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Opposite Effects of BCG on Spleen and Lymph Node Cells: Lymphocyte Proliferation and Immunoglobulin Synthesis

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C57BL/6 mice were immunized intravenously (i.v.), intraperitoneally (i.p.), or subcutaneously with one dose of *Bacillus* Calmette-Guérin (BCG). At various time intervals after injection, the lymphocyte response, as measured by thymidine incorporation into DNA, and the number of immunoglobulin-secreting cells were determined in vitro before and after mitogenic stimulation with phytohemagglutinin, concanavalin A, or lipopolysaccharide. In unstimulated cultures, the spontaneous thymidine incorporation and immunoglobulin synthesis of spleen cells were increased to some extent in mice infected i.p. or i.v. with BCG, as compared with noninfected mice. In contrast, after mitogenic stimulation, a marked depression of the proliferative response of spleen cells to both T- and B-cell mitogens and a marked inhibition of LPS-induced immunoglobulin secretion were observed in mice infected i.v. and to a lesser extent in those infected i.p. The depression of lymphoblastogenesis in spleens was fully established 15 days after infection and persisted for a long period of time. When unfractionated or plastic-adherent spleen cells from BCG-infected mice were cultured with normal spleen cells, a strong depression of their reactivity to phytohemagglutinin, concanavalin A, and lipopolysaccharide was observed. After the removal of cells adherent to plastic, the response was partially restored in the nonadherent population from mice infected i.p., but not in that from mice infected i.v. After mitogenic stimulation, lymph node cells of mice inoculated subcutaneously showed a response to mitogen higher than that of normal cells. These results thus demonstrate that, depending on the route of administration, BCG exerts very different effects.

BCG injected into mice leads to a strong stimulation of the reticuloendothelial system (2) and acts as an adjuvant at both the cellular (3, 14) and the humoral (15) levels. Most importantly, BCG is of considerable value as a nonspecific adjuvant in the immunoprophylaxis and immunotherapy of neoplasia (for reviews, see references 1 and 12). However, the exact mechanisms underlying these effects are far from being elucidated, and several recent in vitro studies have been designed to investigate the cellular basis of the action of BCG.

These studies have shown that in mice, footpad inoculation of BCG caused predominantly a T-cell proliferation in popliteal lymph nodes in vivo (13, 15). However, little is known about the action in vitro of T- or B-cell mitogens on BCG-sensitized lymph node cells. On the other hand, indications are accumulating which suggest that when BCG is administered intravenously (i.v.), the proliferative response in vitro of spleen cells to T-cell mitogens (phytohemagglutinin [PHA] or concanavalin [ConA]) is markedly depressed (5, 11, 16, 20), whereas the response to B-cell mitogens (lipopolysaccharide [LPS] or dextran sulfate) can be either increased (5, 20) or decreased (16). Splenic suppressor cells adherent to plastic and nylon wool were implicated in the in vitro impairment of T-cell functions (5, 16, 20).

In relation to the humoral response of draining regional lymph nodes (4, 15), subcutaneous inoculation of BCG led to a marked increase in subsequent antibody-secreting cell (PFC) responses to thymus-dependent antigens but not to T-independent antigens. The increased lymph node PFC response is believed to the mediated through an activation of T-helper cells by BCG (15). On the other hand, the i.v. injection of BCG led to controversial results. For one group of workers (4), it caused a suppressed response to subsequently injected T-dependent and T-independent antigens, whereas for another group (5), an increased response to both types of antigens was observed, suggesting a direct effect of BCG on B-cells.

The present experiments were performed to obtain a further insight into the mode of action

of BCG. Different routes of administration of this bacillus were investigated as to their effects on (i) the proliferative responses of spleen and lymph node cells to T- and B-cell mitogens, (ii) the immunoglobulin synthesis, irrespective of antibody specificity, by spleen and lymph node cells before and after stimulation in vitro with LPS, and (iii) the kinetics of induction of splenic suppressor cells and some of their biological properties.

The results show that, according to the route of administration, BCG exerts very different effects on the proliferative response and immunoglobulin secretion of spleen and regional lymph node cells.

MATERIALS AND METHODS

Mice. Groups of 8- to 12-week-old female C57BL/6 mice (The Jackson Laboratories, Bar Harbor, Me.) were injected intraperitoneally (i.p.) or i.v. (tail vein) with 1.0 mg (about 10^7 colony-forming units) of a lyophilized preparation of BCG (Institut Armand-Frappier, Ville de Laval, Quebec, Canada) suspended in 0.1 ml of physiological saline or with saline alone (control mice). In subcutaneously injected mice, the same dose of BCG, but suspended in 0.02 ml, was administered into the right hind footpad. Mice were sacrificed by cervical dislocation at various intervals after injection.

