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Mechanism of *Bacillus Calmette-Guérin*-induced Suppression of Metastases in a Poorly Immunogenic Fibrosarcoma[1]

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SUMMARY

Bacillus Calmette-Guérin (BCG) reduces the rate of spontaneous pulmonary metastases from a poorly immunogenic fibrosarcoma. To be effective, BCG (1×10^6 organisms) must be given in admixture with (1×10^6) tumor cells at the time of transplantation. Reduction of metastasis at this dosage of BCG occurs without a change in the size of the primary tumor or the extent of necrosis within it. Tumors transplanted in admixture with spleen cells from BCG-exposed donors reduced the number of metastases, while spleen cells from normal or tumor-bearing donors had no effect on metastases. Fewer total tumor cells and clumps are collected from the venous effluent of tumors transplanted with BCG than from control tumors. The BCG-treated tumors have more host macrophages intimately associated with the effluent tumor cells then did controls. These data indicate that BCG can inhibit the metastatic potential of a weakly immunogenic fibrosarcoma. The mechanism of this effect appears to be a depression of entry of tumor cells into the tumor vascular channels which may be related to the interaction of tumor cells with BCG-stimulated macrophages.

INTRODUCTION

It has been well established that the host immune system can modify the course of the metastatic process (6, 9, 10, 14, 18). Since immune defenses are, in general, effective against a limited number of tumor cells (2, 3, 20, 22), immunotherapy of the metastatic process would seem most successful against tumor cells entering the circulation or against small metastatic foci. Previous studies on the immunotherapy of metastases have been devoted to the mechanism of inhibition of metastasis following i.v. injection of tumor cells in conjunction with bacterial adjuvants (1, 19). However, little information is available on the effect of immunotherapy on the spontaneous metastatic process, in particular, the rate of entry of tumor cells into the vascular channels of the primary tumor. Tumor immunogenicity is a major determinant of the efficacy of immunotherapy (3, 21, 22). Poorly immunogenic transplanted tumor systems have been shown to be more metastatic than are highly immunogenic systems (6, 7, 15, 21). This study concerns the effect of BCG[3] treatment on the metastatic process from a poorly immunogenic transplanted fibrosarcoma. The development of spontaneous metastases was studied after BCG was administered at a variety of sites and times. To verify that an immune mechanism was involved, BCG treatments were combined with or substituted for by spleen cells from normal tumor-bearing or BCG-exposed donors. A major objective of this study was to evaluate the mechanism of inhibition of metastasis in this system. This was accomplished by quantitating the tumor cells and associated host macrophages in the tumor venous effluent following BCG treatment.

MATERIALS AND METHODS

Tumor-Host System. The tumor-host system, C57BL/6J mouse and syngeneic T241 fibrosarcoma, is poorly immunogenic and highly metastatic (9, 16). The origin of this tumor has been described previously (24). Lack of a substantial immunogenicity was defined by: (*a*) failure of the host to retard the growth of as little as 2×10^3 tumor cells 12 days after amputation of a primary tumor. The growth rate of tumors transplanted at graded doses of 2×10^3 to 1×10^5 tumor cells was the same in recipients following tumor amputation as it was in normal mice; (*b*) failure of glutaraldehyde-fixed tumor cells [method of Frost and Sanderson (11)] injected i.p. to retard the growth or metastasis of tumors originating from subsequent transplantations; (*c*) failure of the host to retard concomitant tumor transplants of as little as 2×10^3 cells.

Mycobacteria. BCG No. 8 (Phipps TMC No. 1029) was obtained from the Trudeau Mycobacterial Collection as frozen suspension. Inocula were diluted in 0.9% NaCl solution after thawing, and viable organisms were counted by serial dilutions on Middlebrook's medium at 37° with a CO_2-enriched environment (8).

Spleen Cells. Nonsensitized spleen cells were obtained by pooling excised spleen from a group of noninfected, non-tumor-bearing C57BL/6J mice, and sensitized spleen cells were obtained from a group of mice 24 days after they received the i.m. transplant. A 3rd population of spleen cells was obtained from mice given an i.m. challenge of 1×10^6 viable BCG organisms i.m. 14 days previously. Spleen mince suspended in 0.9% NaCl solution was filtered through a metal sieve with 0.1-mm holes to eliminate larger cell aggregates.

