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INDUCTION OF SUPPRESSOR CELLS BY INTRAVENOUS ADMINISTRATION OF BACILLUS CALMETTE-GUÉRIN AND ITS MODULATION BY CYCLOPHOSPHAMIDE*

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Abstract—Intravenous administration of Bacillus Calmette-Guérin (BCG) activated cells in the bone marrow and induced cells in the spleen, which suppressed the development of cell-mediated immunity by T lymphocytes *in vitro*. The activity of these suppressor cells was dependent on both the dose and the schedule of BCG. A course of cyclophosphamide (60 mg/kg/day) given during the 4 days preceding or following the administration of BCG potentiated the induction of suppressor cells by BCG. If the 4-day course of cyclophosphamide was not initiated until 7 days after the administration of BCG, then the induction of suppressor cells by BCG was partially reversed.

Bacillus Calmette-Guérin (BCG) has been used in the treatment of several forms of cancer. The most notable reports of therapeutic responses with BCG have been obtained from its use in the treatment of melanoma [1], Stage 1 lung cancer [2], and perhaps acute lymphocytic leukemia [3, 4]. It is usually employed after the tumor burden has been reduced by surgery, radiation or chemotherapy, and is administered in combination with or subsequent to chemotherapy. The therapeutic action of BCG has been attributed to a generalized activation of the lymphoreticular system with a subsequent mobilization of both macrophages and T lymphocytes against the weakly immunogenic tumor cells [5], a local, non-specific destruction of tumor cells at the site of the granulomatous response induced by BCG [6], and a more specific immunological activation of lymphocytes against tumor cells which have surface antigens in common with those found on BCG organisms [7]. However, the administration of BCG has not been without its toxic effects [8]. Severe delayed hypersensitivity reactions, hepatic dysfunction, malaise, and in a few cases even tumor enhancement have occurred following treatment with BCG. Further complicating the picture are the facts that there are several strains of BCG in use each with its own level of virulence [9] and that the utilization of BCG has not been standardized in terms of dose, route and schedule.

We have reported recently that the intravenous administration of a large dose of BCG results in the induction of adherent suppressor cells that inhibit the primary immunization of T lymphocytes against alloantigen *in vitro* [10, 11]. The induction of these suppressor cells was not influenced by T cells, and the suppressor cells themselves appeared to be immature monocytes. In this paper we present evidence that these suppressor cells originate in the bone marrow. BCG activates these natural suppressor cells in the bone marrow and induces them to circulate into the spleen. We show further that the administration of cyclophosphamide in temporal proximity to BCG potentiates the activation of suppressor cells by BCG.

EXPERIMENTAL

Animals. C57B1/6 (H-2^b) female mice were obtained from the Charles River Laboratory (Wilmington, MA, U.S.A.). Experimental mice were 8- to 12-weeks-old and weighed 19–23 g. Spleen, bone marrow (two femora and two tibiae per mouse) and thymus were excised from normal or treated mice and suspended under sterile conditions as described previously [12]. The cell number in each suspended organ was determined by microscopic enumeration of an appropriately diluted aliquot of the cells in a hemacytometer.

BCG. Mycobacterium bovis, Tice strain, was obtained as a freeze-dried product from the University of Illinois. Each ampule containing $5 \pm 3 \times 10^8$ viable units was reconstituted by adding 1.0 ml of sterile water immediately before use. Except for those experiments reported in Table 1, mice that received BCG were given 2×10^7 viable units intravenously.

Cyclophosphamide. Cyclophosphamide was obtained in powder form from the Mead Johnson Laboratories, Evansville, IN, and was solubilized with sterile water.