Spleen and lymph node cell preparations. Spleens and popliteal lymph nodes were removed under sterile conditions, collected in RPMI 1640 (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), and finely minced with iris scissors. Tissue fragments were then aspirated repeatedly through a Pasteur pipette, and the whole preparation was passed through four layers of sterile gauze. Contaminating erythrocytes in spleen preparations were lysed by osmotic shock, after which cells were washed three times in the culture medium, counted, and adjusted to 5×10^6 viable cells per ml. In some experiments, the spleen cells were transferred into 100-mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and incubated for 2 h at 37°C in a humidified atmosphere containing 5% CO_2 to remove adherent cells. After incubation, nonadherent cells were harvested by gentle agitation, washed once, and adjusted to 5×10^6 viable cells per ml in culture medium. When needed, plastic-adherent cells were recovered by scraping dishes with a rubber policeman. Viabilities of all these cell preparations were assessed by the trypan blue exclusion test and were usually greater than 95%. Most of the experiments reported in this paper were performed with cell pools from two BCG-infected mice or from two or three normal mice.

Lymphocyte transformation. The responses of spleen and lymph node cells to PHA, Con A, and LPS were determined by measuring the incorporation of [^{3}H]thymidine ([^{3}H]TdR) into DNA. Viable cells (5×10^5) were cultured with optimal concentrations of mitogens in 0.1 ml of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and antibiotics as mentioned above. PHA (GIBCO) was used at a final concentration of 50 μl/ml, Con A (Calbiochem, La Jolla, Calif.) was used at 2 μg/ml, and LPS (LPS O26:B6; Difco Laboratories, Detroit, Mich.) was used at 50 μg/ml. All cultures were set up in triplicate in flat-bottomed tissue culture plates (Linbro Scientific Co., Inc., Hamden, Conn.) and incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO_2. Cultures were pulsed with 1 μCi of [^{3}H]TdR (Amersham/Searle Corp., Oakville, Ontario, Canada; specific activity, 2.0 Ci/mmol) 18 h before the end of the incubation period and harvested with a MASH multiple automated sample harvester (Microbiological Associates, Bethesda, Md.) on glass fiber filters (H. Reeve Angel & Co. Clifton, N.J.). Glass fiber disks were placed in toluene containing 2,5-diphenyloxazole (5 g/liter) plus dimethyl-1,4-bis-(5-phenyloxazolyl)benzene (0.1 g/liter), and the radioactivity was counted in a Beckman DPM-100 scintillation spectrometer. Results are expressed as Δcpm (*i.e.*, mean counts per minute of stimulated triplicate cultures minus mean counts per minute of nonstimulated triplicate cultures).

Induction of immunoglobulin synthesis. The conditions for the in vitro induction of immunoglobulin synthesis by LPS were as described above except that mercaptoethanol (5×10^{-5} M) was added to the culture medium during the 3-day incubation period. Then, the cells were washed twice and suspended at the appropriate concentration in Earle balanced salt solution and assayed for immunoglobulin secretion. Cells secreting immunoglobulin, irrespective of antibody specificity, were enumerated as described by Molinaro et al. (17). Briefly, the cells were incubated successively for 1 h in 0.6% agarose (Industrie Biologique Française, Gennevilliers, France) in 35-mm tissue culture plates with sheep erythrocytes coated with purified rabbit antibodies to whole mouse immunoglobulin, then for 1 h with 0.5 ml of rabbit antibodies to whole mouse immunoglobulin, and finally for 1 h further with sheep erythrocyte-absorbed guinea pig complement.

RESULTS

The injection of 1 mg of BCG either i.p. or i.v. into C57BL/6 mice led to an increase in splenic weight that reached a maximum value (three to four times that of a normal mouse spleen) 18 to 20 days after injection. Thereafter, although it decreased slightly, the weight remained above the normal level for a long period of time, since at day 122 after infection it was still about twice that of a normal spleen. A certain correlation could be established between the total number of extractable spleen cells and splenomegaly. For instance, that number increased by a factor of 2.6 (mean value from eight determinations made between days 16 and 35 after infection) in the spleens of BCG-infected mice as compared with those from control mice. No significant difference in splenic weights and total numbers of extractable spleen cells was found between mice infected i.p. and i.v. (Table 1). However, the

relative proportion of spleen cells that did not adhere to plastic varied according to the immune status of the mice and the route of infection. Thus, it was found that the percentages of spleen cells not adherent to plastic were 75.0 in noninfected mice, 62.8 in mice infected i.p., and 55.6 in mice infected i.v. The differences in nonadherent spleen cells between i.p.- and i.v.-infected mice were not statistically significant, but they both differed significantly from normal cells.

Spontaneous (no mitogen added) TdR incorporation into spleen cells from normal or BCG-infected mice was studied. For these experiments, [^{3}H]TdR was added at the time of culture initiation, and the incorporation was measured 18 h later. Cultures of the same cellular preparations were also incubated for 72 h, [^{3}H]TdR being added 18 h before the end of the incubation period. More TdR was incorporated by normal and infected cells during the first 18 h of incubation than during the last 18 h (Table 2). Moreover, at both incubation periods, although the difference was not statistically significant, infected spleen cells incorporated about twice as much TdR as normal cells did. Essentially the same results were obtained when nonadherent spleen cells were used instead of the unfractionated populations.

TABLE 1. *Total number of cells and number of nonadherent cells per spleen in normal and BCG-infected mice*

Mice	Cells/spleen ($\times 10^7$)[a]		P[b]
	Total	Nonadherent	
Normal	6.07 ± 0.82[a]	4.56 ± 1.27 (75.0)	
BCG infected			
i.p.	14.83 ± 3.29	9.28 ± 3.74 (62.8)	<0.05
i.v.	13.83 ± 3.18	7.70 ± 1.80 (55.6)	<0.01

[a] Mean data from six independent experiments (two mice per experiment) ± standard deviations. Percentages (in parentheses) were calculated from the total numbers of spleen cells.
[b] For the nonadherent cells of infected mice when compared with those of normal mice, using Student's *t* test.