Experimental Groups. As described previously (16) tumor

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cells were transplanted in an i.m. femoral site. In Series A, mice received inoculations of 10^6 tumor cells simultaneously admixed with 10^6 viable BCG organisms suspended in 0.2 ml of 0.9% NaCl solution. In Series B, 0.2-ml suspensions of BCG (10^6 viable organisms) were injected into established tumors (*a*) on Day 4 and (*b*) on Days 4 and 12 following tumor implantation. In Series C, BCG i.m. injections outside the tumor, either at a proximal femoral site or in the contralateral limb, were given at the time of tumor implantation. Additional experiments (Series D) involved inoculations of combinations of 10^7 spleen cells, either from tumor-bearing, BCG-exposed, or normal donors, and admixed with 10^6 tumor cells in a 0.4-ml suspension with or without 10^6 BCG organisms. None of these groups of mice had previously received BCG.

Methods of Assay. The effects of treatment were evaluated by counting the number of pulmonary metastases with lobe transillumination usually 19 days after implantation and in some cases 23 or 26 days after. All lungs were coded and randomized to avoid bias prior to counting. As described previously (16, 17) the lungs were inflated with neutral buffered formalin and allowed to fix and clear for at least 10 days before examination. Individual lobes were then transilluminated under a stereomicroscope, and macroscopic metastases were sized and counted. Macroscopic metastases as small as 0.3 mm within the parenchyma or on the surface are distinguished by this method and verified histologically. Sections of all lobes were stained with hematoxylin and eosin and studied to confirm that all observed gross lesions were composed microscopically of tumor cell deposits. In addition a separate evaluation was made of the number of microscopic metastatic deposits per unit area of lung section. At the time of sacrifice, size was quantified by multiple-axis measurements on the bisection of the excised tumor. Tumor bisections were quantitated by point count for the proportion of tumor that was necrotic (23). Collections of the tumor venous effluent 12 days after implant were made to study the concentrations of tumor effluent cells, cell clumps, and host leukocytes as well as the proportion of effluent tumor cells in contact with the leukocytes. As described previously (16, 17) the tumor vascular bed was perfused at physiological pressure, and the venous effluent is immediately passed through a Nuclepore filter. Examination of the stained filter reveals tumor cells in single and clump form and host mononuclear leukocytes free or attached to tumor cells. Tumor cells are identified by applying quantitative morphological criteria (16).

Statistical Analysis. The null hypothesis of no significant difference between the means of 2 given populations was evaluated by the Student *t* test for compared populations with equal variances (verified by the *F* test). Where variances could not be assumed to be equal, separate confidence intervals were computed for each population and the null hypothesis was tested by checking for overlap of the confidence intervals (5).

RESULTS

BCG decreases the number of spontaneous pulmonary metastases formed from transplanted T241 fibrosarcomas when administered in admixture with the tumor at the time of transplantation (Table 1). The decrease in the number of metastases observed in the BCG-treated group on Day 19 after implantation is statistically significant. This inhibition occurred without change in size of the primary tumor or amount of necrosis (Table 1). This inhibition based on quantitation of pulmonary metastases by macroscopic observation has been confirmed by microscopic study. Microscopic examination of all lungs showed more than a 3-fold reduction in the mean number of small metastatic deposits (0.05 sq mm or less) per unit area of lung lobe in BCG-treated mice as compared to controls. However, no inflammatory cell reaction was noted around pulmonary metastases from BCG-treated tumors. When metastases were assayed in another experiment at a later time (Day 23) following BCG administration admixed with tumor, the inhibition was still present and statistically significant (Table 2).

