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The Effect of *Mycobacterium bovis (Bacillus Calmette-Guérin)* on Macrophage Random Migration, Chemotaxis, and Pinocytosis[1]

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SUMMARY

Macrophage random migration, chemotactic responsiveness, and pinocytosis were studied in the guinea pig after *Bacillus Calmette-Guérin* (BCG) administration. Oil-induced peritoneal exudate cells were evaluated for random migration and chemotaxis in modified Boyden chambers. Pinocytosis was measured by the *in vitro* uptake of colloidal ^{198}Au. There was a parallel increase in random migration and chemotaxis after an i.p. dose of 1.0×10^7 colony-forming units of BCG. This was seen as early as Day 4, sustained through Day 21, and absent by Day 44. A number of different strains and preparations of BCG vaccine exerted a similar augmentation of macrophage mobility. In parallel assays, aliquots of macrophages from BCG-treated animals showed increased pinocytosis, first noted at Day 14, peaking at Day 28, and no longer measurable at Day 44. These alterations in macrophage function following BCG administration may in part contribute to the antitumor effect of BCG adjuvant therapy.

INTRODUCTION

Nonspecific stimulation of the immune response following BCG[2] administration is manifested by heightened reticuloendothelial system activity (4), increased resistance to bacterial infection (10, 16), greater survival following irradiation (4), accelerated graft rejection (3), inhibition of tumor transplants (12, 27), and a regressive effect on established neoplasms (23, 35, 36). Many of these effects may be the consequence of macrophage activation following BCG infection.

In the present study, 3 sensitive parameters of macrophage activation, *i.e.*, random migration, chemotaxis, and pinocytosis of colloidal ^{198}Au, were studied in an effort to examine the effects of *in vivo* BCG administration on guinea pig macrophage function.

MATERIALS AND METHODS

Animals. Female Strain II guinea pigs, 500 to 700 g, were obtained from the NIH Animal Breeding Section and fed food and water *ad libitum*.

BCG Vaccines. The Phipps, Brazil, and Japan strains of *Mycobacterium bovis* (obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, N. Y.) were prepared as previously described (30), counted (28), and stored at −70°. A specially prepared BCG vaccine with extremely high viability, prepared from the Tice strain (TMC 1032) by the Trudeau Institute, was kindly supplied by Dr. G. Kubica and Dr. B. Zbar. For inoculation, the BCG vaccine was thawed quickly in a 37° bath, diluted with buffered PBS, pH 7.2 and injected i.p. in a volume of 1.0 ml. The vaccine was heat inactivated by boiling at 100° for 60 min.

Macrophage Chemotaxis. An adaptation of the techniques of Boyden (6) with modifications (2, 5, 30, 32) was used. Acrylic blind-well chemotaxis chambers were obtained from Neuroprobe Co., Bethesda, Md. These cylindrical wells, 8 mm in diameter and 4.7 mm deep, contain 0.20 ml of chemotactic agent. Polycarbonate filters (5 Å pore size) were obtained from Nucleopore Inc., Pleasanton Calif. PEC that had been induced by injection of 35 ml of light mineral oil (Marcol 52; Humble Oil Co., Houston, Texas) 4 days previously were prepared by the method described by Altman (2) and adjusted to 5×10^6 mononuclear cells per ml in Gey's balanced salt solution with 2% bovine serum albumin. Guinea pig C5a, a positive chemotactic agent, was prepared by the method of Snyderman *et al.* (33) using Lipopolysaccharide W. *Salmonella typhosa* 0901.

Preparation of Chambers. The lower well was filled with either Gey's balanced salt solution or C5a as described (2). The upper well was filled with 0.4 ml of the PEC mixture. All incubations were in a moist 37°, 5% CO_2 incubator for 90 min. The filters were fixed for 15 sec in methanol and stained with Hemal stain (Hemal Stain Co., Danbury, Conn.). All macrophages that migrated completely through the filter onto its distal surface were counted under oil immersion by means of a microgrid eye piece (American Optical Co., Buffalo, N. Y.). Ten microgrid fields in each of 2 perpendicular diameters across the length of each filter were counted. Results were adjusted for the percentage of macrophages in the starting mixture. All values comprise the arithmetic mean of 3 replicate samples.