TABLE 2. *Spontaneous [^{3}H]TdR incorporation (counts per minute) in unstimulated cultures of normal and BCG-infected spleen cells during the first and the last 18 h of a 72-h incubation period*

Spleen cells	[^{3}H]TdR incorporation[a]			
	18-h incubation		72-h incubation	
	cpm	P	cpm	P
Normal	2,625 ± 702		738 ± 317	
BCG infected[b]				
i.p.	5,235 ± 1,251	<0.2	1,374 ± 465	<0.3
i.v.	6,003 ± 1,459	<0.1	1,284 ± 307	<0.3

[a] Mean counts per minute of five experiments ± standard error of the mean; *P* values as measured by Student's *t* test.
[b] BCG was injected between days 15 and 35 before collecting the cells for culture.

The ability of unfractionated spleen cells from mice infected i.p. to respond to PHA, Con A, and LPS was next examined. The lymphocyte blastogenesis induced by the three mitogens was markedly depressed. This inhibition was first detectable 6 to 9 days after infection; it was almost complete at day 15 and persisted till the end of the observation period, 34 days after infection (Fig. 1). Moreover, a few determinations made at day 122 revealed that the responses to Con A and LPS remained low, whereas that to PHA was slightly restored. A similar pattern of hyporesponsiveness (results not shown) was also observed in the spleens of mice injected i.v. with a corresponding dose (1 mg) of BCG.

To see whether that low response could be due to suppressor cells, we first examined the effect of mixing spleen cells just before stimulation with PHA, Con A, and LPS. For these experiments, spleen cells from mice infected i.p. or i.v. 15 to 35 days previously were used at 1:1 ratio of infected to normal spleen cells. Cells from infected mice suppressed the mitogen-induced response of normal cells (Fig. 2). Moreover, the degree of suppression was slightly more marked, but not statistically significant, when spleen cells from mice infected i.v. were used. The addition of normal spleen cells did not significantly modify the response.

In an attempt to evaluate quantitatively the suppressive activity of spleen cells from BCG-infected mice, decreasing numbers of infected cells were mixed with proportionally increasing numbers of normal cells before stimulation with the three mitogens (Fig. 3). As expected, the suppressive activity, expressed in percent inhibition of the response of normal cells, decreased gradually with the decreasing number of infected spleen cells added. However, the pattern of the dose-response curve differed for each mitogen, especially for PHA, where the curve was biphasic. The number of infected spleen cells leading to 50% inhibition of the normal cells varied with the mitogen used, the ratios being about 1:8 for Con A, 1:16 for LPS, and 1:64 for PHA. Essentially the same results (biphasic shape of the PHA curve and relative suppressive activity obtained with each mitogen) were observed in two other experiments in which decreasing numbers of infected cells were added to a constant number (5×10^5) of normal spleen cells. In these experiments, the end point (that is, complete suppression of inhibition) was observed at infected-to-normal cell ratios of 1:40 for Con A and 1:500 for both LPS and PHA.

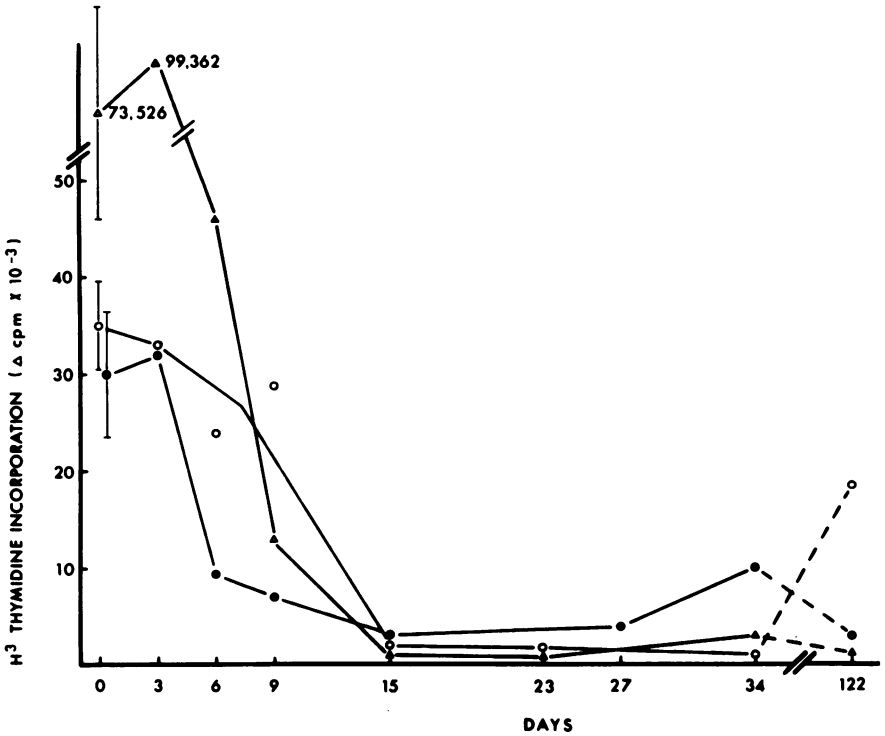

FIG. 1. *Mean incorporation of TdR in response to PHA (○), Con A (△), and LPS (●) of spleen cells of C57BL/6 mice infected i.p. with 1 mg of BCG, as a function of time after injection. The day 0 value represents the mean value of eight separate experiments ± standard error of the mean, whereas values on the other days each represent the mean of two separate experiments (two mice per experiment).*

