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Modulation of the Immune Response of Guinea Pigs by Repeated BCG Scarification[1] (38338)

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Mycobacterium bovis (BCG), a potent immunostimulant, has been shown to improve host immune response to a variety of antigens. Single BCG vaccinations in experimental animals have resulted in enhanced humoral immunity (1), increased antigen clearance and degradation by phagocytosis (2–5), and accelerated allograft reactivity (6, 7). With respect to the latter, there is an extensive literature relevant to the use of BCG, or fractions thereof, as nonspecific stimulants of resistance to syngeneic and autochthonous tumors (for review see Ref. 8). Most of those studies were performed in mice and usually involved a single immunization by intravenous, intraperitoneal, subcutaneous, or intradermal routes of administration. Based on these results, "systemic adjuvant therapy" protocols have been initiated to enhance host response to cancer in man, performed by repeated application of BCG by dermal scarification (9–11).

We felt that it would be important to determine what alterations of immune competence would be derived by repeated BCG scarification in random-bred as well as inbred (syngeneic) guinea pigs. These studies were performed primarily to determine whether repeated scarification would result in functional alterations of immunity similar to those seen after single challenges by conventional routes of administration. In the present study we also assessed the histopathologic alterations of the skin site and regional and peripheral lymph nodes during the course of scarification. Peripheral blood leucocyte changes were also monitored. These studies should be relevant to future application of adjuvant therapy in man.

Materials and Methods. Animals. Male Carworth Duncan–Hartly (CFDH) random-bred guinea pigs and male Sewall–Wright strain 2 inbred guinea pigs were used. The breeding stock of the strain-2 guinea pigs was originally obtained from the Laboratory Aids Branch, Division of Research Services, National Institutes of Health, and was syngeneic as shown by routine skin grafting. The animals weighed 400–600 g and were housed four per cage and fed Wayne guinea pig chow and kale or cabbage.

BCG scarification. Mycobacterium bovis strain BCG was obtained from Dr. R. G. Crispen of the Research Foundation, Chicago, IL. The BCG was Tice strain vaccine T94 (s) 104, lyophilized and reconstituted in water to 1 ml. Viable counts determined by surface growth on Dubos oleic albumin agar demonstrated that there were $2–4 \times 10^8$ viable organisms/ml.

The scarification was performed on areas clipped free of hair, cleansed with acetone, and air-dried. Five parallel and five perpendicular scratches approximately 1.5 cm in length were made on the skin with a 19-gauge needle. Sufficient pressure was exerted on the needles to cause slight oozing of blood. Dilutions of the BCG were prepared so as to apply approximately 5×10^7 viable organisms in 0.1 ml over the freshly scratched area. This application was spread with the edge of a 19-gauge needle to cover the entire scratched area. A Lucite ring which encompassed the scarification site was taped on and maintained for 24 hr. One scarification per week for 6 or 8 weeks was performed posterior to the right and left shoulder and an-

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terior to the right and left hip, sequentially in that order.

Immunization. The test antigen, sheep red blood cells (SRBC), was obtained sterile in Alsever's solution (Baltimore Biological Company, Baltimore, MD), washed three times in 10–20 vol of isotonic saline at 4°, and resuspended in saline to give a 10% suspension. Each animal received 1 ml of this preparation intraperitoneally. Hemagglutinin titers of the serum were determined 6 or 11 days after injection of antigen. Individual serum samples were heat-inactivated at 56° for 30 min to inactivate complement and titrated, using a Cooke microtitrator, by a standard two-fold serial dilution technique.

Allograft rejection. Inbred strain-2 grafts were transplanted to the CFDH random-bred guinea pigs. An area of full-thickness skin approximately 6 × 12 mm was transplanted to the right side of the animal just posterior to the shoulder. Grafting and postoperative dressing were done according to the techniques described by Billingham (12). The dressings were removed after 7 days, and the status of the grafts was assessed three times weekly. A thick, dried graft, curled up at its edges with scab formation, was taken as the end point of allograft rejection. The results were expressed as mean survival time of the grafts.

