THE MODIFYING EFFECT OF BCG ON THE IMMUNOLOGICAL INDUCTION OF T CELLS*

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Lymph nodes draining the site of a BCG infection respond more vigorously to a second antigenic stimulus (1). The augmented response is associated with a higher and more sustained state of delayed-type hypersensitivity (DTH). When BCG is introduced systemically, the reticuloendothelial system (RES) becomes hyperactive (2) and bestows a marked degree of nonspecific resistance to a variety of infectious agents (3). The BCG-infected mouse may also show increased resistance to transplantable tumors, particularly to those with immunogenic properties (4). It is not known whether this systemic effect of BCG is due to augmentation of the immune response to tumor specific antigens, as occurs in BCG stimulated lymph nodes (5), or results from activation of a nonspecific effector mechanism of the sort that confers increased resistance to infection.

Since mice develop classical DTH in response to an appropriate intravenous dose of sheep red blood cells (SRBC) (6), it was possible to use this measure of T-cell activity to investigate the immunological consequences of a systemic BCG infection. It seems that the T-cell blocking factors which are formed by the interaction of antigen and antibody (7) do not function normally in BCG-infected mice. In consequence, the immune response is less subject to feedback inhibition, and immunity (both humoral and cellular) becomes enhanced.

Materials and Methods

Animals.—Specific pathogen-free, male and female mice of the CD-1 strain (Charles River Breeding Laboratories, Wilmington, Mass.) were used at 5 or 6 wk of age.

Organisms.—Two strains of Mycobacterium bovis BCG, BCG Montreal (TMC 1012) and BCG Pasteur (TMC 1011), were obtained from the Trudeau mycobacterial collection (TMC) (8). The organisms were grown as dispersed cultures in Middlebrook's 7H9 medium containing Tween 80 (Difco Laboratories, Inc., Detroit, Mich.). After 5 days' incubation, the cultures were frozen slowly to −70°C and were stored at this temperature. Dosage was based upon viable counts performed by plating on Middlebrook's 7H10 medium.

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1 Abbreviations used in this paper: AS4, absorbed 4-day immune serum; CY, cyclophosphamide; DTH, delayed-type hypersensitivity; RES, reticuloendothelial system; SRBC, sheep red blood cell; TMC, Trudeau mycobacterial collection.
Blocking Serum.—Serum containing specific inhibitors of activated T cells was prepared from mice immunized intravenously with $10^9$ SRBC. The animals were bled after 4 days by heart puncture. The serum, after heating to 56°C for 30 min, was absorbed by incubation for 20 min at 37°C with an equal volume of packed SRBC. The supernate, after centrifugation, was referred to as absorbed 4-day serum or AS4 (7). The batch of AS4 used in the present study had a hemagglutinating titer of 1:1024 before absorption and 1:128 after absorption. It was sterilized by membrane filtration (0.45 μm pore size, Millipore Corp., Bedford, Mass.) and was stored frozen until required.

Other Materials and Techniques.—Preparation of SRBC, methods of immunization and assay for DTH, and method for estimating serum hemagglutinin titers were described in preceding papers (1, 6-8).

RESULTS

The Development of DTH in Response to Intravenous Immunization of Normal and BCG-Infected Mice.—In an earlier study (6), CD-1 mice showed maximum T-cell activity (DTH) on day 4 of the response to intravenous immunization with SRBC. The optimum sensitizing dose of $10^5$ was sharply defined. Higher doses caused increasing titers of hemagglutinating antibody and a corresponding decrease in DTH (7). The pattern of response was quite different in mice which had been infected intravenously with $9 \times 10^7$ BCG Montreal. Fig. 1 shows that vaccinated mice developed higher levels of DTH in response to large and widely varying doses of SRBC.

The Persistence of T-Cell Activity in BCG-Infected Mice.—Normal and BCG-infected mice were immunized with doses of SRBC chosen from either end of the range that gave maximum levels of DTH in BCG-infected mice (Fig. 1). BCG Pasteur ($10^9$) was inoculated intravenously 12 days before injecting
SRBC by the same route. Fig. 2 shows that DTH was higher and persisted longer in the BCG-infected mice. Although not the optimal intravenous dose for sensitizing BCG-infected animals, the higher dose (10⁸) gave a bigger differential effect because it suppressed DTH almost completely in normal mice. This dose was therefore used for most subsequent studies.

