cells through nylon wool. This lack of responsiveness to the B-cell mitogen constitutes a further measure of the efficacy of the nylon wool treatment in removing B-cells from the whole spleen cell population. When PPD was added to unfractionated normal spleen cells, there was a substantial mitogenic response. This effect seems to be in agreement with the work of Sultzer and Nilsson (23), who reported several years ago that PPD can function as a B-cell mitogen in vitro. This response by normal spleen cells could be prevented by first filtering the cells through nylon wool. However, when the responses of unfractionated and nylon wool-enriched spleen cells taken from BCG-infected animals were compared, there was little or no difference in tritium uptake. This indicates that in infected animals, the B-cell component of the response is quite small and, contrary to the recent finding of Sultzer (22), the present data suggest that the T-cells present are also likely to proliferate when exposed to PPD in culture. Sultzer postulated the presence of a suppressor T-cell population which he thought capable of acting on the B-cell popuation. In the present system, the T-cells appear to function by suppressing the corresponding responses of other T-cells which normally respond to the T-cell-sensitizing antigens in the BCG population.

The aerogenically infected mice failed to develop significant tuberculin hypersensitivity, despite the fact that their spleens contained substantial numbers of viable BCG. When these spleen cells were tested in culture, they did not respond to PPD and gave normal responses to PHA. This indicates no suppression of the ability of these cells to respond to PHA, and in the case of PPD it suggests the absence of specifically sensitized T-cells within the spleen at the time of testing. The PHA response shown by the filtered spleen cells was at all times within normal limits.

The present study shows that animals heavily infected with BCG (10^8 cells) are unable to respond to PPD when tested in the footpad. At the same time spleen cells from these animals show an impaired performance in vitro in response to both a specific and a nonspecific mitogen. This impairment has been shown to be due to a suppressor T-cell population in the spleens of heavily infected animals. This is in no

way meant to imply that the suppressor cells detectable in vitro are responsible for the in vivo observation, as the loss of skin reactivity could be due to other phenomena. Work is at present underway to determine whether a cell population capable of in vitro suppression has an effect on the expression of a positive skin response to PPD. It is interesting to note, however, that spleen cells from heavily infected animals do show an initial response to PPD (in vitro) and that this is quickly suppressed. It is therefore possible that this apparently simple suppression is just the tip of a complex series of cell interactions which may involve several T-cell subsets, with the net result appearing as in vitro suppression of lymphocyte transformation. The connection, if any, between in vitro suppression and lack of skin test reactivity awaits the clarification of these interactions.

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