Histology. At various intervals after scarification, groups of guinea pigs were killed, and portions of their scarified skin, as well as the draining lymph nodes, were excised during the course of a complete autopsy. The tissues were fixed for histology in Bouin's fixative, dehydrated, and embedded in paraffin. The tissue blocks were sectioned and stained with hematoxylin and eosin for examination under a light microscope.

Results. Changes in peripheral blood leukocyte total and differential cell counts. At 1, 4, and 6 days after each scarification, two or three CFDH guinea pigs were bled from the foot vein, blood was collected for total leukocyte counts, and smears were prepared for differential cell counts. Subsequently, the same animals were killed and autopsied for histopathologic studies. In Fig. 1 it can be seen that no alteration of total leukocyte count occurred during the week after the first scarification; however, very marked increases in leukocyte counts occurred 24 hr after each of the successive scarifications, followed

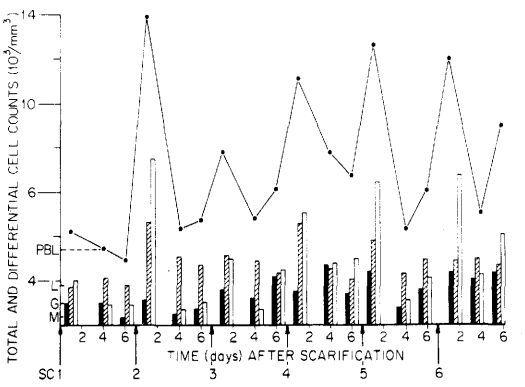

FIG. 1. Mean total peripheral blood leukocyte (PBL) counts (–) and total differential cell counts of 3 guinea pigs per point at 1, 4, and 6 days after six successive dermal scarifications with BCG. Differentials were determined from 200–500 cell counts per guinea pig. ■, monocytes; ▨, lymphocytes; □, granulocytes. The baseline values of mean differential counts for each cell type, lymphocytes (L-), granulocytes (G-) and monocytes (M-), and mean total leukocyte count (---) determined from all sham-scarified controls are indicated on left.

by recovery at 6 days after scarification to higher than normal leukocyte levels. The differential change in the cellular elements of peripheral blood as a result of successive scarifications, while primarily reflecting significant modulation of segmented neutrophils and lymphocytes, was also exemplified by a more gradual elevation of circulating monocytes throughout the 6-week period of treatment. No consistent alterations in circulating eosinophils or basophils were detected in this study.

Humoral immune capacity to SRBC antigens. Three CFDH guinea pigs were given intraperitoneal injections of suspensions of SRBC 24 hr after each scarification and bled from the foot vein at 6 days after scarification, and hemagglutinin titers were determined (Table I). After the second scarification a slight increase in responsiveness occurred; after the fourth a significant decrease in mean hemagglutinin titer was detected; and after the fifth the titer was 25% of normal.

Similar studies were performed in inbred strain-2 guinea pigs. After the second and fifth scarifications with BCG, hemagglutinin titers were compared to those of sham-scarified guinea pigs. The strain-2 guinea pigs demonstrated a lower humoral immune capacity to the 10% SRBC suspensions than that of the CFDH guinea pigs. The mean peak titer measured in the

TABLE I. Humoral and Cell-Mediated Immune Competence after Repeated Dermal Scarification with BCG.[a]

Number of BCG scarifications	Mean hemagglutinin titer to SRBC in CFDH guinea pigs	Number of strain-2 guinea pig responders/total number of animals[d]	Mean rejection time (days) of strain-2 skin grafts on CFDH guinea pigs
1	4.8 ± 0.7	—	13.3 ± 0.7
2	6.3 ± 0.7	1/6	12.0 ± 0.0
3	6.0 ± 0	—	—
4	3.3 ± 0.7[b]	—	9.0 ± 1.0[c]
5	2.7 ± 1.2[b]	0/6	—
6	4.0 ± 0[b]	—	8.8 ± 0.7[c]
7	—	—	—
8	—	—	8.0 ± 0.7[c]
Control	5.4 ± 0.6	6/6	13.3 ± 0.41

[a] Each point represents the mean of 3–4 animals ± SE.

