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Immunology

Systemic bacillus Calmette–Guérin (BCG) activates natural suppressor cells

(*in vitro* immunization/cell-mediated immunity/suppression of immunization by non-T cells)

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ABSTRACT **Addition of normal C57BL/6 mouse bone marrow cells to an *in vitro* culture of normal C57BL/6 spleen cells and allogeneic P815-Y tumor cells inhibited the development of cell-mediated immunity. Bacillus Calmette–Guérin (BCG) enhanced the suppressive activity of these bone marrow cells as early as 2 days after its intravenous administration to donor mice and elicited similar activity in the spleen by 7 days. Concomitant with the appearance of suppressor cells in the spleen there was a decrease in bone marrow cell number and an increase in spleen cell number. While normal spleen cells failed to inhibit immunization, spleen cells from thymectomized, irradiated, bone marrow-reconstituted mice were inhibitory. Administration of BCG further increased the suppressive activity of spleen cells in these T cell-deprived mice. From this evidence it appears that systemic administration of BCG activates natural suppressor cells in the bone marrow and elicits suppressor cells in the spleen through the migration and colonization of the spleen by bone marrow elements.**

Bacillus Calmette–Guérin (BCG) is one of the most widely used biological adjuvants in the immunotherapy of malignancy. Although the mechanism of action of BCG is not completely understood, its therapeutic efficacy has been attributed to activation of the lymphoreticular system. Several investigators have found increased immunological reactivity of both T lymphocytes (1, 2) and macrophages (3, 4), as well as nonadherent cells resembling monocytes (5), after BCG administration. However, because the immune system is a tightly regulated network, activation of its components does not always lead to an increased immune response. Hence, there are several reports that BCG has led to a decrease in immunological responsiveness (6–8). Recently we reported that intravenous administration of a high dose of BCG led to the induction of splenic adherent cells that suppressed *in vitro* immunization to alloantigen (9). In this study we present evidence for the existence of suppressor cells in normal bone marrow—i.e., natural suppressor cells. These cells are stimulated by systemic BCG, and upon stimulation they may well be the source of the suppressor adherent cells found in the spleen.

MATERIALS AND METHODS

C57BL/6 (H-2^b) female mice, 6–10 weeks old, were obtained from Charles River Laboratories (Wilmington, MA). Mice treated with BCG (Tice strain, University of Illinois) were given 2×10^7 viable units intravenously. Bone marrow cells (from the femur and tibia) and spleen cells were obtained and suspended under sterile conditions as previously described (10). Immunization *in vitro* was carried out according to a modification of the procedure of Mishell and Dutton (11). Samples containing 2×10^7 viable spleen cells (H-2^b) were cultured with 2×10^5 irradiated (4000 rads, 40 J/kg) P815-Y mastocytoma cells (H-2^d) in 35 × 10 mm plastic dishes in a total volume of 1 ml. The medium was RPMI 1640 supplemented with heat-inactivated dialyzed fetal calf serum (5%), glutamine (2 mM), sodium pyruvate (1 mM), 100× nonessential amino acids (1%), penicillin (100 units/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (50 μM). Cultures were incubated for 4 days at 37°C in an atmosphere of 10% CO_2 in air and were fed daily with 0.1 ml of RPMI 1640 medium supplemented with penicillin, streptomycin, fetal calf serum (33%), 50× essential amino acids (6%), 100× nonessential amino acids (3%), glutamine (6 mM), sodium bicarbonate (200 μM), and glucose (100 mM). At the end of culture, cells were agitated with a rubber policeman, collected, washed, and counted. After viability was assessed by trypan blue dye exclusion, cells were adjusted to appropriate concentrations in RPMI 1640 medium with 5% fetal calf serum. Cell-mediated immunity against P815-Y target cells was measured using both the 4-hr ^{51}Cr release assay (12) and the 48-hr growth inhibition assay (13). The percent ^{51}Cr released and the percent growth inhibition were calculated in the following way:

$$\%\ ^{51}\text{Cr release} = \frac{\text{supernatant cpm}}{\text{pellet cpm} + \text{supernatant cpm}} \times 100\% \quad [1]$$

$$\%\ \text{growth inhibition} = [1 - (T/N)] \times 100\% \quad [2]$$

in which T is the number of target cells remaining in the presence of test lymphocytes and N is the number of target cells remaining in the presence of normal lymphocytes. Cells were fractionated on a nylon wool column as previously described (14). To prepare T cell-deprived ("B") mice, normal mice were thymectomized, lethally irradiated (750 rads, 7.5 J/kg) 14 days after thymectomy, then reconstituted intravenously with 2×10^7 syngeneic bone marrow cells 24 hr after irradiation.

