Modulation of the Immune Response by BCG: A Review

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Received February 10, 1976

Bacillus Calmette-Guérin (BCG), an attenuated strain of Mycobacterium bovis, was developed as a vaccine against human tuberculosis by Calmette and Guérin at the Pasteur Institute in 1908. BCG has recently regained the spotlight in completely different roles, that of an immunological adjuvant and "antitumor" agent. Investigations with this agent in the immunotherapy of cancer have met with variable success in different animal systems and with limited success in a small number of human trials. The majority of this work has been done on an empirical basis using tumor regression or animal survival as the end point. The effects of BCG on the immune response are multifaceted, and a clearer understanding of its mechanism of action may lead to a more rational basis for its use, not only in cancer immunotherapy, but in other immunologic applications.

Antitumor Effects

In the nearly two decades since BCG was first reported to increase the resistance of rats and mice to a variety of transplantable tumors (7, 17, 60), a large number of experiments has been performed in an attempt to elucidate the mechanism of resistance induced by this agent. In guinea pigs, concomitant intra-dermal administration of BCG and living tumor cells or injection of BCG into small established tumor nodules resulted in the inhibition of tumor growth and the production of systemic immunity to the tumor (68-70). Close contact between the tumor cells and BCG was necessary, and the induced immunity was tumor specific. This systemic resistance could not be induced by other common methods of immunization, such as injection of killed tumor cells or excision of the primary tumor. The efficacy of BCG-tumor vaccines or of intratumoral injections of BCG was more variable in producing tumor regression in mice. Even in those systems where tumor growth was inhibited, the development of systemic immunity was inconsistent (4, 40, 53).

BCG therapy has also been associated with negative effects, including clinical deterioration associated with the appearance of serum blocking factors (40) and hypersensitivity reactions to BCG occasionally resulting in death (38). Increased tumor growth has occurred after BCG injection or sensitization to purified protein derivative (PPD) of Mycobacterium tuberculosis (50, 52). BCG-tumor cell mixtures can
either enhance or reduce artificial experimental pulmonary "metastasis" depending on the antigenicity of the tumors (1). Established solid tumors may be enhanced by the systemic administration of BCG, while leukemias are either inhibited or unaffected (36).

The predominant cell types involved in BCG-mediated augmentation of immunity to solid tumors are lymphocytes and monocytes (18, 19, 48). The site of inhibition is characterized by a localized inflammatory response with intense mononuclear infiltration, leading to a granuloma. Although the inflammatory response to BCG may be necessary for the expression of tumor resistance, it may not be sufficient, since other inflammatory agents do not possess similar effectiveness and may even accelerate tumor growth (19).

Early investigators attributed the effects of BCG to a stimulation of the reticuloendothelial system (RES) and an augmentation of the immune response (2, 6). The evidence presented above suggested that the mechanism of the antitumor activity of locally administered BCG is a two-step phenomenon involving the specific recognition of bacterial antigens by the host and then a granulomatous reaction which non-specifically destroys the tumor cells (an "innocent bystander" effect). Subsequent development of specific immunity might be due to an increased number of activated lymphocytes by tumor antigens. While this hypothesis may be valid for solid tumors, there is a large body of information suggesting that the antitumor effect of BCG is more than simply the result of the local granulomatous response to BCG antigens. Administration of BCG can inhibit the induction of tumors by a variety of oncogenic agents, and mice treated systemically with BCG and subsequently injected with syngeneic leukemia L1210 show an increased survival time (35). Repeated BCG injections prolong the survival of AKR mice, which normally develop a spontaneous leukemia (59). Pretreatment of mice with the methanol-insoluble fraction of BCG (methanol extract residue (MER)), which can mimic most of the adjuvant effects of the whole organism, reduced the incidence of leukemia following inoculation with radiation leukemia virus (20). Unfortunately, immunological correlates are generally not available for these survival studies.

Recent studies have shown that macrophages may play an important role as an effector cell in allograft resistance, and they may also be important in the resistant state induced by BCG. Macrophages nonspecifically activated in vitro and in vivo by a variety of substances including BCG are able to recognize and destroy a variety of neoplastic cells (10, 22–24, 26) but with a few exceptions (39) demonstrate little or no cytopathic effect on nontransformed fibroblast lines. In general, early passages of embryo cells, which showed little contact inhibition, were susceptible to killing by activated macrophages. Later passages were minimally affected (10, 26). BCG-activated adherent peritoneal cells are cytotoxic to tumor lines, while peritoneal exudate cells induced by starch, mineral oil, or thioglycollate are not tumoricidal (10). This is an in vitro correlate of the earlier observations that activation of macrophages or induction of an inflammatory response may be necessary but is not sufficient for the expression of the tumoricidal effects of BCG. It is also of interest to note that the BCG-activated macrophages are apparently able to discriminate between neo-antigens of tumors and allo- and xenoantigens on normal cells.