[b] $P \leq 0.01$ level, as calculated by Dunnett's test for multiple comparison with control.

[c] $P \leq 0.05$ level, as calculated by Dunnett's test for multiple comparison with control.

[d] Responders are animals having a mean hemagglutinin peak titer of 3 $\log_2$ units. Nonresponders had no detectable antibody.

sham-scarified strain-2 controls was 3 $\log_2$ units reached at 11 days after immunization, compared to a mean peak titer of 5.4 $\log_2$ units achieved 6 days after immunization in the CFDH guinea pigs. However, the most interesting indication of the suppression of humoral immune competence as a result of BCG scarification was detected in these inbred strain-2 guinea pigs (Table I). Eleven days after immunization, six out of six responders were detected in the control animals, but only one out of six was detected after the second BCG scarification. After five BCG scarifications no responders were detected, while six out of six responders were detected in the control group. These results demonstrate that repeated scarification with Tice BCG preparation under some circumstances may suppress humoral immune competence to alloantigens.

Allograft rejection. CFDH guinea pigs received full-thickness strain-2 skin grafts after one, two, four, six, and eight scarifications. The results are shown in Table I. Mean rejection time for sham-scarified guinea pigs was 13.3 days. The rejection time in BCG-scarified guinea pigs had decreased to 9 days after the fourth scarification and was 8 days after the eighth scarification. The scarification resulted in a decrease of approximately 40% in allograft rejection time.

Histopathology of skin and regional lymph nodes. Twenty-four hours after the first BCG scarification no marked infiltrative reaction in the skin could be detected. At 4 days perivascular infiltration was prominent in the dermis and adiposus of the skin. Numerous acid-fast organisms were present in the areas of focal infiltration of polymorphonuclear and mononuclear cells. The overall response subsided by 6 days, while occasional small granulomatous reaction centers survived in the adiposus. After the second scarification, infiltration of polymorphonuclear and mononuclear cells could be detected throughout the skin at 24 hr. The general histologic pattern of the skin repeated the events observed after the first scarification. The infiltrative response of the dermis persisted beyond 6 days, however, and could still be detected at 2 weeks. The overall reaction was more intense after the fifth scarification, which was a second scarification of the right upper quadrant, and qualitatively the latter stages of the skin reaction showed a more prominent and persistent granulomatous response.

The reactivity of the regional lymph nodes was quite marked, with a lymphoproliferative response detectable 6 days after the first scarification. This pattern was repeated in regional lymph nodes of the first four scarification sites; however, during the course of scarification of alternate sites, a grossly detectable enlargement with a microscopically detectable marked histiocytic response persisted in the regional lymph node draining the first scarification site. Eventual coalescence of focal granulomas in the cortical and paracortical regions was prominent in

this node during the course of the first two scarifications. Six days after the fourth scarification (Fig. 2a), granulomatous reactivity could be detected in nodes draining the first two scarification sites but not the third or fourth. The intensity of the granulomatous reactivity and the size of the node draining the first site were always greater. By 6 days after the fifth scarification (Fig. 2b), which was a repeated scarification of the right upper quandrant, the enlarged draining node of this region was still quite marked; however, granulomatous reactivity could also be detected in the regional node draining the third scarification site.