Immunization in vitro. The procedures for immunization *in vitro* and quantitation of cell-mediated immunity are depicted in Fig. 1. Spleen cells were immunized *in vitro* according to a modification of the procedure originally described by Mishell and Dutton [13]. Briefly, 2×10^7 viable C57B1/6 spleen cells

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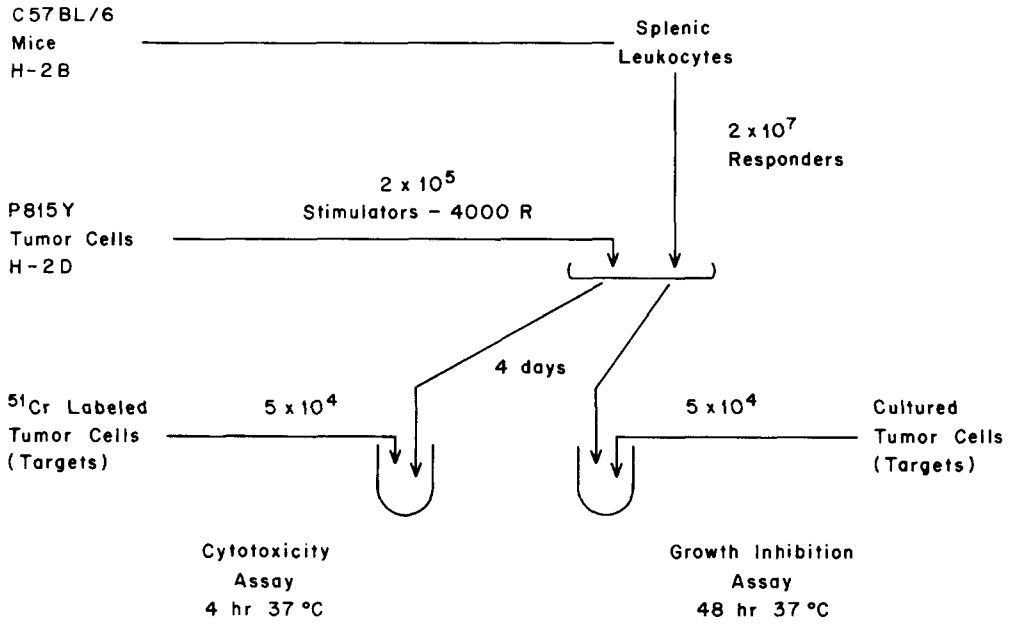

Fig. 1. Methodology for immunization *in vitro* and determination of CMI.

(H-2b) were cultured with 2×10^5 irradiated (4000 rads) P815 mastocytoma cells (H-2d) in 35×10 mm plastic dishes in a total volume of 1 ml. The culture medium was RPMI supplemented with heat-inactivated, dialyzed fetal calf serum (5%), glutamine (2 mM), sodium pyruvate (1 mM), 100× non-essential amino acids (1%), penicillin (100 units/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (5×10^{-5} M). Cultures were incubated for 4 days at 37° in an atmosphere of 10% CO_2 in air, and were fed daily with 0.1 ml of RPMI 1640 medium supplemented as described previously [14]. 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 fresh culture medium.

Assays for cell-mediated Immunity (CMI). CMI against P815-Y target cells was measured using both the 4-hr ^{51}Cr release assay [15] and the 48-hr growth inhibition assay [16]. The per cent ^{51}Cr released and the per cent growth inhibition were calculated in the following ways:

$$(1)\ \%\ ^{51}\text{Cr release} = \frac{\text{cpm in supernatant}}{\text{cpm in pellet} + \text{cpm in supernatant}} \times 100\%$$

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

where T = number of target cells remaining in the presence of test lymphocytes and N = number of target cells remaining in the presence of normal lymphocytes.