On microscopic examination the untreated tumor consisted of solid masses of anaplastic pleomorphic cells. They were round to oval and varied in size from 10 to 36 μm in diameter. The round to oval nuclei show prominent nucleoli and 4 to 5 mitoses per high-power field. Many nuclei are hyperchromatic. The nuclear to cell area ratio varied from 0.4 to 0.7. Connective tissue stroma is sparse. Numerous thin-walled vascular structures are present throughout the tumor with the greatest vascular density existing at the tumor periphery. Necrosis, first seen after 8 days, is patchy and sparse at earlier times (8 to 12 days) but becomes more extensive and centrally located in older tumors (15 to 26 days). The tumor border shows invasion of muscle with degenerating muscle fibers but no connective tissue capsule. An acute inflammatory reaction could be found adjacent to areas of necrosis. Areas of mononuclear cell infiltrate were rare in untreated tumors and were located chiefly at the tumor periphery.

Table 1

Intratumor BCG inoculation, primary implant, and metastases

Effects of 1×10^6 BCG organisms admixed with 1×10^6 tumor cells at the time of transplantation on the number of spontaneous pulmonary metastases.

Treatment	*n*	Primary diameter (mm)	Proportion of necrosis in primary (%)	Lung metastases, 19th day
Intratumor BCG	12	15.4 ± 0.70[a,b]	13.0 ± 1.0[b]	3.01 ± 0.81[b]
0.9% NaCl solution control	12	15.3 ± 0.80	15.0 ± 2.0	5.60 ± 0.52

[a] Mean ± S.E. Not significant.
[b] $p < 0.02$.

The BCG-treated tumors showed an increased mononuclear cell infiltrate in the tumor periphery, but no granulomas are seen and no granulomatous encapsulation is present. Otherwise, the cellular pattern, extent of necrosis, and stromal character over the time interval of 7 to 30 days were not noticeably different between the treated and untreated tumors.

When BCG is inoculated into tumors 4 days after implantation, no inhibition of pulmonary metastases is observed (Table 3). BCG inoculated into the tumor mass on Days 4 and 12 after implantation actually enhanced the number of metastases that appear as compared with noninjected controls (Table 3). This enhancement is no greater than that produced by 0.9% NaCl solution injections alone. We have previously reported the enhancement of metastases produced by the trauma of 0.9% NaCl solution injections in the T241 primary tumor (17).

Extratumor inoculation of BCG into sites adjacent to the tumor in the ipsilateral limb muscle or the contralateral limb at the time of implantation failed to produce inhibition of metastasis when compared to appropriate control groups (Table 4). These data indicate the necessity for intimate contact between BCG organisms and tumor cells at the time of tumor implantation in order to produce an inhibition of metastasis formation.

The administration of spleen cells from tumor-bearing and control hosts admixed with tumor cells at the time of primary implantation into recipients did not affect the metastatic potential of the tumor implant (Table 5). However, when BCG was admixed with both "sensitized" (tumor-bearing donors) or normal spleen cells at the time of tumor cell implantation, a significant decrease in the number of pulmonary metastases was found on Day 19 (Table 5). The fewest numbers of metastases were observed when BCG and "sensitized" spleen cells were used together at the time of tumor implantation. This value is not significantly different from that observed when BCG and normal spleen cells were inoculated or for BCG treatment alone.

The administration of spleen cells from BCG-exposed donors admixed with tumor cells with or without BCG did reduce the number of metastases (Table 6) significantly when compared to untreated controls or tumors treated with spleen cells from tumor-bearing donors. The greatest inhibition of metastasis was seen when the tumor was transplanted with BCG and spleen cells from BCG-exposed donors. This was significantly greater than the inhibition resulting from treatment with transplantation of spleen cells from BCG donors alone (Table 6).

Table 2

Intratumor BCG metastases (Day 23)

Treatment is the same as in Table 1. Metastases are assayed at a later date.

Treatment	*n*	Metastases
BCG	8	4.87 ± 1.39[a,b]
3.9% NaCl solution control	6	12.50 ± 2.07

[a] Mean ± S.E.
[b] $p < 0.01$.

Table 3

Intratumor BCG on Days 4 and 12 and metastases

Effects of 1 × 10^6 BCG organisms or 0.9% NaCl solution injected into the established tumor mass on Days 4 and 12 on the number of pulmonary metastases.

