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Cutaneous Unresponsiveness to *Mycobacterium bovis* BCG in Intravenously Infected Mice

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Mice injected with 1 mg (about 1×10^7 CFU) of *Mycobacterium bovis* BCG in the footpad showed high levels of delayed-type hypersensitivity (DTH) to BCG antigens and a continuous increase in circulating specific anti-immunoglobulin G (IgG) antibodies throughout a 6-week observation period. In contrast, mice injected intravenously with a 1-mg dose failed to mount a DTH and showed a depression in antibody production at week 5 postinfection. A dose-response study revealed that an optimum dose of BCG, when injected intravenously, can induce a small but significant DTH response. The delayed cutaneous unresponsiveness in intravenously infected mice lasted at least 6 weeks and was not antigenically specific, in that it depressed the DTH response to *Corynebacterium parvum*. No simple relationship existed between levels of DTH and the amount of circulating anti-IgG antibodies. Splenectomy and treatment with a high dose of cyclophosphamide before infection, although greatly reducing the humoral response, did not reverse the BCG-induced unresponsiveness nor enhance levels of DTH in mice infected in the footpad. It is concluded that the intravenous infection of mice with BCG exerts a nonspecific inhibitory effect on delayed-type immune reactions by the induction of some type of suppressor mechanisms that are resistant to cyclophosphamide.

In the past few years, evidence has accumulated concerning the presence of suppressor cells (macrophage-like or T lymphocytes or both) in the spleen of mice infected with various species of mycobacteria or inoculated with a massive dose of the BCG strain of *Mycobacterium bovis*. These suppressor cells are not antigenically specific, since they can inhibit the lympho-proliferative responses to polyclonal mitogens (15, 33) and alloantigens (12), as well as to specific antigens (8, 29). In addition, they depress several other in vitro immune functions, such as the generation of cytotoxic T-cell response (17), antibody response to sheep erythrocytes (SRBC) (4, 6), and the expression of natural killing activity (16). So far, no well-defined role has been ascribed to these cells in the in vivo regulation of anti-mycobacterial immune responses, apart from the fact that the loss of the delayed cutaneous tuberculin reaction occurring in mice heavily infected with mycobacteria appears to correspond to the time of the appearance of suppressor cells in their spleen (6, 8).

Some of the above mentioned studies (8, 29, 33) have revealed that the appearance of BCG-induced suppressor cells is directly related to the route of administration of the bacilli. For example, suppressive activity was detectable in mice infected intravenously (i.v.) or intraperitoneally, whereas no such activity was found in the spleen of those infected aerogenically or subcutaneously (s.c.). The present experiments were performed to investigate the effects of doses and routes of infection on the induction of humoral and cellular immune responses to BCG antigens in C57BL/6 mice.

A cutaneous unresponsiveness to BCG antigens followed by a late depression in the synthesis of anti-BCG antibodies occurred in i.v. infected mice, whereas both cellular and humoral responses developed normally in s.c. infected mice. Assuming that the cutaneous unresponsiveness was due to splenic suppressor cells, we performed splenectomy and treated the mice with cyclophosphamide before infection. The results show that both treatments markedly reduced circulating anti-BCG antibodies but failed to prevent the development of the cutaneous unresponsive state.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 female mice, obtained from Charles River Breeding Laboratories, Inc., Wilming-

ton, Mass. and from the Canadian Breeding Farm & Labs Ltd., St. Constant, Quebec, were 2 to 3 months of age at the time of infection. This strain of mice was chosen for the present experiments on the basis of its high susceptibility to BCG (11).

BCG. A lyophilized preparation of the Montreal substrain of BCG (Institut Armand-Frappier) was used. The content of each vial was reconstituted with sterile saline immediately before use. A 1-mg amount of the reconstituted vaccine contained about 1×10^7 CFU when plated on Dubos solid medium, that is, approximately 10% of the total bacterial count.

Infection of mice. The mice were injected i.v. in the tail vein with 1.0 mg of BCG suspended in 0.1 ml of saline or s.c. in the right hind footpad (RHFP) with the same dose suspended in 10 μl of saline.

Antigen preparation. Soluble BCG antigens were prepared as described previously (31). Briefly, these antigens represent unheated soluble constituents isolated from mechanically disrupted bacillary bodies and purified by ammonium sulfate precipitation at 50% saturation. After exhaustive dialysis against distilled water, the preparation was lyophilized. The same preparation of antigens containing about 80% proteins as measured by the method of Lowry et al. (21) was used throughout the present study.

Sonicated BCG was prepared by the technique of Harboe et al. (14) with a suspension of 60 mg of bacilli in 10 ml of Hanks balanced salt solution (HBSS). In some experiments, the whole sonic extract was used for the elicitation of the delayed footpad reaction. After sonication, some preparations were centrifuged at 20,000 × *g* for 30 min to remove bacillary debris, and appropriate dilutions of the supernatant were used for the detection of anti-BCG antibodies in the sera of infected mice.