Effect of Varying the Dose of BCG on the T-Cell Response to SRBC.—It is of potential practical importance to determine whether the immunopotentiating effect of BCG depends on dose, and how long the effect lasts. Mice were therefore infected intravenously with varying doses of BCG. Subgroups of five normal or BCG-infected mice were tested at weekly intervals for immunological responsiveness to a standard intravenous injection of 10⁸ SRBC. DTH was measured 4 days later, but the reactions are recorded in Fig. 3 against the day of immunization. The highest dose of BCG caused a very prompt change in the level of T-cell activity generated in response to a dose of SRBC that again failed to sensitize uninfected controls. The effect was not sustained, perhaps because the highest dose of BCG was debilitating. It may be significant, however, that mice of this group also failed to react to tuberculin during the phase of depressed responsiveness to SRBC.

The somewhat aberrant behavior of the most heavily infected mice did not obscure a systematic dose-effect relationship (Fig. 3). T-cell responses were

Fig. 2. Development and decay of DTH in normal mice (——) and mice infected intravenously with 6 X 10⁶ BCG Montreal (——) 12 days before intravenous sensitization with 10⁶ (Δ, ▲) or 10⁸ (○, ●) SRBC. Tests for DTH were performed on the days indicated; they were read 24 h later. Means of 5 ± SEM.
The indicated doses of BCG Pasteur were injected intravenously into four groups of mice. At weekly intervals, five mice from each group, and from a group of untreated controls (O—O), were injected intravenously with $10^8$ SRBC. DTH was measured 4 days later with a standard eliciting dose ($10^9$) of SRBC in one hind footpad. The results are recorded for each treatment group. As expected, no DTH occurred in untreated controls; but high levels of DTH appeared in BCG-treated animals. Maximum potentiation of the T-cell response to SRBC was reached at different times in different treatment groups, but the peak values were linearly related to the dose of BCG used to modify the host response. This is shown on the right, together with SEM for the values plotted.

Additive Effects of BCG and Cyclophosphamide (CY)—Mice were infected intravenously with BCG Pasteur ($10^7$). Half, and an equal number of uninfected mice, were given CY (200 mg/kg) 10 days later. After another 2 days, mice of the four treatment groups were subdivided and immunized (in groups of five) with varying intravenous doses of SRBC. All mice were tested for DTH 6 days later. In addition, the mice immunized with $10^5$ SRBC were bled immediately after the reading of footpad reactions. Hemagglutinin titers were determined on the pooled serum from each treatment group.

The effect of separate and combined treatment with BCG and CY, which enhances T-cell activity by inhibiting antibody production (9), is compared in Fig. 4. The optimum sensitizing dose of SRBC in untreated CD-1 mice was again $10^5$, but the peak level of DTH was lower than that recorded in the previous experiments (Fig. 1) because tests were not made until day 6. By this time, DTH has passed its peak in normal but not in BCG-infected animals (see below). The BCG-infected animals again showed potentiation of DTH, but
Fig. 4. Levels of DTH developed in response to varying intravenous doses of SRBC by normal mice, mice inoculated intravenously with \(10^7\) BCG 14 days before sensitization, mice treated with cyclophosphamide (200 mg/kg) 2 days before sensitization, and mice given both forms of treatment. Means of 5 ± SEM. The reciprocal of the hemagglutinin titer, found on day 7 in pooled serum obtained from mice which received the largest (\(10^9\)) dose of SRBC, is given at the end of each curve.

only with high doses of SRBC. This and other observations suggest that the effective antigenicity of an inoculum of SRBC may be somewhat diminished at this (earlier) stage of a BCG infection. An augmented T-cell response was nonetheless apparent. It was accompanied by increased antibody production, as evidenced by the hemagglutinin titers recorded at the end of each curve in Fig. 4.