Table 1. Effect of i.v. treatment of mice with BCG on the *in vitro* generation of an immune response by their spleen cells*

No. of BCG organisms	Spleen cell no. $\times 10^8$	% Specific ^{51}Cr release	% Specific growth inhibition
Control	1.8 ± 0.3	65 ± 3	80 ± 11
1×10^6	1.6 ± 0.3	66 ± 4	75 ± 9
5×10^6	1.8 ± 0.2	63 ± 4	76 ± 11
1×10^7	3.0 ± 0.5	15 ± 7	26 ± 8
2×10^7	4.1 ± 0.5	5 ± 2	0
5×10^7	Lethal dose		

*C57B1/6 mice were injected intravenously with the indicated doses of BCG. Mice were killed 8 days after treatment. Their spleens were excised and teased into suspension. Twenty million spleen cells from each treatment group were immunized *in vitro* against 2×10^5 P815-Y cells. The CMI of the immunized spleen cells was measured by the ^{51}Cr release assay with an effector to target cell ratio of 100:1 and by the growth inhibition assay with an effector to target ratio of 10:1. Values are mean ± S. D.

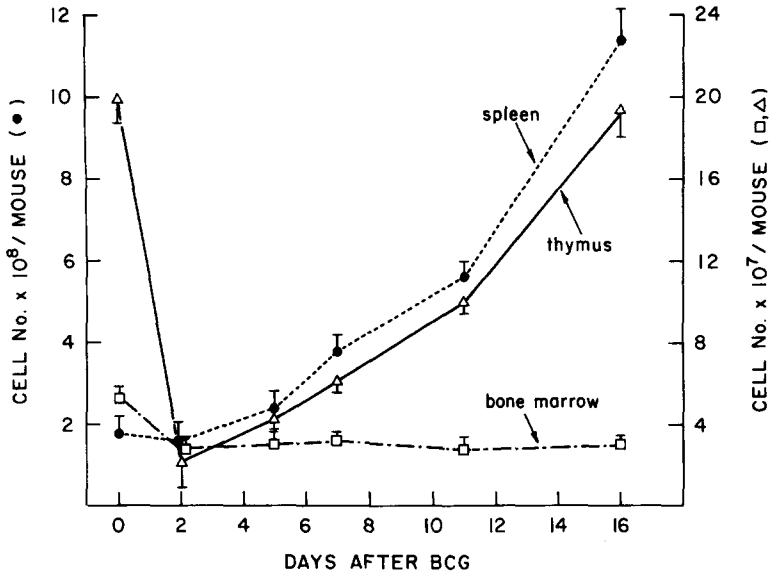

Fig. 2. Effect of BCG on the number of cells in mouse spleen, bone marrow and thymus. C57B1/6 mice were injected intravenously with 2×10^7 viable units of BCG. At various times after treatment at least three identically treated mice were killed, and the spleen, bone marrow (two femora and two tibiae per mouse) and thymus from each were excised and teased into single cell suspension. The cell number in each tissue was determined microscopically, the mean cell number ± the standard deviation in each organ was calculated, and the values are expressed as mean cell number per mouse.

RESULTS

Induction of splenic suppressor cells with increasing doses of BCG. As shown in Table 1, an increase in cell number was found within the spleen of mice that had received BCG intravenously 8 days earlier. The increase was manifested in mice receiving 1×10^7 viable units of BCG and was proportional to the amount of BCG given. The lethal dose was 5×10^7 viable units of BCG. When these cells were immunized against alloantigens *in vitro*, the CMI which developed was reduced markedly compared to that obtained after the immunization of normal spleen cells. In fact, no significant CMI developed after attempts to immunize spleen cells from mice that had received 2×10^7 viable units of BCG, as assayed by both ^{51}Cr release and growth inhibition assays.

Schedule of BCG-induced changes in spleen, bone marrow and thymus. In mice receiving 2×10^7 viable units of BCG, the increase in spleen cell number was not apparent until 7 days after treatment (Fig. 2). This increase continued until 16 days after treatment, at which point it reached its maximum, remaining at approximately this level for another 45 days. In contrast, BCG administration resulted in a marked reduction in cell number in both bone marrow and thymus (Fig. 2). Within 2 days after treatment with BCG, there was a 40 per cent decrease in the number of bone marrow cells and a 90 per cent decrease in the number of thymus cells. The decrease in bone marrow cells persisted and was still apparent 16 days after BCG, whereas in the thymus a gradual recovery in cellularity began 5 days after BCG, but cellularity did not return to normal levels until 16 days after BCG.