Test for DTH. Delayed-type hypersensitivity (DTH) was evaluated by the footpad swelling test. Three weeks after infection, that is, at the time when the tuberculin reaction in s.c. infected mice reached its maximum value (Fig. 1), the mice were challenged in the left hind footpad (LHFP) with 20 μg of soluble BCG antigens or, when indicated, with 20 μg of sonicated BCG in a 10-μl volume of HBSS. The thickness of the LHFP was measured with a dial-gauge calipers before the challenge and at 4, 24, and 48 h later. The increase in footpad thickness was calculated and expressed in 0.1-mm units. Except when otherwise stated, no animal was challenged more than once.

Antibody measurement. Serum antibodies to the antigens of BCG were measured by means of a solid-phase radioimmunoassay using ^{125}I-labeled protein A. Since protein A binds to the Fc region of mouse immunoglobulin G (IgG) (18), obviously only this class of anti-BCG antibodies was measured in the present study. The microtechnique of Marier et al. (22) was used without modification. The wells of polyvinyl chloride disposable microtiter plates (Linbro Scientific Co., Inc., Hamden, Conn.) were coated with the supernatant fraction of sonicated BCG at a 1:25 dilution. All sera were tested in triplicate at a 1:20 dilution. ^{125}I-labeled protein A (50 mCi/mg, labeled with Bolton and Hunter reagent) was obtained from Amersham Corp., Oakville, Ontario, Canada, and a quantity corresponding to about 10^5 cpm was added per well of microtiter plate. The radioactivity bound was measured in an auto gamma scintillation spectrometer. For each antibody determination, the maximal binding activity against BCG antigens was established with a pool of 10 sera from BCG-hyperimmunized mice. The binding activity (i.e., antibody activity) of the sera to be tested was expressed as a percentage of the maximal binding activity and was calculated as follows. The % binding activity = (cpm of tested serum − background cpm)/(cpm of hyperimmune serum − background cpm) × 100, where the background counts per minute represents the count obtained in antigen-coated wells run in parallel but without the addition of any serum.

All determinations were performed before skin testing since the amount of BCG antigens (20 μg) used for challenges provoked a secondary antibody response in BCG-infected mice.

Sensitization and DTH to SRBC and to *Corynebacterium parvum*. The mice were sensitized in one rear footpad with 5×10^8 SRBC or 0.7 mg of *C. parvum* (Burroughs Wellcome Co., Research Triangle Park, N.C.) suspended in 20 μl of saline. Six to seven days later, the animals were challenged in the opposite footpad with 1×10^8 SRBC or 7 μg of *C. parvum*, and the reactions were measured 24 and 48 h later.

Cy treatment. Cyclophosphamide (Cy) monohydrate (Sigma Chemical Co., St. Louis, Mo.) was dissolved in HBSS and injected intraperitoneally into the mice at a dose of 200 mg/kg of body weight 2 days before infection or sensitization.

Splenectomy. Two to three weeks before infection, the mice were splenectomized and sham-splenectomized under pentobarbital anesthesia through a left subcostal incision.

Statistical analysis. Each experimental group comprised 5 to 10 mice. The results are expressed as the arithmetic mean of data obtained in individual experiments or of results obtained in replicate experiments plus or minus the standard error of the mean (SEM). Statistical significance was determined by Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Kinetics of evolution of DTH and anti-BCG antibody responses in mice infected s.c. or i.v. with BCG. Groups of C57BL/6 mice were infected s.c. via the RHFP or i.v. with 1.0 mg of living BCG. At 1-week intervals thereafter, one group of s.c. infected and one group of i.v. infected mice were challenged in the LHFP with BCG soluble antigens to evaluate the level of DTH response. The s.c. infected mice developed a strong cutaneous reaction, which was first detected 2 weeks after infection; it reached a maximum value at 3 weeks and then decreased slowly during the following weeks (Fig. 1A). The intensity of this reaction was found to increase during the first 24 h after the challenge and to persist at about the same level for the next 24 h. Thereafter, it decreased slowly and usually disappeared at 96 h. Such a kinetics therefore suggests that the cutaneous reaction detected in s.c. infected mice is really of the tuberculin type. In contrast, during the 6-week observation period, the mice infected i.v. failed to develop a

positive cutaneous reaction at 24 and 48 h after the challenge. In fact, the small detectable skin reaction seen at 2 to 4 weeks did not significantly differ from the background value, that is, the low nonspecific inflammatory reaction provoked by the inoculation of noninfected control mice with the antigens. Moreover, at 2 to 4 h after the challenge, no significant increase in footpad thickness was observed, thus eliminating the presence of an immediated-type reaction. The use of a larger dose of soluble antigens (i.e., 80 instead of 20 μg) or of a whole sonic extract of BCG did not provoke delayed footpad swelling in i.v. infected mice, whereas under both of these conditions, the level of cutaneous reactions in s.c. infected mice was increased significantly (data not shown).