Thus there was a prolonged responsiveness of the regional lymph nodes, and throughout the course of the first six scarifications the most highly reactive regional node was that draining the first scarification site. The progressive development of a major histiocytosis, with obliteration and loss of lymphoproliferative regions as well as medullary sinuses, eventually could be detected in the second and third regional nodes. These results indicate that while a rather acute reactivity of the skin site occurs as a function of scarification, with eventual recovery, the responsiveness of the regional lymph nodes is much more progressive and apparently influenced by subsequent BCG scarification of other sites.

Discussion. An elevation of the peripheral blood leukocyte (PBL) counts 24 hr after the second and each successive BCG scarification is indicative of an acute systemic effect, possibly extrusion of bone marrow reserves of mononuclear and polymorphonuclear cells. The PBL counts generally stabilized at a level approximately twofold above normal, and this hematogenous effect of repeated dermal challenge with BCG is in agreement with one report in man (13). While differential cell counts demonstrate marked variability in levels of lymphocytes and polymorphonuclear cells, there was a more gradual elevation of circulating monocytes throughout the course of treatment.

Histologically, no mononuclear or polymorphonuclear cell infiltration could be detected in the skin 24 hr after the first scarification. Infiltration was established 24 hr after each successive scarification. This correlates with the acute PBL response, and may be indicative of peripheral blood cell mobilization as a consequence of BCG challenge. Also, the more persistent and prominent granulomatous reaction of the skin site at the later scarifications may be a function of recruitment in the skin of circulating monocytes, which were elevated during this period.

The immediate lymphoproliferative response of regional lymph nodes with more progressive but eventually generalized histiocytosis was a most dramatic alteration of lymphatic tissue as a result of repeated BCG scarification. The discrepant enlargement of the first regional lymph node and its prominent granulomatous response during the course of the first four scarifications, followed by a similar pattern in the second and then third regional lymph nodes, definitely indicate that these nodes are responsive to subsequent distant BCG scarification. Thus, flaring of previous scarifications, activation and enlargement of draining lymph nodes, and flaring of nodes draining sites of previous scarifications were observed in the animals in this study. Similar reactions and responses are commonly observed in human subjects receiving BCG by scarification (J. U. Gutterman, G. M. Mavligit, and E. M. Hersh, unpublished data). Careful histologic studies would be of interest in such patients.

The immune assays in guinea pigs undergoing repeated BCG dermal scarification indicate a suppression of humoral immune competence to SRBC alloantigen. Although there have been many reports of enhanced humoral immunity as a function of single BCG challenge, Mathé *et al.* (14) have found that this response is variable during adjuvant therapy in experimental systems. Antigenic competition is one likely explanation for a suppressive effect resulting from repeated dermal scarification with viable BCG organisms. Antigenic competition after low doses of BCG has been reported (15); and, although competition has been more readily achieved in experimental systems with respect to humoral immunity, some limited data exist regarding antigenic competition with respect to cell-mediated immunity (16). It is interesting to note, however, that in our guinea pig model allograft reactivity generally associated with T-cell activity were augmented as a function of BCG scarification in both random-bred and inbred guinea pigs. Thus, while BCG treatment may have a depressive effect on humoral immune competence in this guinea pig model, it is capable of enhancing cell-mediated immunity as measured by allograft rejection.