Macrophage Colloidal ^{198}Au Uptake. A modification of

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[2] The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; PEC, peritoneal exudate cells; RPMI, Roswell Park Memorial Institute; CFU, colony-forming units; PBS, phosphate-buffered 0.85% NaCl solution, pH 7.2.

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the techniques of Davies *et al.* (9) and Meade *et al.* (19) was used. Radioactive ^{198}Au with a specific activity of 3.5 mCi/mg and a particle size of 5 to 20 nm was obtained from Amersham/Searle Co. (Arlington Heights, Ill.). A working gold solution was prepared by diluting the stock gold to 1:50 with RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.).

Samples of PEC were washed twice in RPMI 1640 and adjusted for the number of macrophages. Incubation cultures were prepared by adding 0.1 ml of the working gold solution to 5×10^6 macrophages in 1.0-ml volumes of incubation media [RPMI 1640, supplemented with 5% heat-inactivated fetal calf serum, streptomycin (50 mg/ml), penicillin (50 units/ml), and glutamine (2 mM)]. Incubations were performed in 12- × 75-mm polypropylene test tubes (Falcon Plastics, Oxnard, Calif.) and rocked gently (20/min) at 37° in 5% moist CO_2 for varying periods of time. Control samples for each time period, consisting of 0.1 ml of the working gold colloid solution without additional cells, were incubated simultaneously. At the end of each incubation period, samples were harvested by vigorously resuspending the cell-gold mixtures in 3.0 ml cold PBS and centrifuging at $400 \times g$ for 10 min. Supernatants were aspirated, and this wash procedure was repeated 3 times. Cell pellets, in the original incubation tubes, were counted in a γ counter (Model 1185; Searle Analytic Inc., Silver Spring, Md.) for 1 min. Unwashed control samples were counted simultaneously to determine the total radioactive gold present. All samples were performed in quadruplicate.

An arithmetic mean value for the total cpm of each group of replicates was obtained. Results are also described in terms of differences in the percentage of colloidal gold taken up by the different sample groups. This latter value was obtained by expressing the total cpm of the sample groups as a percentage of the total cpm of colloidal ^{198}Au added.

RESULTS

In the following experiments, 2 parameters of macrophage movement were assessed, random migration and chemotaxis. Random migration, sometimes referred to as spontaneous motility, was measured in the absence of a chemotactic gradient (media alone). Chemotaxis, or the response of cells to a chemotactic gradient, was measured with C5a as a chemotactic stimulant.

Changes in Macrophage Random Migration following BCG Administration

To determine the optimal dose of BCG, the following experiment was performed. Groups of guinea pigs received either 1.0×10^7, 10^5, or 10^3 CFU of Phipps BCG or 1.0 ml 0.85% NaCl solution i.p. and were sacrificed at Day 16 or Day 44. The results of the random migration assay for Day 16 (Chart 1) show that migration with 10^7 CFU BCG results in an approximate doubling of the spontaneous motility of the macrcphages of the treated animals. Lower immunizing doses of BCG were less effective in altering the random migration of the macrophage.

Duration of Increased Random Migration following BCG Administration

Groups of animals received 10^7 CFU of Phipps BCG and several guinea pigs from each group were sacrificed on Day 4, 7, 14, 21, 28, or 44. Random migration of macrophages from the BCG-treated animals was markedly increased by Day 4. This effect was sustained for at least 3 weeks but had markedly dissipated by Day 28 (Chart 2).