As reported elsewhere (9), CY interferes with T-cell blocking by suppressing antibody formation. Its contrasting effects on DTH and antibody formation were seen again in the data of Fig. 4. When combined with BCG, CY caused a further augmentation of the T-cell response. The reason for this enormous increase in T-cell activity is revealed by the hemagglutinin titers found in the pooled serum of the mice which were immunized with \(10^9\) SRBC and bled on day 7: antibody formation was markedly elevated in BCG-infected animals; but whether infected with BCG or not, CY-treated mice failed to give a humoral response.
In an experiment of similar design, the separate and combined effects of BCG and CY on the response to \(10^8\) SRBC were studied over an extended time-course. The results recorded in Fig. 5 show that DTH appeared fleetingly in untreated mice, but that a high and long-lasting state of DTH developed in all treated animals. Two phases of hypersensitivity were apparent: an intense but short-lived hypersensitivity that gave way within 10 days to a more sustained but lower level of reactivity. Fig. 6 gives an indication of the magnitude of the DTH reactions elicited in mice treated with BCG and CY. The reaction depicted was 48 h old. Persistent swelling is a feature of reactions elicited in CY-treated mice (10).

**BCG-Induced Resistance to T-Cell Blocking.**—It has been inferred that DTH fails to develop or quickly disappears in animals immunized with large...
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Fig. 6. Footpad reaction 48 h after injecting eliciting dose of SRBC on day 6 in mouse of experiment recorded in Fig. 5. It had received BCG and CY. Mice of this group had a mean recorded increase in footpad thickness of 24 ± 0.9 at 24 h. When the reaction was photographed at 48 h, the difference in thickness between right and left footpads was 15 (= 1.5 mm). The edema which characterizes such severe reactions involves the whole extremity.

doses of antigen because activated T cells become blocked by immune complexes (7, 9). Inasmuch as DTH develops in BCG-infected mice despite very high levels of serum antibody, and as CY further enhances the T-cell activity of BCG-infected mice (Figs. 4, 5), the same blocking mechanism must operate, to some extent at least, in BCG-infected animals. If so, the high levels of DTH developed by BCG-infected mice could be due as much to increased production of activated T cells (1) as to the absence of blocking by immune complexes. To investigate this question, the inhibitory activity of serum containing blocking factors was compared in normal and BCG-infected animals.

AS4 (7) was tested for its capacity to block both the induction and expression of DTH. Blocking of induction was tested in normal and BCG-infected mice which had received 10^7 BCG Pasteur intravenously 12 days previously. The sensitizing inoculum of 10^8 SRBC was given in one hind footpad to untreated mice and to mice given a single intravenous dose of AS4 (0.2 ml) at the time of sensitization. Others received three doses (each of 0.2 ml) of AS4 which were given 12 h before, with, and 12 h after sensitization. DTH was tested in the opposite footpad 5 days after sensitization.

The capacity of AS4 to interfere with the expression of DTH was compared in normal and
BCG-infected mice which had also been infected with $10^7$ BCG Pasteur 12 days before the sensitizing footpad inoculation of $10^6$ SRBC. DTH was measured 5 days after sensitization in untreated mice and in mice treated with 0.2 ml of normal mouse serum (absorbed with SRBC) or AS4 given intravenously 1 h before footpad testing.

Fig. 7 shows that absorbed immune serum (AS4), which completely blocked the induction of DTH in normal mice, did not interfere with its development in BCG-infected mice. Even three doses given at 12-h intervals had little effect. The blocking serum also interfered, but to a less extent, with an estab-

![Figure 7](image.png)

**Fig. 7.** Left: Effect of AS4 on the induction of DTH to SRBC in normal or BCG-infected mice. DTH was measured 5 days after a footpad sensitizing dose of $10^6$ SRBC in animals (NS) given 0.2 ml of normal mouse serum absorbed with an equal volume of SRBC and in similar animals which had received one (1 X AS4) or 3 (3 X AS4) doses (0.2 ml) of AS4. The latter were given intravenously 12 h before, together with, and 12 h after the sensitizing dose of SRBC. Means of 5 ± SEM. Right: Effect of AS4 (0.2 ml) on established DTH in normal or BCG-infected mice. BCG was given intravenously 12 days before a sensitizing footpad injection of SRBC ($10^6$). Footpad tests for DTH were performed 5 days after sensitization. Controls received 0.2 ml of normal serum (NS) which was also absorbed with SRBC. Means of 5 ± SEM.

lished state of hypersensitivity. It caused average falls of 72% and 21% in normal and BCG-infected mice, respectively ($P < 0.025$).