Two groups of seven mice were infected, one i.v. and the other in the RHFP, with 1.0 mg of BCG, and at 1-week intervals for 6 weeks thereafter, each mouse was bled via the retro-orbital plexus for the measurement of antibody activity (Fig. 1B). A small activity (usually less than 10%) was detected in the serum of some of the noninfected control mice. In both groups of infected mice, a significant response ($P < 0.001$, when compared with the controls) was detectable at 2 weeks after infection. Thereafter, the humoral responses increased gradually but to a lower extent in the i.v. infected mice, so that at 6 weeks postinfection the difference between the two groups was highly significant ($P < 0.02$).

Effect of dose of BCG on cutaneous unresponsiveness in i.v. infected mice. To investigate whether the cutaneous unresponsiveness in i.v. infected mice represented a state of immune paralysis or tolerance due to the fact that a too large dose of bacilli was inoculated, we infected

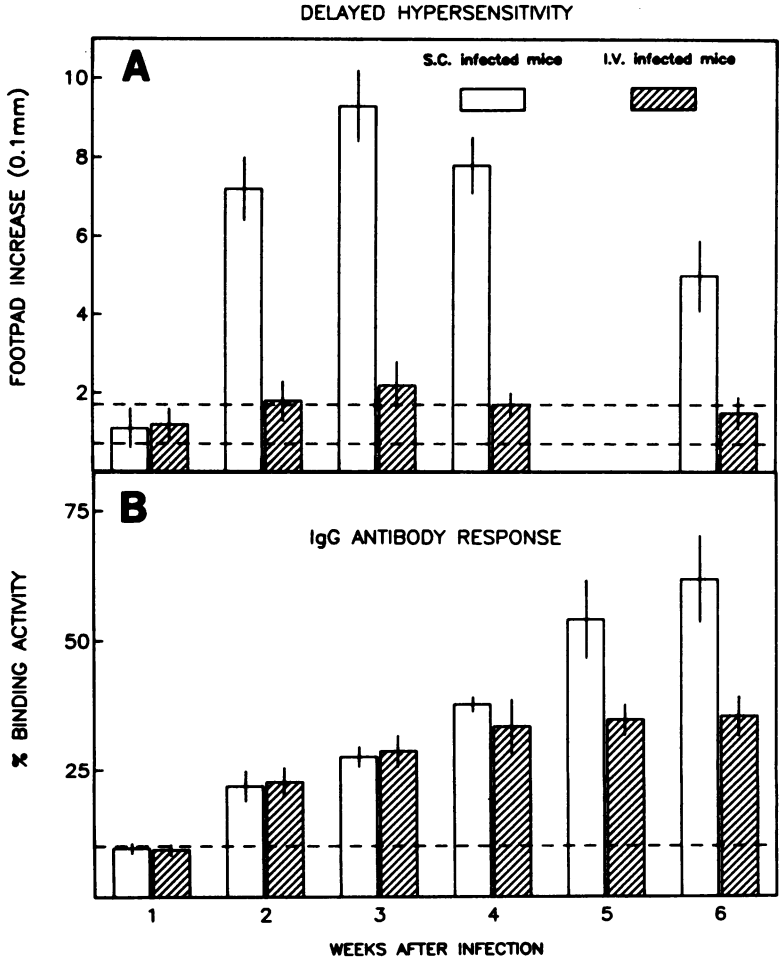

FIG. 1. Kinetics of appearance of DTH reaction (A) and antibody activity (B) in mice infected i.v. and s.c. Each bar represents the mean value of 10 mice (A) or 7 mice (B), and the SEMs are indicated by the vertical lines. The horizontal broken lines represent the range of background reactions in the control mice.

TABLE 1. Effect of dose of BCG on DTH and IgG antibody responses in i.v. and s.c. infected mice at 3 weeks after infection

Dose of BCG inoculated (mg)	i.v. infected mice		s.c. infected mice	
	48-h footpad increase[a]	Antibody activity[b]	48-h footpad increase[a]	Antibody activity[b]
1.0	2.2 ± 0.6	28.7 ± 3.0	9.8 ± 1.7	30.1 ± 3.5
0.1	3.3 ± 0.3[c]	24.2 ± 2.1	9.7 ± 1.9	31.7 ± 3.7
0.01	3.6 ± 0.4[c]	18.6 ± 1.7	8.7 ± 1.1	17.9 ± 2.4
0.001	2.1 ± 0.7	7.5 ± 0.9	6.9 ± 1.4	12.8 ± 2.5
0.0001	2.2 ± 0.4	7.8 ± 1.1	4.3 ± 1.0	5.3 ± 0.8
Control	1.3 ± 0.3	6.1 ± 0.8	1.5 ± 0.5	5.9 ± 0.7

[a] Mean increase in footpad thickness (0.1 mm) plus or minus the SEM obtained from groups of 10 mice.
[b] Mean percentage of binding activity of corresponding groups of mice plus or minus the SEM.
[c] Statistically different from the control ($P < 0.01$).