Changes in Macrophage Chemotaxis following BCG Administration

Duration of Action. Macrophages were obtained at various intervals from guinea pigs that received 1.0×10^7 Phipps BCG. Chemotaxis was measured on Days 4, 7, 14, 21, 28, and 44. As can be seen in Chart 2, a definite increase in chemotactic responsiveness was seen by Day 4, sustained through Day 21, but was absent by Day 44. Although this increase paralleled the observed increase in random migration, the increment for chemotaxis was uniformly greater.

Effect of Incubation of Macrophages *in Vitro* with BCG. Normal guinea pig PEC were incubated with either 1.0×10^6 washed Phipps BCG (spun at $7000 \times g$ 3 times with PBS) or

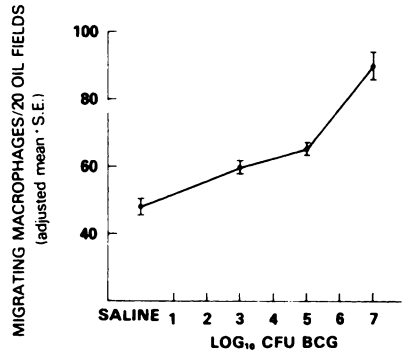

Chart 1. Dose response effect of BCG on macrophage random migration. *Saline*, 0.85% NaCl solution.

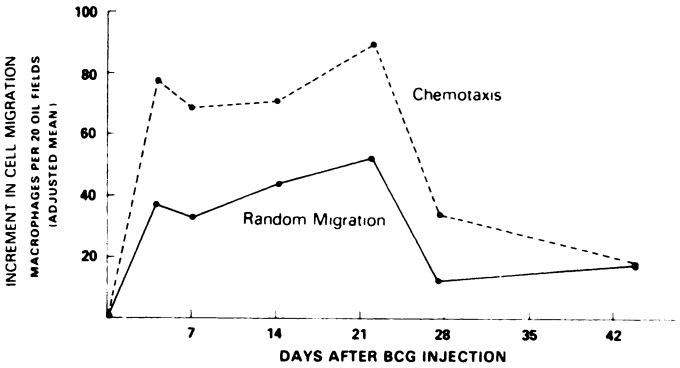

Chart 2. Time course effect of BCG on macrophage random migration and chemotaxis. *Points*, increment of random migration or chemotaxis compared to the mean control value for each day.

1.0×10^6 CFU of BCG diluted from the stock suspension as usual. Incubations were performed at 10 min, 60 min, or 24 hr at 37°. No increases in chemotactic responsiveness or random migration were seen following incubation with either preparation at the 3 time intervals tested.

The Effect of Different Strains and Preparations of BCG Vaccine on Random Migration and Chemotaxis

To determine whether different strains or preparations of BCG produce different immunostimulatory effects in these systems, equal doses (10^7 CFU) of the Phipps, Brazil, and Japan strains of BCG and a "high viable" preparation (>90%) of the Tice vaccine (21) was administered i.p. In addition, the Brazil BCG was heat inactivated and similarly administered. Results are summarized in Table 1. All vaccines increased both random motility and chemotactic responsiveness by Day 16. This effect was almost gone by Day 28. No significant differences among any of the vaccines or preparations were detected.

Changes in Uptake of Colloidal ^{198}Au following BCG Administration

Kinetics. Aliquots of macrophages from the previously described random migration and chemotaxis experiments were assayed for uptake of colloidal ^{198}Au *in vitro*. A comparison in the kinetics of colloidal ^{198}Au uptake between BCG and control macrophages was performed. Results for Day 14 (Chart 3) show that BCG administration caused increased uptake at 1, 5, and 24 hr. The greatest differences were seen after 24 hr of incubation.

Time Course. Macrophages, obtained at the intervals described for the time course chemotaxis study, were assayed for their ability to pinocytose colloidal ^{198}Au. Cells from both the 0.85% NaCl solution- and BCG-treated animals were incubated with colloidal gold and harvested at 24 hr. Macrophages from the BCG injection-treated guinea pigs manifested an increase in colloidal gold uptake when compared to controls (Chart 4). This increase was first measured at Day 14 and peaked at Day 28. Augmentation was no longer measurable at Day 44.