Table 2. Effect of spleen, bone marrow and thymus cells from BCG-treated mice on the immunization of normal spleen cells against alloantigen *in vitro**

Days after BCG	% Specific ^{51}Cr release with sensitized spleen cells (65 ± 3); immunized in the presence of:			% Growth inhibition with sensitized spleen cells (81 ± 7); immunized in the presence of:		
	Spleen	Bone marrow	Thymus	Spleen	Bone marrow	Thymus
No BCG	66 ± 2	38 ± 8	62 ± 3	82 ± 8	45 ± 9	74 ± 9
2	64 ± 3	9 ± 4	66 ± 5	88 ± 6	10 ± 5	70 ± 11
7	45 ± 5	5 ± 3	59 ± 4	60 ± 5	8 ± 4	79 ± 8
11	33 ± 6	7 ± 2	67 ± 3	37 ± 11	19 ± 10	85 ± 8
16	31 ± 5	3 ± 2	64 ± 3	31 ± 8	12 ± 4	80 ± 12

*C57B1/6 mice were given 2×10^7 viable units of BCG intravenously. At the indicated times after treatment, spleen, bone marrow and thymus were excised, and single cell suspensions from each tissue were prepared. Six million of these cells were added to 2×10^7 normal C57B1/6 spleen cells, and this mixture was cultured for 4 days with 2×10^5 killed P815-Y cells. At the end of the culture period, the CMI of the sensitized spleen cells was determined using an effector to target cell ratio of 100:1 in the ^{51}Cr release assay and 10:1 in the growth inhibition assay. Values are means ± S. D.

Table 3. Effect of spleen cells from mice treated with BCG or with BCG preceded by cyclophosphamide on the *in vitro* immunization of normal spleen cells against alloantigen*

Additional spleen cells from mice treated with:	Cell no. × 10^8 per mouse spleen	Killed on day	% Specific ^{51}Cr release	% Specific growth inhibition
Control	1.6 ± 0.2	7	65 ± 2	85 ± 5
BCG (day 0)	4.0 ± 0.4	7	47 ± 5	60 ± 5
CTX (days −4 to −1), BCG day 0	3.6 ± 0.5	7	13 ± 1	14 ± 9
CTX (days −4 to −1)	1.5 ± 0.2	7	66 ± 5	89 ± 4
BCG (day 0)	11.0 ± 1.3	14	35 ± 4	41 ± 6
CTX (days −4 to −1), BCG day 0	7.5 ± 0.9	14	26 ± 2	32 ± 3
CTX (days −4 to −1)	1.3 ± 0.1	14	67 ± 1	83 ± 6

*C57B1/6 mice were injected intravenously with 2 × 10^7 viable units of BCG on day 0. On the indicated days, mice were given cyclophosphamide (CTX), 60 mg/kg, i.p. Mice were killed on days 7 or 14, at least two spleens from each treatment group were pooled, and 6 × 10^6 cells from these pools were added to 2 × 10^7 normal spleen cells. This mixture was cultured for 4 days with 2 × 10^5 killed P815-Y cells and the CMI was determined as described. The values were obtained from two experiments. The mean of duplicate samples within each experiment was calculated; the mean ± range of these values is reported.