groups of mice i.v. (and s.c. as controls) with decreasing doses of BCG 3 weeks before the challenge (Table 1). In two independent experiments, a small skin reaction was observed in the groups inoculated i.v. with 0.1 and 0.01 mg. These reactions differed ($P < 0.01$) from the background reaction but did not statistically differ from those detected in the other experimental groups. All groups of mice that were infected in the footpad mounted a large tuberculin reaction, the intensity of which was found to be directly related to the sensitizing dose. Since the mice infected i.v. with the smallest doses might require more time to become fully sensitized, all groups were skin tested again 3 weeks later, that is, 6 weeks postinfection, in the opposite footpad with BCG antigens. Only the group previously inoculated with 0.1 mg still showed a small cutaneous reaction, and the intensity of this reaction was about the same order of magnitude as at 3 weeks (data not shown). These results thus indicated that although an optimum dose of BCG, when given i.v., can induce a small but distinct cutaneous reaction, the degree of sensitization never reached that of s.c. infected mice.

Twenty-four hours before the 3-week footpad test, all groups of mice were bled for the determination of antibody activity (Table 1). As seen, 0.01 and 0.001 mg of BCG represented the minimum dose leading to a IgG antibody response in i.v. and s.c. infected mice, respectively. The dose leading to an optimum antibody response appeared to be 0.1 mg, regardless of the route of administration. As expected, between these optimum and minimum doses, the humoral response was dose related.

TABLE 2. Delayed cutaneous reaction induced in mice unresponsive to BCG

Group	Mice[a]	Induction (BCG s.c.)[b]	Skin test[c]	48-h footpad increase[d]
1	Normal	−	+	1.40 ± 0.4
2	Normal	+	+	6.63 ± 0.7
3	Unresponsive	+	+	3.90 ± 0.3[e]
4	Unresponsive	−	+	2.13 ± 0.3

[a] Unresponsive mice were those infected i.v. with BCG 3 weeks earlier.
[b] Unresponsive or age-matched normal mice were injected or not with 1.0 mg of BCG in the RHFP.
[c] Three weeks after the induction, all groups of mice were skin tested with 20 μg of BCG antigens inoculated in the LHFP.
[d] Mean increase in footpad thickness (0.1 mm) plus or minus the SEM obtained in three separate experiments; each experiment comprised five mice per group.
[e] Statistically different from group 2 ($P < 0.05$) and group 4 ($P < 0.05$).

Attempts to induce a delayed footpad reaction to BCG antigens in unresponsive mice. As seen above, the mice infected i.v. with 1.0 mg of BCG failed to develop a significant DTH reaction at the footpad level. Experiments were next performed to investigate whether these unresponsive mice would respond to an inductive dose of BCG. Thus, the mice infected i.v. 3 weeks earlier were injected in the RHFP with 1.0 mg of bacilli, and 3 weeks later, they were challenged with the specific antigens in the opposite footpad. Pooled data obtained from three consecutive experiments that gave essentially the same results are shown in Table 2. Unresponsive mice, when inoculated s.c. with BCG (group 3), mounted a delayed footpad reaction that was significantly ($P < 0.05$) smaller than the one induced in normal mice (group 2). However, although smaller, this reaction was statistically ($P < 0.05$) higher than the one observed in untreated, unresponsive mice (group 4). Thus, under these experimental conditions, the induction of a DTH reaction to BCG was not com-

TABLE 3. Effect of BCG administered i.v. on the induction of DTH reactions to SRBC and to *C. parvum* in C57BL/6 mice

BCG pretreatment[a]	Sensitizing antigen	Increase in footpad thickness[b] at (h):	
		24	48
−	SRBC	4.9 ± 1.3	1.5 ± 0.6
+	SRBC	5.3 ± 1.2 (NS)	2.5 ± 0.8 (NS)
−	*C. parvum*	5.9 ± 0.9	6.3 ± 1.0
+	*C. parvum*	3.1 ± 0.8 (<0.05)	2.3 ± 0.7 (<0.02)

[a] BCG was administered i.v. 2 weeks before the sensitization with SRBC and *C. parvum* via one hind footpad.
[b] Each value represents the arithmetic mean of increase in footpad thickness (0.1 mm) plus or minus the SEM obtained from five to six mice. The *P* value when compared with BCG-untreated control mice is given in parentheses. NS, Not significant.

pletely suppressed in unresponsive mice. On the other hand, in two other experiments not reported here, in which the mice were injected concomitantly with 1.0 mg i.v. and 1.0 mg in the footpad, a complete suppression of the induction of the cutaneous reaction was obtained. These results agreed well with those of Lefford and Mackaness (20), who reported that a concomitant i.v. inoculation of *M. lepraemurium* suppressed granulomata of a cell-mediated-immunity type that is normally induced when mice are infected in the footpad.