The possible mechanisms underlying the action of BCG against tumors may be illuminated by the results of immunological studies with that agent in other systems. This seems to be a valid approach because tumor immunology is not a discipline with unique principles or circumstances but simply a branch of general immunology. Thus, information relating to allograft rejection, other varieties of cell-mediated im-
munity, or even antibody synthesis may well be applicable to immunity against tumors. In addition, recent technological advances in the measurement of immunity in vitro have allowed the conclusions drawn from studies on the survival of animals to be reexamined on a more sophisticated level.

Effects on Cell-Mediated Immunity

BCG is widely used as an immunizing agent against tuberculosis. Although the precise mechanisms of immunity to tuberculosis are not known, BCG is presumed to protect by virtue of a specific antimycobacterial immune response evoked in the vaccinated subject (34). In addition, however, alveolar macrophages from rabbits injected with BCG have an increased ability to inhibit nonspecifically the growth of intracellular organisms, with an accompanying increase in the content of lysosomal hydrolases (32). Nonspecific resistance (cytotoxicity) to unrelated bacteria can also be induced in macrophages from animals immunized with Mycobacterium tuberculosis, as well as with Listeria monocytogenes or Brucella abortus. The rate of onset and strength of resistance is dependent upon the vaccinating dose of BCG. Nonspecific killing is dependent upon development of a delayed sensitivity reaction to BCG. However, heat-killed organisms alone are ineffective in the induction of nonspecific cytotoxicity of microorganisms. This requires the persistence of the inducing mycobacteria, either through chronic infection with living bacteria or injection of killed organisms in complete Freund’s adjuvant (53). Protection against M. tuberculosis infection can be conferred with BCG cell walls attached to oil droplets or a combination of a peptidoglycolipid cell wall skeleton plus a lipid component (52). Mice inoculated with BCG have an increased rate of clearance of Escherichia coli endotoxin and carbon particles by macrophages of the reticuloendothelial system (28). BCG-infected animals also clear Salmonella enteritidis more rapidly than control animals, and they catabolize denatured albumin more rapidly. Not all of the underlying immunological mechanisms of nonspecific antimicrobial immunity are understood, since BCG-activated alveolar macrophages have an enhanced ability to kill L. monocytogenes but not Yersinia tularensis (46). This indicates that, although activated macrophages demonstrate an increased resistance to a variety of intracellular organisms ranging from bacteria to viruses, the scope of the nonspecific resistance is incomplete.

The adjuvant (helper) effect of an active tuberculous infection on cell-mediated immunity was first noted by Dienes and Schoenheit, who noted that increased delayed hypersensitivity reactions (DHR) to egg albumin and timothy pollen could be induced by injection of the antigen into a tuberculoid lesion (12). BCG or its derivatives can potentiate cell-mediated immunity to protein antigens (41), to haptens conjugated to normally nonimmunogenic carrier molecules (30), and to poorly immunogenic tumor lines (21). The wax D fractions from several human types of M. tuberculosis, when injected together with a protein antigen in a water-in-oil emulsion, could mimic the adjuvant effect of the whole killed mycobacteria, including the production of corneal hypersensitivity (64). Wax D fractions of several bovine strains of mycobacteria, including BCG, were not active. The difference appeared to be the presence of a peptide moiety in the human wax D which was absent from the wax D fractions of bovine, as well as the inactive wax D fractions from avian and saprophytic strains of M. tuberculosis (62, 64).