The effect of depleting BCG-infected spleen cells of plastic-adherent cells was next examined to study the nature of the suppressor cells. The responses to PHA, Con A, and LPS of unfractionated and nonadherent spleen cells from normal mice and from mice infected i.p. or i.v. with BCG are shown in Fig. 4. In normal mice, the depletion of adherent cells generally caused a slight decrease in the response to LPS, and in some experiments it also increased the responses to PHA and Con A. In mice infected i.p. with BCG, the removal of adherent cells resulted in a significant recovery of the response, but the recovery was never complete by comparison with the response of normal nonadherent cells. In contrast, no such recovery was noted with nonadherent cells from mice infected i.v. The addition of 5×10^{-5} M mercaptoethanol in the culture medium to substitute for the possible deficiency of macrophages in the nonadherent cell population did not substantially modify the above results.

In an attempt to define the suppressor cells, adherent cells from BCG-infected mice were also removed on a nylon wool column (8) instead of plastic dishes as used above. Preliminary results indicated that in mice infected i.p. or i.v., the restoration was complete (that is, the response of nonadherent cells was similar to that of normal nonadherent cells), provided that the culture medium was supplemented with 5×10^{-5} M mercaptoethanol.

Plastic-adherent and nonadherent cells from normal and BCG-infected mice were added to normal spleen cells to compare their suppressive activities (Table 3). It was observed that at the 1:1 ratio used, the adherent spleen cells from infected mice completely suppressed the incorporation of [^{3}H]TdR by normal cells, whereas adherent cells from normal mice were without any significant effect except for a depression of the PHA response. With the nonadherent spleen cells, a significant degree of suppression was still detectable, but to a lesser extent than that obtained with the adherent cells. Moreover, it appears that the greatest suppressive activity was present amongst the nonadherent cells from mice infected i.v. with BCG.

To evaluate the effect of BCG on a functional response rather than on cell proliferation, the number of immunoglobulin PFCs was measured before and after in vitro stimulation of spleen

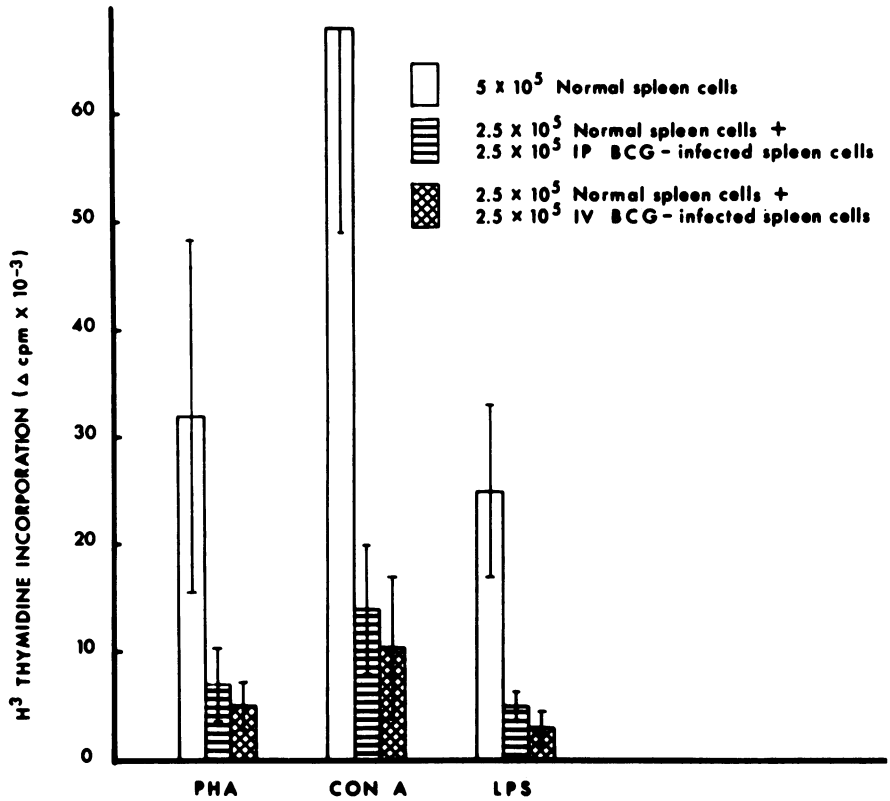

FIG. 2. *Effect of spleen cells from BCG-infected mice on the PHA-, Con A-, and LPS-induced responses of normal cells (mean data from seven experiments ± standard error of the mean).*