Specificity of cutaneous unresponsiveness to BCG antigens. To study whether the cutaneous unresponsiveness was antigenically specific, we sensitized mice infected i.v. with BCG 2 weeks earlier and uninfected mice (as controls) with SRBC or *C. parvum* in one rear footpad and challenged them 6 to 7 days later with the appropriate antigen in the opposite footpad. The levels of induced DTH reaction to SRBC were the same whether or not the mice were pretreated with BCG, whereas the level of cutaneous reaction to *C. parvum* was significantly depressed ($P < 0.05$) in BCG-pretreated mice (Table 3). Interestingly, the drop in the footpad reaction at 48 h occurred more rapidly in SRBC- than in *C. parvum*-sensitized mice.

Attempts to block the expression of the delayed footpad reaction. For these experiments, mice that had been sensitized via the footpad 3 weeks earlier were infected i.v. with BCG, and 3 weeks later, they were skin tested with specific antigens (Table 4). A significant ($P < 0.01$) depression in the footpad thickness was observed in presensitized mice treated i.v. with BCG (group 3) by comparison with the cutaneous reaction of untreated presensitized mice (group 4). However, a comparison of the cutaneous reactions of group 2 and group 3 revealed that, under these experimental conditions, the desensitization was incomplete.

Footpad reactions of mice treated with Cy before infection with BCG. The treatment of mice with Cy 2 days before immunization usually enhances cell-mediated immune responses, presumably by depleting or inactivating the precursors of suppressor cells (2) or by inhibiting antibody synthesis (19) or both. Cy was used in the present study in an attempt to reverse the BCG-induced unresponsive state. Cy treatment neither abolished the unresponsiveness of i.v. infected mice nor improved the cutaneous reaction of mice infected in the footpad (Table 5). The same results were obtained in four additional experiments. The use of sonicated BCG instead of soluble antigens for challenging Cy- and i.v.-treated mice also did not elicit a positive skin reaction. On the other hand, pretreatment with Cy highly depressed the humoral response

TABLE 4. Effect of i.v. administration of BCG on expression of delayed footpad reaction in BCG-sensitized mice

Group	Mice[a]	Suppression[b] (BCG i.v.)	Skin test[c]	48-h footpad increase[d]
1	Normal	−	+	1.10 ± 0.3
2	Normal	+	+	2.08 ± 0.4
3	Sensitized	+	+	3.83 ± 0.2[e]
4	Sensitized	−	+	7.50 ± 0.8

[a] Sensitized mice are those injected with 1.0 mg of BCG in the RHFP 3 weeks earlier.
[b] Sensitized and age-matched normal mice were infected i.v. with 1.0 mg of BCG.
[c] All groups of mice were skin tested in the LHFP with 20 μg of BCG antigens 3 weeks after the i.v. infection.
[d] Mean increase in footpad thickness (0.1 mm) plus or minus the SEM obtained in three separate experiments; each experiment comprised five mice per group.
[e] Statistically different from group 2 ($P < 0.05$) and group 4 ($P < 0.01$).

TABLE 5. Effects of Cy on footpad reactions to BCG and SRBC and on anti-BCG antibodies

Mouse treatment		Footpad increase[c] at (h):		Antibody activity (% binding ± SEM)[d]
Cy[a]	Antigen and route[b]	24	48	
−	BCG i.v.	3.0 ± 0.7	2.5 ± 0.8	23.3 ± 2.1
+	BCG i.v.	2.7 ± 0.6	1.9 ± 0.7	10.4 ± 4.3 (<0.05)
−	BCG s.c.	9.5 ± 0.9	8.8 ± 1.3	32.2 ± 1.4
+	BCG s.c.	10.5 ± 1.2	8.5 ± 0.4	11.0 ± 2.4 (<0.001)
−	None	1.8 ± 0.4	1.6 ± 0.3	2.3 ± 0.1
+	None	1.0 ± 0.2	0.8 ± 0.1	0
−	SRBC s.c.	6.2 ± 1.5	1.4 ± 0.4	ND[e]
+	SRBC s.c.	16.0 ± 1.8	5.8 ± 1.6	ND

[a] Cy (200 mg/kg of body weight) was administered intraperitoneally 2 days before infection or sensitization.
[b] The mice were challenged with soluble BCG antigens 3 weeks after infection or with SRBC 6 days after sensitization.
[c] Mean increase in footpad thickness (0.1 mm) plus or minus the SEM obtained from five mice per group.
[d] The *P* value when compared with untreated infected mice is given in parentheses.
[e] ND, Not determined.

at 3 weeks postinfection in both groups of BCG-infected mice; and, in addition, it completely eliminated the small amount of natural antibodies in the serum of normal mice. The depression of the anti-BCG activity, although less intense, was still detectable 6 weeks postinfection, indicating the long-lasting effect of Cy on this type of response (data not shown).

The SRBC model was used to verify the enhancing effect of Cy on another delayed cutaneous reaction. Thus, Cy-pretreated mice and untreated C57BL/6 mice were sensitized in one rear footpad with SRBC and 6 days later they were challenged in the contralateral footpad with SRBC (Table 5). Cy-treated mice mounted a very strong cutaneous reaction, which reached its maximum value at 24 h postchallenge and markedly differed ($P < 0.01$) from that of untreated sensitized mice. At 48 h, the drop in the footpad reaction was much faster in the SRBC than in the BCG model.