RESULTS

As shown in Table 1, significant suppression of cell-mediated immunity occurred as a result of adding 6×10^6 normal syngeneic bone marrow cells to splenic lymphocytes before immunization *in vitro*. No suppression resulted from the addition of 6×10^6 normal spleen cells. Both bone marrow and spleen cells obtained from mice treated 10 days earlier with BCG suppressed the *in vitro* generation of sensitized effector cells. The inhibitory influence of BCG-treated bone marrow appeared to be greater than that of BCG-treated spleen, and both were more inhibitory than normal bone marrow. Bone marrow cells harvested 2 or 10 days after BCG were equally suppressive. However, splenic suppression did not develop until 7 days after

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Abbreviations: BCG, bacillus Calmette–Guérin; T cell, thymus-derived lymphocyte; B mice, thymectomized, irradiated, bone marrow-reconstituted mice.

Table 1. Effect of bone marrow and spleen cells from normal or BCG-treated mice on the immunization of normal spleen cells against alloantigen *in vitro*

Additional cells*	% specific ^{51}Cr release 100:1	% specific ^{51}Cr release 10:1	% growth inhibition 10:1	% growth inhibition 1:1
None	62 ± 6	37 ± 4	80 ± 10	35 ± 7
Normal B.M.	38 ± 8	14 ± 6	45 ± 9	4 ± 3
Normal Spl.	60 ± 5	33 ± 4	84 ± 9	41 ± 5
BCG B.M. (day 2)	9 ± 4	2 ± 1	10 ± 5	0
BCG B.M. (day 7)	5 ± 3	5 ± 3	8 ± 4	3 ± 2
BCG B.M. (day 10)	7 ± 2	2 ± 2	19 ± 10	0
BCG Spl. (day 2)	64 ± 3	31 ± 4	88 ± 6	40 ± 5
BCG Spl. (day 7)	45 ± 5	23 ± 6	60 ± 5	30 ± 8
BCG Spl. (day 10)	30 ± 9	5 ± 3	28 ± 11	0

Twenty million (2×10^7) spleen cells from normal C57BL/6 mice were cultured with 2×10^5 killed P815-Y cells for 4 days. Additional cells were obtained from normal C57BL/6 mice or from C57BL/6 mice treated intravenously with 2×10^7 BCG organisms 2, 7, or 10 days previously. In each case 6×10^6 additional cells were added at the initiation of culture. Cell-mediated immunity that developed in these cultures was assessed with a 4-hr ^{51}Cr release assay and a 48-hr growth inhibition assay with the effector-to-target-cell ratios indicated. Values reported are the mean ± the standard deviation of at least four replicate samples obtained from two or more experiments.
* B.M., bone marrow; Spl., spleen.

BCG. None of these populations was inhibitory when added to already sensitized effector cells (data not shown).

BCG also had a marked influence on the number of cells in both the bone marrow and spleen (Fig. 1). Within 2 days after the administration of BCG, there was a 40% decrease in the number of cells found in the bone marrow, and this decrease was still apparent 16 days after BCG. On the other hand, the number of spleen cells began to increase 7 days after BCG and continued to increase sharply for another 9 days.

In our previous work (9) and that of others (6) the spleen cell population from BCG-treated mice that suppressed *in vitro* immunization was localized to a nylon wool adherent fraction. The suppressive activity of nylon wool-adherent spleen cells from B mice and from B mice treated with BCG is shown in Table 2. Addition of 10^7 adherent cells from B mice caused a significant reduction in the immunization of normal spleen cells. Addition of a similar number of adherent cells from normal spleen had no effect. BCG treatment of B mice further enhanced the suppressive activity within their spleens such that 10^7 adherent cells almost completely prevented the *in vitro* immunization of normal spleen cells.

Table 2. Effect of nylon wool-adherent spleen cells from untreated or BCG-treated B mice on the *in vitro* immunization of spleen cells from normal mice against alloantigen

Additional adherent cells	% specific ^{51}Cr release 100:1	% specific ^{51}Cr release 10:1	% growth inhibition 10:1	% growth inhibition 1:1
None	64 ± 2	37 ± 3	84 ± 6	32 ± 9
Normal speen (1×10^7)	63 ± 2	40 ± 2	80 ± 5	28 ± 7
B spleen (6×10^6)	67 ± 3	45 ± 5	78 ± 4	21 ± 6
B spleen (1×10^7)	51 ± 5	25 ± 6	60 ± 8	6 ± 4
BCG B spleen (6×10^6)	15 ± 3	8 ± 1	30 ± 5	0
BCG B spleen (1×10^7)	7 ± 2	3 ± 1	0	0