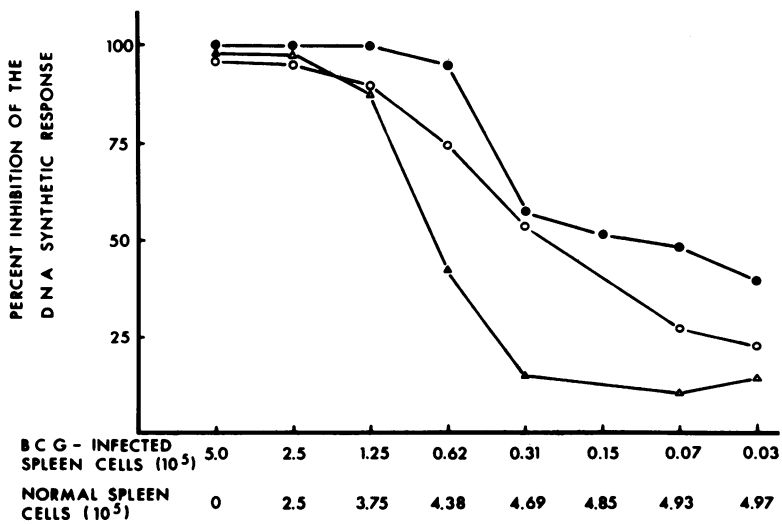

FIG. 3. *Effect of varying concentrations of sensitized spleen cells on the responses of normal cells to PHA (●), Con A (△), and LPS (○) (mean data from two separate experiments, two mice infected 21 days previously with BCG per experiment).*

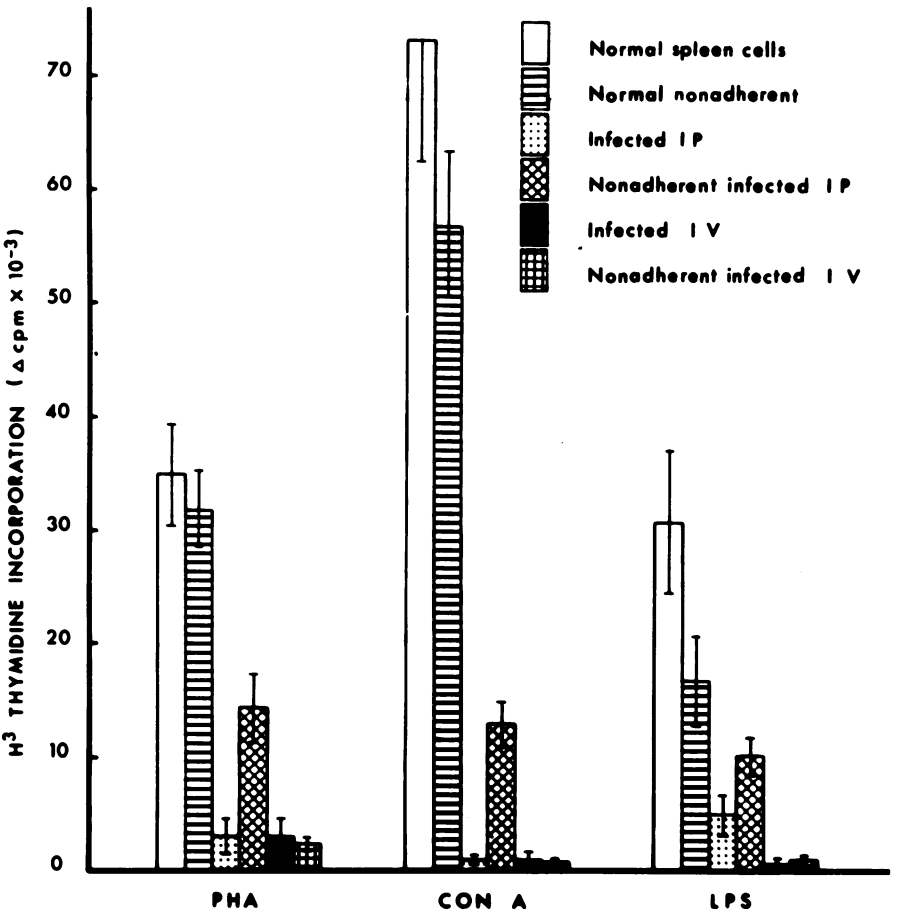

FIG. 4. *Mean DNA-synthetic responses to PHA, Con A, and LPS of unfractionated and nonadherent spleen cells from normal and BCG-infected C57BL/6 mice (mean data from eight separate experiments ± standard error of the mean).*

cells with LPS for 3 days. i.p. and i.v. inoculation of BCG increased the number of immunoglobulin PFCs initially present in the spleen (background PFCs) (Table 4). However, after stimulation in vitro with LPS, the effect was opposite; the response of mice injected i.p. was moderately decreased, and that of mice injected i.v. was practically nil. Removal of plastic-adherent cells restored completely the response in mice infected i.p. and, to a lesser extent, that in mice infected i.v. The extent of restoration appeared to be qualitatively correlated with the efficiency of the separation. Thus, in the i.v.-infected group in experiment 1 (Table 4), there was a significant decrease in background PFC and a good restoration on the response to LPS, whereas in experiment 2, there was no decrease and a poor restoration of the response.