The suppressive influence of cells in the spleen, bone marrow and thymus was determined by adding them to cultures of normal spleen and immunizing this mixture against P815-Y. As shown in Table 2, significant suppression of CMI 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 normal spleen or normal thymus cells. Treatment of mice with BCG markedly enhanced the suppressive activity in bone marrow and induced suppressive activity in spleen. More than 4-fold augmentation of suppression by bone marrow cells occurred 2 days after administration of BCG, and this increased suppressive activity was still apparent 14 days later. However, suppressive activity by spleen cells was not apparent until 7 days after BCG. Suppression by the spleen was even more pronounced 11 and 16 days after BCG. Although not shown, the suppressor cell activity that was found in the spleens of mice 60 days after treatment with 2 × 10^7 viable units of BCG was approximately the same as that found 16 days after treatment. Unlike bone marrow and spleen, thymus cells from BCG-treated mice did not suppress the immunization of normal spleen cells.

Potentiation by cyclophosphamide (CTX) of the BCG-induced development of splenic suppressor cells. Mice were pretreated with a 4-day course of CTX, 60 mg/kg/day, and then given BCG 24 hr after the last dose of CTX. As shown in Table 3, pretreatment with CTX had no effect on the BCG-induced increase in spleen cell number, when spleens were harvested 7 days after BCG administration. However, CTX pretreatment did result in a 4-fold increase in the suppressor cell activity induced by BCG within these spleens. When spleens were harvested 14 days after BCG, CTX pretreatment reduced the increase in spleen cell number but had only a slight augmentative effect on the suppressor cell activity induced by BCG. Similar results were obtained when mice were treated with a single dose of CTX, 200 mg/kg, 2 days before the administration of BCG.

Mice were also given CTX after BCG and the changes in spleen cellularity and suppressor cell activity were analyzed 14 days after BCG (Table 4). In mice given CTX on days 2–5 or 7–10, the BCG-induced increase in spleen cellularity was reduced by 60 and 70 per cent respectively. Treatment with CTX on days 2–5 after BCG also augmented the BCG-induced increase in splenic suppressor cell activity. However, in mice given CTX on days 7–10 after BCG, the induction of suppressor cells by BCG was partially reduced.

DISCUSSION

Intravenous treatment of mice with BCG resulted in marked changes in spleen, bone marrow and thymus.

Table 4. Effect of spleen cells from mice treated with BCG or with BCG followed by cyclophosphamide on the *in vitro* immunization of normal spleen cells against alloantigen*

Additional spleen cells from mice treated with:	Cell no. × 10^8 per mouse spleen	Killed on day	% Specific ^{51}CR release	% Specific growth inhibition
Control	1.7 ± 0.2	14	63 ± 2	79 ± 5
BCG (day 0)	9.8 ± 0.7	14	32 ± 5	33 ± 8
BCG (day 0), CTX (days 2–5)	3.9 ± 0.4	14	21 ± 2	18 ± 6
BCG (day 0), CTX (days 7–10)	2.9 ± 0.5	14	45 ± 1	36 ± 5

*C57B1/6 mice were injected intravenously with 2 × 10^7 viable units of BCG on day 0. On the indicated days, mice were given cyclophosphamide (CTX), 60 mg/kg, i.p. Mice were killed on day 14, at least two spleens from each treatment group were pooled, and 6 × 10^6 cells from these pools were added to 2 × 10^7 normal spleen cells. This mixture was cultured for 4 days with 2 × 10^5 killed P815-Y cells and the CMI was determined as described. The values were obtained from two experiments. The mean of duplicate samples within each experiment was calculated; the mean ± range of these values is reported.

These changes were dependent on both the dose and the schedule of BCG. The development of splenomegaly and the induction of splenic suppressor cells were directly proportional to the dose of BCG. A marked reduction in the cellularity of both the bone marrow and thymus occurred within 2 days after the administration of BCG, whereas in the spleen an increase in cellularity occurred 7 days after BCG. Similarly, suppressor cells were activated in the bone marrow 2 days after BCG, but they did not develop in the spleen until 7 days after BCG. There was no evidence of suppressor cell activity in the thymus at any time after BCG. Moreover, previous studies by our group have shown that BCG induced the development of suppressor cells in thymus-deprived mice [10]. However, given the marked change in thymus cellularity after treatment with BCG, it is quite likely that the thymus plays a significant role in the mediation of other events elicited by BCG *in vivo*.