Treatment	*n*	Assay day	Metastases
BCG, Day 4	8	19	4.37 ± 0.88[a,b]
0.9% NaCl solution control, Day 4	8	19	5.12 ± 1.02
BCG, Days 4 and 12	8	26	42.16 ± 8.29[b]
0.9% NaCl solution control, Days 4 and 12	8	26	39.85 ± 7.26
No treatment	5	26	20.20 ± 3.04

[a] Mean ± S.E.
[b] Not significant compared to 0.9% NaCl solution controls.

Table 4

Extratumor BCG and metastases (Day 19)

Effects of 1 × 10^6 BCG organisms or 0.9% NaCl solution injected into extratumor sites at the time of transplantation on the number of pulmonary metastases.

Treatment	*n*	Metastases
BCG, ipsilateral	8	3.66 ± 0.74[a,b]
0.9% NaCl solution, ipsilateral	8	3.80 ± 0.76
BCG, contralateral	8	3.91 ± 0.75[b]
0.9% NaCl solution, contralateral	8	5.25 ± 1.19

[a] Mean ± S.E.
[b] No significant differences are noted between treated and 0.9% NaCl solution groups.

Table 5

Intratumor BCG, spleen cells, and metastases

Effects of 1 × 10^6 BCG organisms, spleen cells from tumor-bearing donors ("sensitized") or spleen cells from normal donors (nonsensitized) admixed with the tumor cells at the time of transplantation on the number of pulmonary metastases.

Group	Treatment	*n*	Metastases on Day 19
1	BCG + "senstitized" spleen cells	10	2.10 ± 0.43[a,b]
2	"Sensitized" spleen cells only	10	5.55 ± 1.14
3	BCG + nonsensitized spleen cells	10	3.44 ± 0.60[b]
4	Nonsensitized spleen cells	10	4.62 ± 0.80
5	0.9% NaCl solution control	12	5.60 ± 0.52

[a] Mean ± S.E.
[b] Significantly different from Group 5 ($p < 0.05$). No significant difference ($p > 0.05$) between groups 1 and 3 or between groups 2 and 4.

Table 6

Spleen cell and metastases

Effects of 1 × 10^6 BCG organisms, spleen cells from tumor-bearing donors ("sensitized"), and spleen cells from BCG-exposed donors (BCG spleen cells), admixed with the tumor cells at the time of transplantation on the number of pulmonary metastases.

Group	Treatment	*n*	Metastases on Day 26
A	Control	8	39.5 ± 2.8[a]
B	Tumor "sensitized"	8	31.5 ± 2.4
C	BCG spleen cells	8	18.8 ± 2.5[b]
D	BCG spleen cells + BCG	10	9.7 ± 3.3[b,c]

[a] Mean ± S.E.
[b] Significantly different from Group A; $p < 0.01$.
[c] Significantly different from Group C; $p < 0.05$.

Study of the cellular population characteristics in the tumor venous effluent of the BCG and control mice demonstrated several factors possibly responsible for the decrease in metastases in the BCG-treated group. As shown in Table 6, significantly less tumor cells were present in the venous effluent of the BCG-treated group than in that of controls. Furthermore, a significantly smaller proportion of these tumor cells were in clump form. The decreased number of total tumor cell clumps was associated with no difference in the number, density, or size distribution of perfused tumor vessels [measured by methods described previously (16)] observed microscopically between treated and control tumors; [6.3 ± 2.6 (S.D.) vessels/sq mm untreated *versus* 5.9 ± 2.8 vessels/sq mm BCG-treated]. Large quantities of what appeared to be macrophages (approximately 100 cells/ml) were consistently seen in the venous effluent of BCG - treated tumors, but they were rare in the effluent of control tumors (approximately 5 cells/ml). The proportion of effluent tumor cells found in intimate contact with mononuclear host cells was significantly greater from BCG-treated tumors (Table 7). In ancillary experiments the tumor venous effluent was incubated with latex particles. Most (80 to 90%) of the mononuclear cells attached to tumor cells had the morphological appearance of macrophages and could phagocytize latex particles. Usually, more than 1 macrophage was found clustered around single tumor cells.