Popliteal lymph node cells were also investigated for the presence of suppressor cells after footpad inoculation of mice with 1 mg of BCG. The results of two separate experiments on the incorporation of TdR after mitogen stimulation, the first 14 days and the second 33 days after infection, are shown in Table 5. Although popliteal lymph nodes were enlarged, no suppressor cells could be detected. On the contrary, sensitized cells showed a marked proliferation in the absence and in the presence of all three mitogens by comparison with normal node cells. Interestingly enough, B-cell function, as measured by the proliferative response to LPS, was more enhanced than T-cell function. It should be noted that, compared with normal spleen cells, the cells from mice infected in the footpad showed neither stimulation nor inhibition when cultured with any of the three mitogens (data not shown).

The effect of footpad infection with BCG on LPS-induced immunoglobulin synthesis is

TABLE 3. *Effect of plastic-adherent and nonadherent spleen cells from BCG-infected mice on PHA-, Con A-, and LPS-induced response of normal unfractionated spleen cells*

Cell cultures[a]	[^{3}H]TdR incorporation (Δcpm) induced with:		
	PHA	Con A	LPS
Normal cells alone	36,295[b]	47,407	14,086
Normal + i.p.-infected adherent cells	0	0	471
Normal + normal adherent cells	10,889	49,443	18,961
Normal cells alone	32,256 ± 6,156[c]	68,152 ± 18,957	25,466 ± 8,193
Normal + i.p.-infected nonadherent cells	20,931 ± 5,646	22,664 ± 4,639	8,699 ± 1,582
Normal + i.v.-infected nonadherent cells	16,440 ± 1,772	7,557 ± 3,015	8,023 ± 2,650

[a] Ratios of normal to infected adherent or nonadherent cells were 1:1.
[b] Mean Δcpm (i.e., mean counts per minute of stimulated triplicate cultures minus mean counts per minute of nonstimulated triplicate cultures) of two experiments (two mice per experiment).
[c] Mean Δcpm of six experiments ± standard error of the mean (two mice per experiment).

TABLE 4. *Number of spleen PFC in normal and BCG-infected mice before and after LPS stimulation in vitro*

Expt[a]	Cells cultured	PFC/10^6 spleen cells cultured[b]	
		Before LPS stimulation	After LPS stimulation[c]
1	Unfractionated		
	Normal	910 ± 110	26,200 ± 5,100
	i.p.	1,850 ± 350	15,500 ± 710
	i.v.	2,650 ± 210	100 ± 80
	Nonadherent		
	Normal	1,040 ± 60	35,000 ± 4,200
	i.p.	1,070 ± 30	41,000 ± 1,400
	i.v.	790 ± 120	17,500 ± 4.900
2	Unfractionated		
	Normal	660 ± 20	18,400 ± 1,700
	i.p.	3,175 ± 250	4,400 ± 850
	i.v.	2,390 ± 230	200 ± 280
	Nonadherent		
	Normal	300 ± 70	14,200 ± 280
	i.p.	1,812 ± 160	15,300 ± 420
	i.v.	3,625 ± 140	2,200 ± 280

[a] BCG was injected 16 and 18 days before collecting the cells for culture in experiments 1 and 2, respectively.
[b] Mean PFC of triplicate cultures ± standard error.
[c] PFC measured after 3 days of culture in the presence of 10 μg of LPS per ml.

TABLE 5. *In vitro response to mitogens in popliteal lymph node cells from mice inoculated in the footpad*

Expt[a]	Cells cultured	TdR incorporated (cpm)[b]			
		Unstimulated cells	Cells stimulated with:		
			PHA	Con A	LPS
1	Normal	492	8,006	11,193	875
	BCG infected	4,076	78,280	28,736	38,168
2	Normal	153	2,036	8,330	1,105
	BCG infected	3,536	14,874	22,011	26,833

[a] In experiments 1 and 2, mice were tested, respectively, 14 and 33 days after BCG injection.
[b] Mean counts per minute of triplicate cultures; the standard errors, being smaller than 10%, were not included in this table.

TABLE 6. *Number of immunoglobulin PFCs obtained from normal and footpad-infected mice before and after LPS stimulation*

Cells tested	PFC/10^6 cells cultured[a]	
	Before LPS stimulation	After LPS stimulation[b]
Normal spleen	850 ± 42	260,000 ± 32,000
BCG-infected spleen[c]	930 ± 40	975,000 ± 60,000
Normal lymph nodes	50 ± 14	363,000 ± 38,000
BCG-infected lymph nodes[d]	170 ± 43	1,960,000 ± 70,000

[a] Mean PFC of triplicate cultures ± standard error.
[b] Cells were stimulated with 50 μg of LPS for 5 days at 10^4/ml in RPMI medium containing 5 × 10^{-5} M mercaptoethanol and 18% fetal bovine serum.
[c] Cells obtained from mice injected with 1 mg of BCG in the footpad 33 days before culture.
[d] Popliteal lymph nodes stimulated as above.

shown in Table 6. The numbers of immunoglobulin PFCs in the spleens and lymph nodes of normal and infected mice were measured before and after 5 days of culture. It can be seen that normal lymph nodes initially contained many fewer immunoglobulin PFCs than spleens did. Infection with BCG in the footpad selectively increased the number of background PFCs in the nodes without significantly affecting the number of PFCs in the spleens. The effect of footpad infection on the response of the cells to LPS was the opposite of what was observed after i.p. and i.v. injection. There was no indication of suppressor activity, neither in the spleen nor in the lymph nodes, but, instead, the response was increased, and as might be expected, to a higher degree in the lymph nodes than in the spleen.