Effects of splenectomy on cutaneous unresponsiveness of i.v. infected mice. Evidence is accumulating that the spleen is a major source of suppressor cells (25) or is required for suppressor cell induction (30). In a final series of experiments, cellular and humoral responses to BCG in splenectomized mice were compared with those of sham-operated control mice (Table 6). As shown, splenectomy was without any significant effect either on the unresponsiveness of i.v. infected mice or on the intensity of the cutaneous reaction in s.c. infected ones. On the other hand, irrespective of the route of infection, the humoral antibody response was significantly depressed in splenectomized mice.

DISCUSSION

The present study has shown that the development of cellular and humoral immune responses to mycobacterial antigens in C57BL/6 mice is greatly influenced by the route of administration of a high dose of BCG. Indeed, a s.c. (footpad) inoculation provoked a large DTH reaction at the footpad level and a continuous increase in the specific IgG antibody response throughout

TABLE 6. Effects of splenectomy on levels of cutaneous reaction and antibody activity in BCG-infected mice

Route of inoculation of BCG[a]	Mouse treatment	48-h footpad increase[b] (0.1 mm ± SEM)	Antibody activity[c] (% binding ± SEM)
i.v.	Sham-operated	2.9 ± 0.08	44.7 ± 5.1
	Splenectomized	2.7 ± 0.11 (NS)	26.8 ± 3.9 (<0.05)
s.c.	Sham-operated	8.0 ± 0.28	46.5 ± 5.0
	Splenectomized	7.6 ± 0.25 (NS)	28.1 ± 3.4 (<0.05)

[a] BCG was inoculated 2 weeks after the surgical operation of mice.
[b] The mice (10 per group) were challenged in the LHFP with soluble BCG antigens 3 weeks after infection. The *P* value when compared with sham-operated mice is given in parentheses. NS, Not significant.
[c] The antibody activity was measured 1 week after challenge, that is, 4 weeks after infection. The *P* value when compared with sham-operated mice is given in parentheses.

the whole observation period. In contrast, the i.v. route of infection failed to induce significant levels of DTH and greatly reduced the production of anti-BCG antibodies at 5 to 6 weeks after infection. Several groups of workers had observed a failure to mount a delayed cutaneous reaction in mice or guinea pigs heavily infected i.v. with mycobacteria (7, 8, 23, 28, 29). The present study has extended these results by showing that the cutaneous unresponsiveness was not really dose dependent, was of long duration, not antigenically specific, was possibly accompanied by a late depression of IgG antibody synthesis, and finally, was insensitive to Cy and to splenectomy. The failure to mount DTH has also been reported in mice infected i.v. with several types of viruses and parasites (26).

A previous report by Collins and Mackaness (7) had shown that, in i.v. inoculated CD-1 mice, 10^8 viable BCG provoked unresponsiveness, whereas 10^6 did not. In the present study, 10^7 CFU of BCG were suppressive, whereas smaller doses (10^6 and 10^5) induced significant but smaller cutaneous responses than those induced in mice inoculated s.c. with corresponding doses (Table 1). The slight variations in the levels of DTH responses between both studies could be explained by differences either in the susceptibility of the mice to BCG infection (11) or in the nature of the sensitizing material. Since a lyophilized preparation of BCG contains a higher proportion of dead bacilli than does a freshly harvested one, further experiments will be needed to clarify this point.

The immunological unresponsiveness observed in this study might represent a form of partial tolerance called split tolerance or immune deviation (1), since only the cutaneous reaction was effectively depressed, whereas IgG antibody synthesis, when compared with that of s.c. infected mice, remained intact, at least early after infection. The reasons for the late reduction of antibody production are presently unknown. It is likely, however, that the mechanisms involved in the regulation of DTH differ from those regulating antibody production, and both types of mechanisms would operate at different times after infection.

With various sensitizing agents, such as purified proteins in incomplete Freund adjuvant, hapten-carrier conjugates, heterologous erythrocytes, etc., a reciprocal relationship usually exists between DTH and antibody production (13, 24). This does not seem to be the case with mycobacterial agents since the tuberculin reaction was detectable even in the presence of circulating IgG antibodies; the largest skin reactions occurred in the mice showing the highest antibody activity (that is, in s.c. infected mice); and finally, both splenectomy and Cy pretreatment, although reducing significantly the amount of circulating antibodies, did not enhance the delayed footpad reaction. Obviously, no absolute conclusion can be reached about this interrelationship since it is still unknown whether the mycobacterial antigenic determinants responsible for the development of the cellular response are the same as or differ from those leading to antibody production. On the other hand, this does not mean that the presence of circulating antibodies cannot affect the induction or the expression of the tuberculin reaction. Preliminary experiments done in this laboratory have indicated that the amount of the various subclasses of IgG antibodies to BCG varied with the route of inoculation, and as already observed with other murine DTH experimental models, IgG1 can depress the DTH reaction (9).