Another manifestation of cell-mediated immunity, which can be profoundly affected by BCG or MER administration, is the rejection of skin grafts (2, 51, 55, 60, 71). The survival of dispersed syngeneic or allogeneic spleen cells in vivo, as
measured by their ability to synthesize antibodies, is not affected by prior treatment of recipient mice with BCG. In contrast, BCG markedly decreases the survival of solid spleen allografts and isografts (8). The initial take of grafts is not influenced by BCG pretreatment, and it has been suggested that an effect on the proliferation of immunocompetent lymphocytes after their recognition of antigen is of most importance. Accelerated rejection would result from a more rapid sensitization and an expanded pool of reactive cells. Accelerated rejection of syngeneic male to female tail skin grafts was produced when BCG was administered 21 or 28 days before grafting, but treatment with BCG 36 and 7 days before grafting resulted in a prolonged skin graft survival (71). The accelerated rejection but not the prolongation was observed with allogeneic skin grafts. Nonspecific stimulation of the reticuloendothelial system was not the major mode of action, since the twice-immunized mice had a more pronounced increase in reticuloendothelial phagocytic activity than singly immunized animals. In addition, the maximal resistance to isografts induced by MER administration occurs after reticuloendothelial activity has returned to normal, and splenectomy between BCG administration and skin grafting abolishes the accelerated rejection (2).

BCG pretreatment in vivo significantly increased spleen cell-mediated cytotoxicity of suboptimally immunized Swiss mice to allogeneic L1210, as measured in vitro. Interestingly, the administration of BCG alone produced "pseudoimmune" spleen cells with a high level of cytotoxicity towards L1210 (43). The pseudoimmune cells were not contained in the glass-adherent population and are probably not typical macrophages. However, they are found in thymus-deprived mice and are not thymus-dependent (T) lymphocytes either (Kinder and Mitchell, unpublished data). MER pretreatment increased the cytotoxic efficacy of lymphocytes from specifically immunized donors, and the heightened effect could be transferred passively in this system by both lymphoid cells and macrophages (30). MER pretreatment increased the numbers of cells forming rosettes with sheep erythrocytes in normal and immunized spleens. Both cells bearing the thymus-specific ς antigen and those without were increased (61). The stimulatory effects of BCG and its derivative, MER, are most marked in animals given small immunizing doses of antigen, and the elevated levels of immunity are generally maintained for several weeks.

Effects on Antibody Synthesis

Humoral antibody synthesis is increased in a variety of systems by BCG treatment (42, 50, 54, 56). An active tuberculous infection enhances antibody formation to egg albumin and timothy pollen injected into the lesion (12). BCG pretreatment increased DNA synthesis in lymph node cells, and there was a good correlation between increased cell proliferation and the adjuvant effect for both antibody response and the development of DHR to sheep red blood cells (42). This is in agreement with the correlation between the increased antibody levels after injection of egg albumin with the wax D fraction of M. tuberculosis and a proliferation of plasma cell elements in the lymph nodes, spleen, and liver (63).

The work done by Yashphe and her collaborators indicated that MER either directly or indirectly increased the number of cells which is capable or responding to an antigenic stimulus (66, 67). MER pretreatment also appears to expand and/or make more efficient the memory cell pool as well as the antibody-forming cell pool (61). There is some evidence that some of MER's activity may be at the stage of antigen processing by macrophages (61). As with the effects on DHR, the MER adjuvancy for antibody production is more pronounced at low antigen doses or with
weaker antigenic materials. Unfortunately, the effects of adjuvant administration are not easily predictable, since MER pretreatment in guinea pigs could selectively favor humoral antibody formation or delayed hypersensitivity to hapten–protein conjugates, depending on the precise conditions of immunization (61).

Very few adjuvants, other than those directly mitogenic for antibody-forming cell precursors ("bursa"-dependent lymphocytes, B-cells), appear to exert their effects by a direct action on B-cells. There is no direct evidence at this time that BCG or its derivatives are B-cell mitogens. In fact, since other adjuvants require the presence of specific T-cells to be effective, it is likely that BCG and MER also may enhance antibody synthesis by stimulating T-cells to produce soluble factors that stimulate B-cells (37).

Reversal of Immunosuppression

Perhaps one of the most interesting and potentially exploitable effects of BCG or MER is their ability to reverse or protect against the immunosuppressive effects of various agents. MER largely or completely reversed the immunosuppressive effects of antithymocyte serum on the circulating antibody response to sheep red blood cells, whether the MER was given before or after the antithymocyte serum treatment. In addition, MER markedly protected against the depressive effects of cortisone acetate on the appearance of rosette-forming cells after immunization with sheep red cells (61). The immunosuppressive effect of antilymphocyte serum on the immunization against allogeneic cells, as measured by the cytotoxic activity of spleen cells against $^5$Cr-labeled target cells, was similarly affected (29).