DISCUSSION

It is well known that after BCG immunization, enlargement of the spleen and lymph nodes occurs about 2 weeks after injection, and this enlargement, which may persist for several weeks, is associated with an increased number of both macrophages (histiocytes) and lymphocytes (6, 16, 18). One might expect that these cells would be more responsive to the specific antigens (BCG or purified protein derivative) and to nonspecific polyclonal mitogens in culture. This was verified for popliteal lymph node cells when BCG was inoculated subcutaneously into the footpads, but not for spleen cells when BCG was administered systemically (i.p. and i.v.). Instead, a marked depression of lectin-induced transformation occurred with the spleen cells from infected mice.

In unstimulated cultures, sensitized spleen cells incorporated about twice as much TdR as normal spleen cells (Table 2), thus confirming previous findings (5, 16). Moreover, before LPS stimulation, these cells were secreting more immunoglobulins (Table 4). These results may reflect an ongoing in vivo proliferation of spleen B-lymphocytes (plasmacytes) induced by BCG when administered i.v. and i.p. (20). On the other hand, when cultured in the presence of mitogens, immune splenic lymphocytes were highly refractory to stimulation by T- and B-cell mitogens. Several workers (5, 11, 20) have shown that BCG induced in mice or guinea pigs a marked depression of spleen reactivity to T-cell mitogens (PHA or Con A) but not to B-cell mitogens (LPS or dextran sulfate), where an increased response was observed. These results are partly at variance with ours and those of other workers (16), where a depression of the reactivity of spleen cells to B-cell mitogens has been observed. This discrepancy could partly be explained by the dose and/or the immunogenicity of the particular strain of BCG investigated. For instance, *Corynebacterium parvum*, another immunopotentiating agent, was found to depress spleen reactivity to both T- and B-cell mitogens when inoculated i.v. in large doses, whereas at low doses, it depressed the reactivity to T-cell mitogens only (10). The conclusion that the reactivity to B-cell mitogens is decreased in BCG-infected mice also finds support in the present study, which shows that after in vitro LPS stimulation, the induction of immunoglobulin secretion was markedly depressed in the spleens of these mice. Therefore, both T- and B-cell activities would be susceptible to suppression. However, since unstimulated spleen cells from infected mice incorporated more TdR and secreted more immunoglobulins by comparison with mitogen-stimulated cultures, one interpretation could be that the suppressor cells were inactive at the time of culture initiation but were activated by the lectins during the 3-day incubation period. Another interpretation would be that 3 days was an inappropriate incubation time for testing the effect of mitogenic agents on BCG-stimulated spleen cells, since optimum background stimulation occurred earlier (Table 2). Whatever the mechanism, it still appears that BCG infection changes the response of lymphocytes to T- and B-cell mitogens.

The decreased responsiveness in vitro of spleen cells from mice treated i.v. and i.p. with BCG was shown to be due to an active suppression of the response rather than to the absence or the refractoriness of infected cells. Indeed, the addition of infected cells to normal spleen cells led to the inhibition of their mitogen-induced proliferation. Suppressor cells, which could play a role as regulators of immune responses, have been previously demonstrated after injection of BCG (5, 11, 16, 20), *C. parvum* (10, 19), or *Trypanosoma brucei* (7) and in the spleen of mice bearing a variety of tumors (9). With mice infected i.v., the incomplete restoration of the response after the removal of plastic-adherent cells (Fi.g 4) might be quantitative. This could be explained by the inability of the plastic-adherent technique to adequately remove adherent cells from the spleens of mice infected i.v. This interpretation finds support from preliminary experiments in which infected spleen cells depleted of the adherent population by passage through a nylon wool column were completely devoid of suppressive activity. Although we demonstrated that suppressor cells adhere to plastic and nylon, it cannot be concluded from our work whether they represent a subpopulation of T-suppressor cells with adhering properties (21), B-suppressor cells (3), or suppressor macrophages (5, 9). Moreover, it is not known whether the suppression of the T- and B-lymphocyte functions is caused by the same or two different populations of suppressor cells.

It is likely that the i.v. route is better than the i.p. route for the induction of suppressor cells in mice, whereas the footpad route, at the dose used in this study, was found ineffective. An interesting fact was revealed by this study: the splenic suppressor cells, once induced, can persist for several weeks in BCG-sensitized mice. No suppression of the mitogen-induced response was detected in the thymuses of mice infected either i.p. or i.v. with 1 mg of BCG.

An attempt to evaluate quantitatively the relative number of splenic suppressor cells would indicate that these cells seem very active, since

inhibition of lymphoblastic transformation was still effective at low ratios of infected to normal cells; and one has to consider that not all of the sensitized cells were suppressive. Moreover, the activity of the suppressor cells could be dependent on the mitogen used in culture (Fig. 3). More work has to be done to obtain a better understanding of these phenomena as well as the mechanism (direct cell contact or via soluble mediators) by which suppressor cells depress T- and B-cell responses.