DISCUSSION

The results of this study indicate that BCG immunotherapy can decrease the number of spontaneous metastatic lesions from a weakly immunogenic fibrosarcoma without affecting the size and growth of the primary implant or the extent of necrosis in the primary. It is possible that a minimal tumor immunogenicity is required for BCG-induced tumor regression. In this system appropriate BCG therapy significantly reduces the number of tumor cells and clumps that escape from the primary tumor into the vascular space. The finding that host immune responses can modify the rate of hematogenous tumor cell liberation supplements the work of James and Salsbury (14). These investigators found an increased concentration of tumor cells in the blood of mice harboring the poorly immunogenic Lewis lung carcinoma after treatment with antithymocyte globulin. This form of immune suppression was associated with an increased rate of formation of metastasis with no alteration in the size of the primary tumor. In our studies an augmentation of the immune response by BCG produced a decrease in metastases without affecting the size of the implanted primary. The result is consistent with the observations of James and Salsbury, assuming that the number of metastases varies in an inverse manner with the immune response. The absence of any effect on the primary tumor in this poorly immunogenic system may reflect a resistant state of these cells to the effects of BCG treatment. The reasons are not understood, but they may result from several causes. The qualities of the tumor cells released intravascularly may differ from tumor cells in the primary mass and thus respond differently to the immunological processes. Alternatively, the tumor cells shed into the vascular space are fewer in number and may be more susceptible to interaction with macrophages, lymphocytes, or humoral immune defenses than are tumor cells within the primary tumor mass. Our data do not offer any means of evaluating the importance of either of these potential mechanisms.

Table 7

BCG and tumor venous effluent

Effects of 1×10^6 BCG organisms transplanted in admixture with the tumor cells on the (*a*) concentration of venous effluent tumor cells, (*b*) percentage of effluent tumor cells in clump form, and (*c*) proportion of tumor cells in intimate contact with macrophages.

Treatment	*n*	Tumor cells/ml	Tumor cell clumps	Associated macrophages
BCG	6	16.0 ± 5.9[a, b]	13.0 ± 7.0[b]	0.18 ± 0.05[b]
Control	5	38.0 ± 6.1	43.0 ± 4.0	0.04 ± 0.02

[a] Mean ± S.E.

[b] Significantly different; $p < 0.05$.

BCG must come into intimate contact with the tumor cells at the time of implantation to produce any effect on metastases. When administered at other sites and times, BCG produces no inhibitory effect. The BCG therapy is effective only at the time of implantation, possibly because the time necessary for the mobilization of immune cells is proportionately much greater than the time necessary for tumor growth and the development of metastases. If the growth and dissemination of the primary tumor are rapid during the immunoresponsive interval, the immunological defenses may be inadequate to deal with the expanded tumor burden. Our previous studies have shown that the tumor used in our studies exhibits an exponential increase in the rate of dissemination over the 5- to 12-day interval postimplant (16). BCG injected within the tumor at this time fails to reduce metastatic potential. The results of spleen cell admixture with the tumor cells at the time of transplantation provide some information on the existence and specificity of the immune reaction involved. The presence of an immune mechanism was verified by the reduction of metastases from tumor transplanted with spleen cells from BCG-exposed donors. The absence of any effect from normal spleen cells or spleen cells from tumor-bearing animals (admixed with the tumor cells with or without BCG) compared to appropriate controls argues in favor of a tumor nonspecific immune reaction similar to that reported by Bernstein *et al.* (4), Bartlett *et al.* (2), and Zbar *et al.* (27). Their investigations show that intratumor BCG or lymphocytes from mycobacteria-exposed donors can induce the local accumulation of macrophages. These macrophages can cause tumor rejection without requiring the specific recognition of tumor antigens.

In the present experiments the decrease in effluent tumor cells and clumps following BCG treatment was associated with a decrease in metastases. The mechanism of this effect may be related to the macrophages found in the effluent. The work of Hibbs (13) and others (8, 12, 26) has shown that BCG-activated macrophages can be cytotoxic or cytostatic for tumor cells. Wood and Gillespie (25) have shown that fibrosarcomas possessing a greater macrophage content were less metastatic. They dissociated cells in the excised primary tumor and identified macrophages by their ability to

adhere to glass. We have taken these findings a step further and quantitatively studied the *in vivo* macrophages associated with metastasizing tumor cells. The results suggest an inhibitory interaction of BCG-activated macrophages with tumor cells entering the vascular space.

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