The BCG-induced unresponsiveness was found to persist for at least 6 weeks. During this period of time, however, the induction of DTH to tuberculin was completely or partially suppressed when an inductive dose of BCG was administered at the same time as or 3 weeks after, respectively, the tolerogenic dose of bacilli, thus implying that the activity of the inhibitor mechanisms triggered by the i.v. inoculation of BCG decreased with time. In addition, a partial but significant desensitization was observed 3 weeks after an i.v. inoculation of bacilli into BCG-sensitized mice. Considered together, these results strongly suggested that an i.v. injection of BCG can act at both the inductive and the expressive phases of the tuberculin reaction.

Several hypotheses, such as reticuloendothelial blockade (27), perturbation of lymphocyte traffic (5), antigenic competition (10), and the presence of suppressor cells (6, 23, 32), have been put forward to explain the cutaneous unresponsiveness to mycobacteria in heavily infected mice. Two approaches were used in the present study to determine whether suppressor cells were involved in BCG-induced anergy. One was to remove the organ apparently implicated in the induction of these cells (25, 30). Splenectomy did not prevent the development of the unresponsive state. It should be noted, however, that not only does BCG induce nonspecific suppressor cells at the spleen level, as demonstrated in vitro, it also activates the nonspecific suppressor cells naturally present in the bone marrow (3). More recently, we have confirmed this last finding and observed, in addition, that bone marrow activation also occurred in splenectomized mice (unpublished data), suggesting that suppressor cells of mitogen-induced blastogenesis could be activated elsewhere than in the spleen.

Another approach was to treat mice with high

doses of Cy before infection in an attempt to eliminate the precursors of suppressor T lymphocytes (2). Repeatedly, such pretreatment neither reversed the cutaneous unresponsiveness in i.v. infected mice nor improved the footpad reaction in s.c. infected mice, thus suggesting that the suppressor mechanisms of BCG-induced anergy, regardless of their nature, are Cy resistant. These findings contrast with those of other investigators who reported an enhancement of the tuberculin reaction in mycobacteria-infected and Cy-pretreated mice (23) and guinea pigs (10). Attempts to conciliate these divergent results by reducing the Cy dose or the BCG dose in C57BL/6 mice or by using another inbred strain (C3H/He mice) have failed. On the other hand, under the same experimental conditions, Cy markedly improved the delayed cutaneous reaction induced by SRBC, thus implying that the DTH to mycobacteria, i.e., the classical DTH, really differs from the DTH to SRBC, which would correspond to a cutaneous basophil type of hypersensitivity (10). The difference between the kinetics of cutaneous reactions to infection agents (BCG and *C. parvum*) and to SRBC (Tables 3 and 5) would support this interpretation. In addition, the fact that mice treated i.v. with BCG can mount a DTH reaction to SRBC but not to *C. parvum* would indicate that the mechanisms involved in the induction of the various types of DTH also differ.

The anergy that develops in i.v. infected mice could also be caused by the clonal deletion of those cells that naturally react with BCG antigens. This latter mechanism could operate alone or in association with suppressor cells. Adoptive cell transfer experiments, which represent one of the next logical steps in these studies, are in progress to answer this question.