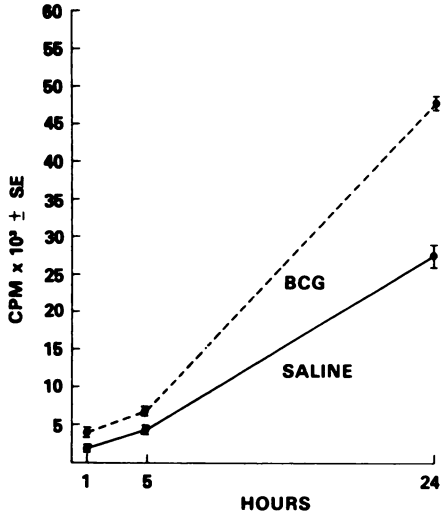

Chart 3. Comparison in kinetics of colloidal ^{198}Au uptake by macrophages from guinea pigs given BCG and control guinea pigs given injections of 0.85% NaCl solution (*saline*). Results are those of animals given injections 14 days earlier.

Effect of Incubation of Macrophages *in Vitro* with BCG. Macrophages preincubated with either BCG or washed BCG, as described previously, showed no increase in colloidal ^{198}Au uptake following a 24-hr period.

DISCUSSION

BCG has been utilized extensively in experimental and, more recently, clinical trials of cancer immunotherapy. Although the precise mechanisms by which BCG treatment results in tumor regression are not completely understood, considerable evidence suggests that the effect of BCG on host macrophages may be a primary factor. Animals given BCG have increased resistance to *in vivo* challenge, both

Table 1

Effect of various preparations and strains of BGG on macrophage random migration and chemotactic responsiveness

Guinea pigs given injections of	Experiment	Day 14 Random migration (media)	Day 14 % increment	Day 14 Chemotaxis (C5a) %		Day 28 Random migration media	Day 28 % increment	Day 28 Chemotaxis (C5a) %	
Control	1	43 ± 3[a]		87 ± 4		ND[b]			
0.85% NaCl solution	2	59 ± 2		111 ± 3		60 ± 4		105 ± 4	
Brazil BCG	1	64 ± 2	49.7	135 ± 5	54.4	67 ± 3	11.3	104 ± 4	(0)
	2	96 ± 3	61.4	174 ± 3	57.0				
Brazil BCG	1	73 ± 2	68.8	142 ± 3	63.0	58 ± 2	0	123 ± 3	(17.8)
Heat killed	2	107 ± 3	80.1	187 ± 3	68.8				
Japan BCG	1	72 ± 3	70.0	188 ± 3	80.8	56 ± 3	0	112 ± 3	(7.5)
	2	100 ± 3	68.6	153 ± 3	40.0				
Tice BCG	1	65 ± 4	51.9	136 ± 2	55.6	63 ± 3	4.8	117 ± 2	(11.7)
No. 1032	2	100 ± 2	69.3	182 ± 4	64.3				

[a] Mean ± S.E.
[b] ND, not done.

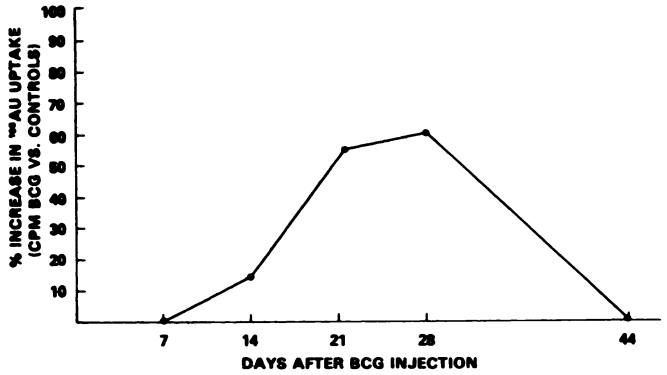

Chart 4. Time course effect of BCG on macrophage colloidal ^{198}Au uptake. *Points,* percentage increase in colloidal ^{198}Au uptake compared to control values for each day.