Twenty million spleen cells from normal C57BL/6 mice were cultured with 2×10^5 killed P815-Y cells for 4 days. Additional cells were obtained from the spleen of normal mice, B mice, or BCG-treated B mice and added in the number indicated in parentheses. Mice that had been thymectomized, lethally irradiated, and bone marrow-reconstituted were termed B mice. Nylon wool-adherent spleen cells were obtained from BCG-treated B mice 10 days after the intravenous administration of 2×10^7 BCG organisms. Values reported are the mean ± the standard deviation of four replicate samples obtained from two experiments.

DISCUSSION

It is clear from the above data that there are cells resident in normal bone marrow that suppress the generation of cells effective in cell-mediated immunity. Earlier, Singhal *et al.* (15) showed a reduction in the development of IgM plaque-forming cells from the spleen *in vitro*, after addition of normal syngeneic bone marrow cells. It is apparent, too, that BCG increases the suppressive activity of this bone marrow population. The origin of suppressor cells found in the spleen of mice given intravenous BCG is open to conjecture. One possibility supported by the data is that stimulated suppressor cells migrate from the bone marrow and colonize the spleen. The persistent reduction in the number of bone marrow cells and gradual increase in the number of spleen cells suggests such a migration. There is also a rapid stimulation of suppressor cells in the bone marrow, as early as two days after BCG, and an apparent lack of suppressors in the spleen until 7 days after BCG. Moreover, the absence of suppressor activity in the normal spleen and the presence of such activity in spleen of bone marrow-reconstituted B mice lends further credence to the concept that suppression in the spleen stems from colonization with bone marrow cells. On the other hand, it cannot be ruled out that the development of suppressor cells is a direct myelopoietic effect of BCG on stem cells in both bone marrow and spleen, and that these cells re-

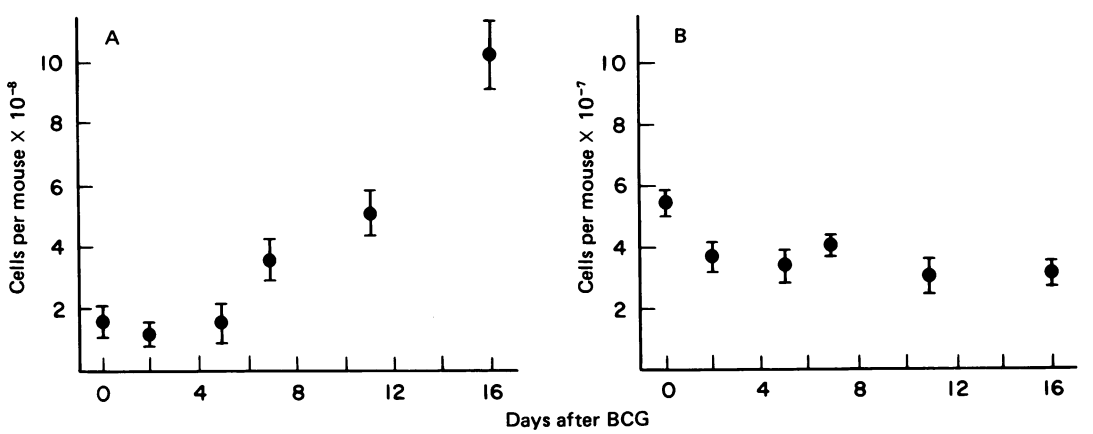

FIG. 1. Changes in cellularity in spleen (*A*) and bone marrow (*B*) after systemic BCG. C57BL/6 mice were injected intravenously with 2×10^7 BCG organisms. At the indicated times bone marrow (two femora and two tibiae) and spleen were excised from each mouse, and the cell number in these tissues was determined. Each value is the mean cell number per mouse ± the standard deviation obtained from at least three mice.

spond with different kinetics. It is clear that, regardless of their origin, BCG-induced suppressor adherent cells can develop independently of the thymus, because B mice also generated such cells.

In summary, suppressor cells that inhibit *in vitro* immunization are present in normal bone marrow. BCG increases the activity of the natural suppressor cells and elicits similar cells in the spleen, perhaps through migration of bone marrow elements. While the overall effect of BCG *in vivo* is usually one of adjuvancy, these results underscore BCG's ability to stimulate intrinsic suppressor elements of immunity as well. It is conceivable that adjuvancy itself may always be the resultant of concomitant stimulation of both helper and suppressor forces.

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