**Effect of Systemic BCG on a Regional Response to SRBC.**—The experiments recorded in Fig. 7 show that a systemic infection with BCG scarcely affected the level of DTH induced by footpad sensitization. Potentiated immune responses are achieved with BCG only if the antigen enters lymphoid tissue that is under active stimulation by BCG (1). This point, which bears on the mode of action of BCG, is further illustrated in Fig. 8. When BCG was injected into one hind footpad, it had little effect on the response to SRBC injected into one front footpad (Fig. 8 A). But when BCG was injected into the plantar surface of a rear footpad, and SRBC were introduced subcutaneously on the
dorsal aspect of the same ankle, a marked potentiation of the T-cell response occurred (Fig. 8 B). Sensitivity was greater in mice immunized in the rear footpad than in mice given an equivalent dose of SRBC in a front footpad. This difference in response is consistently seen and is not surprising in view of the sensitivity of the T-cell response to antigen dose (5) and the fact that lymph from the front footpad is probably shared among several lymph nodes.

The Effect of Blocking Serum on Mice Sensitized Intravenously.—Since blocking serum did not interfere with the induction of DTH in BCG-infected mice (Fig. 7), why should T-cell activity be increased by CY in BCG-infected mice (Fig. 4)? It was possible, in view of the role played by the spleen in causing suppression of T cells (5), that the specifically activated T cells formed by BCG-infected mice might not be fully protected against blocking by immune complexes.

Blocking of activated T cells by AS4 was attempted in normal and BCG-infected mice given $10^7$ BCG Pasteur 14 days before sensitization. Normal mice received $10^5$ SRBC, but the BCG-infected animals were given $10^6$ SRBC because they sometimes require a larger intravenous dose of antigen to provoke a comparable immune response. Mice of both groups
were subdivided on day 5, one half being injected intravenously with AS4 (0.2 ml) 1 h before footpad testing for DTH; those of the other group received absorbed, nonimmune mouse serum.

The expression of DTH was largely suppressed by AS4 in mice which had been sensitized to SRBC intravenously (Fig. 10). Blocking also occurred in BCG-infected mice but was significantly less ($P < 0.001$).

DISCUSSION

While studying the tumor-suppressive effects of BCG, Old and his colleagues (11) observed that simple splenectomy was sometimes as effective as BCG in causing tumor regression. They thought that BCG might "promote the regression of Sarcoma 180 by producing a functional splenectomy", and suggested that the phenomenon might be due to impaired production of enhancing antibody. Lagrange et al. showed in another report (6) that splenectomy did modify the immune response in a way that could promote antitumor immunity: spleenless mice, being virtually unable to make antibodies, did not suffer from T-cell blocking when immunized intravenously with large doses of SRBC. They behaved, in fact, like CY-treated mice, which give enhanced T-cell responses for a similar reason (9). The present experiments show, however, that BCG has an even greater enhancing effect on T-cell activity, but it is accomplished without interfering with antibody production. Indeed, serum antibody levels, like the number of antibody producing cells in a regional node (1), were markedly increased by BCG (Fig. 4). There is no reason to suppose, therefore, that the BCG-infected spleen is functionally impaired. The increased resistance to tumors and the enhanced T-cell responses of BCG-infected mice must be due, therefore, to other causes.

Earlier studies have shown (6, 7, 9) that the mediators of DTH are blocked early in the immune response by a product formed when antigen and antibody interact. Although there is no direct evidence implicating antigen-antibody complexes, indications favor them for a role as the physiological inhibitors of T cells (7, 9). They function to control the cellular response to T-cell-dependent antigens, making it self-limited. The mechanism which thus regulates the immune response clearly does not operate normally in BCG-infected mice, for T-cell activity and antibody production were both increased by BCG (Fig. 4). At least a partial explanation lies in the finding that blocking factors do not inhibit T cells effectively in the presence of a recent BCG infection (Figs. 7, 9). Perhaps they are disposed of more rapidly by an activated RES.