with certain tumor cell types and with facultative intracellular microorganisms like *Listeria monocytogenes* (12, 17, 26, 27). In addition, isolated macrophages from these animals have enhanced tumoricidal and bacterial activity *in vitro* (1, 31). Studies have also demonstrated that BCG-activated macrophages have enhanced ability to collaborate with immunocompetent cells in the production of specific antibody, as well as possessing increased concentrations of lysosomal enzymes, increased respiratory and glycolytic activity, and augmented phagocytosis (8, 11, 24, 25). The present report provides evidence for 3 additional functional changes in BCG-activated macrophages; increased random migration (nondirectional movement), increased chemotactic responsiveness (directional movement), and increased cellular pinocytic activity.

The accumulation of phagocytic and cytotoxic cells at the site of tumor invasion is believed necessary for such cells to exert their tumoricidal effect. Thus, the ability of BCG to enhance macrophage motility may be directly related to its observed *in vivo* antitumor activity.

Spontaneous macrophage motility as assayed *in vitro* in this study could be thought of as a correlate of random inflammatory cell migratory movement throughout the tissues of the body. Such enhanced random movement observed in BCG-treated macrophages may allow a broader range of migration and increased opportunity for contact with invading tumor cells. Macrophage chemotactic responsiveness is a clearly separable type of cell migration, being specific directional movement in response to a chemotactic gradient. Chemotactic substances may be produced following the interaction of serum complement components with antigen-antibody complexes as well as by immunologically activated lymphoid cells in the form of chemotactic lymphokines. Again, the enhanced chemotactic responsiveness observed in the macrophages from BCG-treated animals would potentially aid in their accumulation at sites of tumor invasion. Thus, our chemotaxis findings confirm the recent studies of Meltzer *et al.* (20).

The increase in uptake of colloidal gold by macrophages from BCG-treated animals is also a reflection of macrophage activation (9, 19). Colloidal gold is selectively taken up by macrophages (29) by the process of pinocytosis, and pinocytosis is important in the formulation of lysosomes (7). This phenomenon may also be closely related to the tumoricidal action of activated macrophages, since it has been suggested recently that macrophages mediate the lysis of target tumor cells by direct secretion of lysosomes into the cytoplasm of the target cell (15).

Each of these 3 assays detects different functional alterations in macrophages following BCG treatment, and there is evidence to suggest that they are separable effects rather than a broad expression of a single physiological alteration. The time course of functional change in migration assays shows pronounced increases by Day 4, while pinocytosis is first noted to be enhanced at Day 14 after BCG immunization. Random cell migration is also distinguishable from directed movement in several respects. Extensive studies (21) of leukocyte movement in a number of different disease states have demonstrated discrepancies in these 2 parameters of cell migration. Furthermore, the increase in macrophage random migration following BCG administration contrasts with the results obtained with other immune adjuvants.[3] With agents such as *Corynebacterium parvum,* increases in migration were similar to those seen following BCG migration, but increases in macrophage random migration were quantitatively much less and did not parallel the changes in chemotactic responsiveness.

Although it has been reported (13, 14, 18, 22) that various strains and preparations of BCG vaccine differ in their immunogenicity, immunostimulatory, and antitumor properties, we were unable to detect such differences using our techniques. Thus, strains of high (Brazil), intermediate (Phipps), and low (Japan) virulence and of high (Tice) and low (heated) viability stimulate random migration and chemotaxis to a similar degree and with similar kinetics. This is additional evidence that the various effects on macrophage function following activation are distinct and separable entities.

More extensive studies of the physiological alterations in macrophages following treatment with immunostimulators should be performed to determine whether these functional changes are due either to qualitative differences inherent in each of the stimulators or to quantitative differences in the degree of activation induced in the macrophage.

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