Other studies have emphasized the importance of the route of administration of BCG in modulating the development of CMI. Hawrylko [17] and Doft *et al.* [18] have both shown that BCG enhanced T cell activity when it was given subcutaneously, but suppressed this activity when it was given intravenously. Our studies [10] and those of Klimpel and Henney [19] have shown the induction of splenic suppressor cells after the intravenous or intraperitoneal administration of BCG. On the other hand, Braun *et al.* [20] and Davies and Sabbadini [21] have shown that T cell activity was increased after systemic treatment with a high dose of BCG. In each of the above studies a different strain of BCG was used, which suggests that this difference may be of considerable importance in determining the *in vivo* activity of BCG.

The suppressor cells described here originated in the bone marrow. There were "natural suppressor cells" resident in normal bone marrow that suppressed the sensitization of cells effective in CMI. Earlier, Singhal *et al.* [22] showed a reduction in the development of IgM plaque-forming cells from the spleen *in vitro*, after the addition of normal syngeneic bone marrow cells. It is clear from our data that BCG enhanced the suppressive activity of this bone marrow population. The data also suggest that BCG induced this population to migrate from the bone marrow and colonize the spleen. The persistent reduction in the number of bone marrow cells and the gradual increase in the number of spleen cells suggest such a migration. Also, there was a rapid stimulation of suppressor cells in the bone marrow, as early as 2 days after BCG, and an apparent lack of suppressors in the spleen until 7 days after BCG. Furthermore, when CTX was given from days 7 to 10 after BCG, the BCG-induced increase in spleen cell number and the induction of splenic suppressor cell activity were partially inhibited. This agrees further with the idea that, under the influence of BCG, activated suppressor cells from the bone marrow circulate into the spleen and proliferate there, resulting in both splenomegaly and splenic suppressor cells.

CTX potentiated the induction of splenic suppressor cells by BCG when it was given either shortly before or shortly after the administration of BCG. This potentiation was very clear when suppressor cell activity was assayed on day 7, but was less apparent 14 days after BCG. This suggests that the mice were capable of recovering from this potentiation. Suppression of bone marrow function by CTX has been well established [23]. Since the splenic suppressor cells did seem to stem from the bone marrow, it would appear that CTX left the bone marrow in a condition in which suppressor cell activity was more readily stimulated by BCG, perhaps by eliminating "irrelevant" proliferating stem cells. This may also account for the reduction in the BCG-induced splenomegaly seen on day 14 in mice pretreated with CTX. In fact, we have preliminary evidence which indicates that bone marrow cells obtained from mice 1 or 2 days after treatment with 250 mg/kg of CTX are 20 per cent more active than normal bone marrow cells in suppressing the *in vitro* generation of sensitized spleen cells. Further investigations into the mechanism of this potentiation are currently being carried out.

The *in vivo* role of these BCG-induced suppressor cells is not clear at this time. As putative members of the monocyte-macrophage series, they could have been elicited into the circulation to handle the large number of circulating BCG organisms or to modulate other cells which were responding to the BCG stimulus. It is also not easy to predict how these suppressor cells might respond to the presence of tumor cells. While they might well inhibit the development of CMI against a growing tumor and thereby enhance tumor growth, it is entirely possible that by their very nature as macrophages they might non-specifically suppress tumor cell growth. However, it is clear that through the appropriate scheduling of cytotoxic chemotherapy, such as with CTX, the induction of these suppressor cells can be modulated. Although the overall effect of BCG *in vivo* is usually one of adjuvancy, that adjuvancy may always be the resultant of concomitant stimulation of both helper and suppressor forces. As we develop a better understanding of the role of these helper and suppressor forces, the direction in which to modulate these forces will become easier to determine and the therapeutic potential of BCG will become easier to harness.

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