In contrast to sensitized spleen cells, popliteal lymph node cells from mice injected in the footpad were highly active in response to T- and B-cell mitogens. These results would indicate, first, that lymph node T- and B-cells participate in the host immune response to BCG and, second, that suppressor cells, similar to those observed in the spleen, are absent in the draining lymph node. Since living BCG was repeatedly isolated from the spleens as well as from draining lymph nodes of sensitized mice (unpublished results) it seems unlikely that the presence of these bacilli in cultures would be responsible for the opposite results obtained from these two lymphoid organs.

The opposite effects observed in this study when spleen cells are compared with lymph nodes cells in BCG-sensitized mice emphasize the well-known fact that the route of injection of the immunizing agent affects greatly the type of immune response, and BCG is not an exception to this general rule.

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LITERATURE CITED

1. **Bast, R. C., B. Zbar, T. Borsos, and H. J. Rapp.** 1974. BCG and cancer. N. Engl. J. Med. **290**:1413–1420, 1458–1469.
2. **Biozzi, G., C. Stiffel, B. N. Halpern, and D. Mouton.** 1960. Recherche sur le mécanisme de l'immunité non-spécifique produite par les mycobactéries. Rev. Fr. Etud. Clin. Biol. **5**:876–890.
3. **Bona, C.** 1976. Suppression in PPD-induced blast specific response by B-derived lymphocytes, p. 179–185. *In* G. Lamoureux, R. Turcotte, and V. Portelance (ed.), BCG in cancer immunotherapy. Grune & Stratton, New York.
4. **Doft, B. H., B. Merchant, L. Johannessen, S. D. Chaparas, and N. A. Sher.** 1976. Contrasting effects of BCG on spleen and lymph node antibody in nude and normal mice. J. Immunol. **117**:1638–1643.
5. **Florentin, I., R. Huchet, M. Bruley-Rosset, O. Halle-Pannenko, and G. Mathe.** 1976. Studies on the mechanisms of action of BCG. Cancer Immunol. Immunother. **1**:31–39.
6. **Hanna, M. G., B. Zbar, and H. J. Rapp.** 1972. Histopathology of tumor regression after intralesional injection of *Mycobacterium bovis*. I. Tumor growth and metastasis. J. Natl. Cancer Inst. **48**:1441–1455.
7. **Jayawardena, A. N., and B. H. Waksman.** 1977. Suppressor cells in experimental trypanosomiasis. Nature (London) **265**:539–541.
8. **Julius, M. H., E. Simpson, and L. A. Hertzenberg.** 1973. A rapid method for the isolation of functional thymus derived murine lymphocytes. Eur. J. Immunol. **3**:645–649.
9. **Kirchner, H., M. Glaser, H. T. Holden, B. R. Fernbach, and R. B. Herberman.** 1976. Suppressor cells in tumor bearing mice and rats. Biomedicine **24**:371–374.
10. **Kirchmer, H., H. T. Holden, and R. B. Herberman.** 1975. Splenic suppressor macrophages induced in mice by injection of *Corynebacterium parvum*. J. Immunol. **115**:1212–1216.
11. **Lamoureux, G., and R. Poisson.** 1974. BCG and immunological anergy. Lancet **i**:989–990.
12. **Laucius, J. F., A. J. Bodurtha, M. J. Mastrangelo, and R. H. Creeck.** 1974. Bacillus Calmette-Guérin in the treatment of neoplastic disease. RES J. Reticuloendothel. Soc. **16**:347–373.
13. **Mackaness, G. B., D. J. Auclair, and P. H. Lagrange.** 1973. Immunopotentiation with BCG. I. Immune response to different strains and preparation. J. Natl. Cancer Inst. **51**:1655–1667.
14. **Mackaness, G. B., P. H. Lagrange, and T. Ishibaski.** 1974. The modifying effect of BCG on the immunological induction of T cells. J. Exp. Med. **139**:1540–1552.
15. **Miller, T. E., G. B. Mackaness, and P. H. Lagrange.** 1973. Immunopotentiation with BCG. II. Modulation of the response to sheep red blood cells. J. Natl. Cancer Inst. **51**:1669–1676.
16. **Mitchell, M. S., D. Kirkpatrick, M. B. Mokyr, and I. Gery.** 1973. On the mode of action of BCG. Nature (London) New Biol. **243**:216–218.
17. **Molinaro, G. A., E. Maron, W. C. Eby, and S. Dray.** 1975. A general method for enumerating single cells secreting antigen: albumin-secreting hepatocytes detected as plaque-forming cells. Eur. J. Immunol. **5**:771–774.
18. **Rappaport, H., and A. Khalil.** 1976. The immunoproliferative response of mice to intravenously or subcutaneously injected BCG. Cancer Immunol. Immunother. **1**:45–49.
19. **Scott, M. T.** 1972. Biological effects of the adjuvant *Corynebacterium parvum*: I. Inhibition of PHA, mixed lymphocyte and GVH reactivity. Cell Immunol. **5**:459–468.
20. **Sultzer, B. M.** 1978. Infection with Bacillus Calmette-Guérin activates murine thymus-independent (B) lymphocytes. J. Immunol. **120**:254–261.
21. **Waksman, B. H.** 1977. Tolerance, the thymus and suppressor T cells. Clin. Exp. Immunol. **28**:363–374.