BCG pretreatment increased the number of plaque-forming cells directed against sheep red cells and could partially protect against or reverse the immunosuppressive effect of methylcholanthrene (57). The combination of BCG and methylcholanthrene resulted in approximately the same number of plaque-forming cells as in the immunized controls. BCG can also confer long-lasting protection against the immunosuppressive effects of cytarabine (46). Spleen cell-mediated cytotoxicity to allogeneic leukemia L1210 was decreased by cytarabine alone, but was the same as in immunized controls in the group given both BCG and cytarabine. Treatment with BCG after chemotherapy caused a transient, but significant, reversal of the immunosuppression (46). The effector cell population elicited by BCG in this protective action appears to be a non-thymus-dependent, nonadherent phagocytic cell, probably of the monocytic cell series.

BCG pretreatment was apparently able to protect against the immunosuppressive effects of cyclophosphamide, since mice could be made resistant to tumor induction by a murine sarcoma virus (MSV-M) or could be immunized against challenge by a MSV-induced tumor even in the face of cyclophosphamide chemotherapy (25, 27). These effects may be due in part to the induction of interferon production in addition to the nonspecific stimulatory effects on the immune response (16). The therapeutic benefit from high toxic doses of cyclophosphamide against syngeneic L1210 leukemia could be increased by subsequent treatment with BCG (35). The increased efficacy of combined therapy in this system was attributed to a myeloprotective property of BCG, since BCG can stimulate bone marrow macrophage colony production but may well be due to reversal of immunosuppression, too (65). However, in contrast to the work with the MSV system, BCG pretreatment increased the number of drug-related deaths following high-dose cyclophosphamide treatment.

A similar reversal of an immunosuppressed state was seen in the antigen–antibody-induced suppression of macrophage receptors for cytophilic antibody (44).
This suppression could be prevented or reversed by the injection of BCG organisms 5 days before or after the suppressive treatment (Mitchell and Mokyr, unpublished data). Since "suppressor" T-cells appear to be involved in the inhibition phenomenon (13), it remains to be seen whether the protection and reversal effected by BCG is mediated through T-cells or is a direct effect on the macrophage membrane. Direct membrane effects may play a role in the resistance of spleen cells to immunosuppression by antilymphocyte serum, since isolated cells treated with MER in vivo were more resistant to the in vitro killing by antilymphocyte serum and complement (29).

Other Effects of BCG on Lymphoid Cells

Another index of immunological reactivity is the ability of lymphocytes to respond to mitogens. Prior administration of BCG has been reported to increase the response of monkey lymphocytes to the T-cell mitogen, phytohemagglutinin (11), and MER enhanced the spleen cell response to the B-cell mitogen lipopolysaccharide (LPS) and of lymph node cells to both LPS and concanavalin A, another T-cell mitogen (14). However, we have found that BCG can decrease the responsiveness to mitogens of mouse spleen cells (43, 46). Although the baseline incorporation of [3H]thymidine was increased tenfold in spleens from BCG-treated mice (43), the subsequent response to phytohemagglutinin, concanavalin A, and lipopolysaccharide was reduced below the response of normal spleen cells.

Spleen cells or thymocytes are stimulated to produce DNA when treated with BCG in vitro. The in vitro stimulation of thymocytes has an absolute requirement for macrophages acting in the "afferent" arm of immunization (45). Further evidence for the ability of BCG to promote intercellular cooperation during an immune response can be found in studies on the recently described lymphocyte-activating factor (15, 43). Adherent spleen cells from BCG immunized mice produced more of the factor than normal cells. Production of lymphocyte-activating factor by adherent spleen cells could also be enhanced by in vitro stimulation by BCG, and this effect was further amplified by the presence of nonadherent spleen cells, suggesting a mutual stimulation of macrophages and lymphocytes (45). The production of this mediator from MER-treated peritoneal cells was also increased (14). Our current presumption is that lymphocyte-activating factor is at least one substance produced by macrophages in response to adjuvants such as BCG. These substances may expand and activate populations of T-cells and may thus help to explain the potency of adjuvants in increasing the responsiveness of immunocompetent cells to an antigen introduced into the system.

Regardless of their future use in immunotherapy, BCG and its derivatives can play an important role as prototypes in the study of adjuvancy. Recent advances in obtaining purified cell populations and in tissue culture techniques make it possible to dissect their actions in stimulating humoral and cell-mediated immunity more completely. The nature of cell-to-cell interactions in immunity may be better elucidated through such studies, providing information that goes well beyond the immediate field of "applied immunology."

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