Increased blood clearance rates (2) and enhanced resistance to nonspecific infections (3) are well established features of the host response to BCG. It is significant, therefore, that the two forms of resistance—to nonspecific infection (3) and to T-cell blocking (Fig. 3)—should develop at comparable rates and to an extent that varies with the infecting dose of BCG. It is also significant that maximum enhancement of the T-cell response to SRBC and of resistance to
Fig. 9. Effect of blocking serum (0.2 ml of AS4) on DTH established by intravenous sensitization with \(10^5\) and \(10^6\) SRBC in normal and BCG-infected mice, respectively. The BCG (\(10^7\) BCG Pasteur) were given intravenously 14 days before sensitization; and tests for hypersensitivity were performed 5 days after sensitization. AS4 was injected 1 h before the eliciting dose of SRBC (\(10^6\)). The difference in the degree of suppression achieved in normal and BCG-infected mice was significant (\(P < 0.001\)). Means of 5 ± SEM.

infection were reached at different times with different doses of BCG. This is because BCG grows in the mouse until the antigenic stimulus is sufficient to provoke an adequate defense, an event which occurs sooner with larger doses of BCG (3, 12). Antimicrobial immunity and stimulation of the RES are both a consequence of the action of mycobacterial antigens on specifically sensitized tissues. It follows that the altered T-cell responses observed in BCG-infected mice are a direct consequence of the host's immunological reaction to infection.

The mechanism whereby a BCG infection interferes with the action of blocking factors, though seemingly related to RES activation, is not precisely known. It is evidently not due, however, to the formation of "unblocking" antibodies (13) because the activated T cells of BCG-infected mice were protected from the immediate effects of blocking serum administered passively (Fig. 9). The increased clearing capacity of the RES in BCG infected mice (2) offers a plausible explanation. If the hyperactive phagocytic elements of the RES were to provide an enlarged display of surface receptors that could compete successfully for immune complexes, T cells would be free to circulate in a functionally active state for longer periods of time in BCG-infected mice. The clearance of blocking factors from the circulation of BCG-infected mice is under investigation.

The observations of Fig. 4 suggest that BCG may act in two ways to potentiate T-cell responses. In many similar experiments, BCG and CY have
always produced additive effects. If immune complexes are rapidly removed from the circulation of BCG-infected animals, and this were the only reason for the increase in T-cell activity, one would not expect CY to raise the level of DTH above that achieved in uninfected mice (Fig. 4). The fact is, however, that BCG and CY do have a synergistic effect. This means that the absolute output of activated T cells must be larger than normal in BCG-infected mice. It is equally true, however, that T cells are under some constraint in BCG-infected animals, otherwise CY could not lift them to an even higher plane of activity. Since CY, given before immunization, has very little effect on the ultimate levels of cellular proliferation or of plaque-forming cell production in a lymph node stimulated with SRBC (9), yet permits a greatly exaggerated and long-sustained state of DTH when combined with BCG (Figs. 4, 5, 6), it follows that the duration of the SRBC-specific T-cell response, and the output of activated T cells, must both be markedly increased by BCG.

When studying the immunopotentiating effect of BCG on the regional response to SRBC (1), T-cell responses were found to increase for about 2 wk, rising in proportion to the rate of cell proliferation provoked by BCG in the responding node. It may be, therefore, that the second way in which BCG affects the immune response is through the facilitating effect that one immune response exerts upon another. North et al. (14) have shown that the rate of cell proliferation in the spleens of mice infected intravenously with virulent tubercle bacilli also increased for about 2 wk, so that the effect of BCG on the immunological responsiveness of the spleen is probably similar to that in regional lymph nodes (1), and allied to the allogenic effect (15). It must be admitted, however, that immune complexes formed locally in spleen or lymph node might be disposed of in situ by elements of the RES made hyperactive by BCG. In either case, it is certain that one effect of BCG is strictly local, for potentiation of DTH occurred only when BCG and SRBC had access to the spleen (cf. Figs. 1 and 7) or the same lymph node (Fig. 8). Increased resistance to blocking factors that reach the circulation may thus be merely a systemic manifestation of a process that also occurs locally in lymphoid tissues primed with BCG.

**SUMMARY**

The inhibition of activated T cells by products of the humoral immune response is almost abolished by systemic infection with BCG. As a result, BCG-infected mice develop very high levels of delayed-type hypersensitivity (DTH) in response to doses of sheep red blood cells (SRBC) that cause complete suppression of DTH in normal mice. This systemic effect of BCG is dose-dependent, and lasts for about 3 wk. Its main effect is to counteract the inhibition of T cells by products of the humoral response. As a result, and in contrast to the T-cell-potentiating effects of cyclophosphamide (CY) which depend on a diminished production of antibodies, increased levels of DTH in BCG-infected mice are associated with increased antibody production. Since BCG and CY act in different ways, their effects are additive. Very remarkable levels of DTH are achieved when they are used in combination.
REFERENCES