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LITERATURE CITED

1. **Asherson, G. L.** 1967. Antigen-mediated depression of delayed hypersensitivity. Br. Med. Bull. **23**:24–29.
2. **Askenase, P. W., B. J. Hayden, and R. K. Gershon.** 1975. Augmentation of delayed-type hypersensitivity by doses of cyclophosphamide which do not affect antibody response. J. Exp. Med. **141**:697–702.
3. **Bennett, J. A., V. S. Rao, and M. S. Mitchell.** 1978. Systemic bacillus Calmette-Guérin (BCG) activates natural suppressor cells. Proc. Natl. Acad. Sci. U.S.A. **75**:5142–5144.
4. **Brown, C. A., I. N. Brown, and V. S. Sljivic.** 1979. Suppressed or enhanced antibody responses *in vitro* after BCG treatment of mice: importance of BCG viability. Immunology **38**:481–488.
5. **Bullock, W. E.** 1976. Perturbation of lymphocyte circulation in experimental murine leprosy. II. Nature of the defect. J. Immunol. **117**:1171–1178.
6. **Bullock, W. E., E. M. Carlson, and R. K. Gershon.** 1978. The evolution of immunosuppressive cell populations in experimental mycobacterial infection. J. Immunol. **120**:1709–1716.
7. **Collins, F. M., and G. B. Mackaness.** 1970. The relationship of delayed hypersensitivity to acquired antituberculosis immunity. I. Tuberculin sensitivity and resistance to reinfection in BCG-vaccinated mice. Cell. Immunol. **1**:253–265.
8. **Collins, F. M., and S. R. Watson.** 1979. Suppressor T-cells in BCG-infected mice. Infect. Immun. **25**:491–496.
9. **Crowle, A. J., and C. C. Hu.** 1965. Inhibition of the development of delayed hypersensitivity by treatment with antiserum. J. Immunol. **94**:555–562.
10. **Dwyer, J. M., D. Parker, and J. L. Turk.** 1981. Suppression of delayed hypersensitivity to tuberculin by antigenic competition. A positive immunoregulatory mechanism sensitive to cyclophosphamide. Immunology **42**:549–559.
11. **Forget, A., E. Skamene, P. Gros, A. C. Miailhe, and R. Turcotte.** 1981. Differences in response among inbred mouse strains to infection with small doses of *Mycobacterium bovis* BCG. Infect. Immun. **32**:42–47.
12. **Geffard, M., and S. Orbach-Arbouys.** 1976. Enhancement of T suppressor activity in mice by high doses of BCG. Cancer Immunol. Immunother. **1**:41–43.
13. **Gray, D. F., and C. Cheers.** 1969. The sequence of enhanced cellular activity and protective humoral factors in murine pertussis immunity. Immunology **17**:889–896.
14. **Harboe, M., O. Closs, G. Bjune, G. Kronvall, and N. H. Axelsen.** 1978. *Mycobacterium leprae* specific antibodies detected by radioimmunoassay. Scand. J. Immunol. **7**:111–120.
15. **Huchet, R., and I. Florentin.** 1976. Studies on the mechanism of action of BCG, p. 85. *In* G. Lamoureux, R. Turcotte, and V. Portelance (ed.), BCG in cancer immunotherapy. Grune and Stratton, New York.
16. **Ito, M., P. Ralph, and M. A. S. Moore.** 1980. Suppression of spleen natural killing activity induced by BCG. Clin. Immunol. Immunopathol. **16**:30–38.
17. **Klimpel, G. R., and C. S. Henney.** 1978. BCG-induced suppressor cells. Demonstration of a macrophage-like suppressor cell that inhibits cytotoxic T cell generation *in vitro*. J. Immunol. **120**:563–569.
18. **Kronvall, G., and R. C. Williams.** 1969. Differences in anti-protein A activity among IgG subgroups. J. Immunol. **102**:828–833.
19. **Lagrange, P. H., G. B. Mackaness, and T. E. Miller.** 1974. Potentiation of T-cell-mediated immunity by selective suppression of antibody formation with cyclophosphamide. J. Exp. Med. **139**:1529–1539.
20. **Lefford, M. J., and G. B. Mackaness.** 1977. Suppression of immunity to *Mycobacterium lepraemurium* infection. Infect. Immun. **18**:363–369.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **Marier, R., M. Jansen, and V. Y. Andriole.** 1969. A new method for measuring antibody using radiolabeled protein A in a solid-phase radioimmunoassay. J. Immunol. Methods **19**:41–49.
23. **Nakamura, R. M., and T. Tokunaga.** 1980. Induction of suppressor T cells in delayed-type hypersensitivity to *Mycobacterium bovis* BCG in low-responder mice. Infect. Immun. **28**:331–335.
24. **Neta, R., and S. B. Salvin.** 1973. Specific depression of delayed hypersensitivity to purified proteins, with relation to production of circulating antibody. Cell. Immunol. **9**:242–250.
25. **Pierce, C. W., and J. A. Kapp.** 1976. Regulation of immune responses by suppressor T cells. Contemp. Top. Immunobiol. **5**:91–99.
26. **Playfair, J. H. L.** 1982. Workshop report: suppressor cells in infectious disease. Parasite Immunol. **4**:299–304.
27. **Rook, G. A. W.** 1975. The immunological consequences of antigen overload in experimental mycobacterial infections

in mice. Clin. Exp. Immunol. 19:167–177.
28. **Shepard, C. C., L. L. Walker, R. M. Van Landingham, and S. Z. Ye.** 1982. Sensitization or tolerance to *Mycobacterium leprae* antigen by route of injection. Infect. Immun. 38:673–680.
29. **Sultzer, B. M.** 1978. Infection with bacillus Calmette-Guérin activates murine thymus-independent (B) lymphocytes. J. Immunol. 120:254–261.
30. **Sy, M. S., S. D. Miller, H. B. Kowach, and H. N. Claman.** 1977. A splenic requirement for the generation of suppressor T cells. J. Immunol. 119:2095–2099.
31. **Turcotte, R.** 1969. Yield of non-dialyzable mycobacterial constituents during the growth cycle. Can. J. Microbiol. 15:35–41.
32. **Turcotte, R.** 1980. Influence of route of *Mycobacterium lepraemurium* injection on susceptibility to mouse leprosy and on lymphoblastic transformation. Infect. Immun. 28:660–668.
33. **Turcotte, R., L. Lafleur, and M. Labrèche.** 1978. Opposite effects of BCG on spleen and lymph node cells; lymphocyte proliferation and immunoglobulin synthesis. Infect. Immun. 21:696–704.