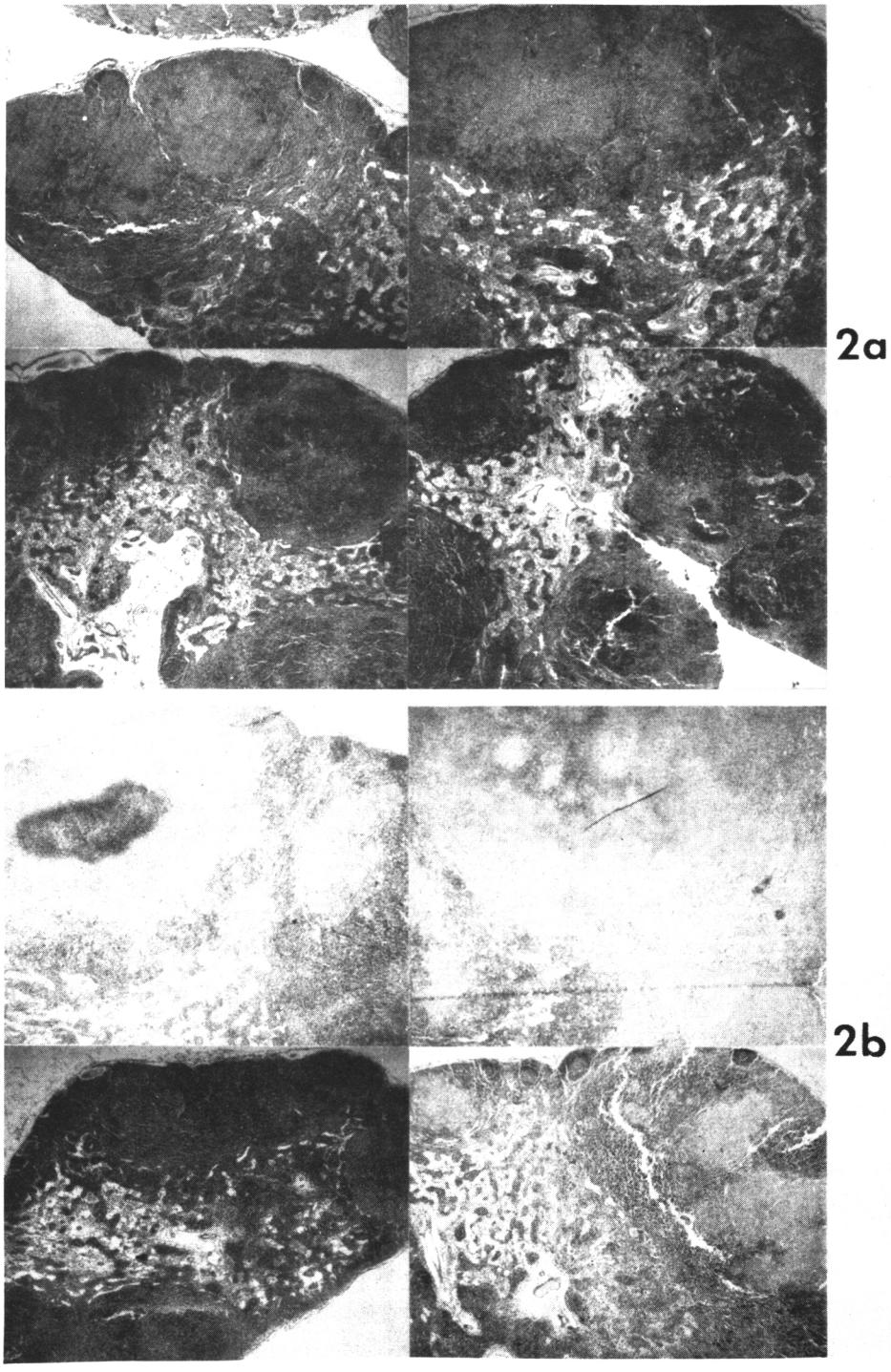

FIG. 2. Regional lymph nodes draining sites of scarification. (a) 6 days after the fourth scarification. *Top right and left*, scarifications 1 and 2. *Bottom right and left*, scarifications 3 and 4. Note coalescing granulomas in top right and left nodes. (b) 6 days after the fifth scarification. Note histiocytic node with obliterated sinuses and minimal residual lymphocytic tissue in top right node. Note abscess and prominent histiocytic obliteration of cortical region in top left node.

The possibility of split-mode immunomodulation in nonspecific adjuvant therapy was recently suggested from studies by Mathé *et al.* (14). Factors which may affect one and not another component of the immune system are dose of viable organisms, ratio of viable to nonviable BCG organisms, strain, and schedule and route of administration. Certainly several reports have demonstrated that results with this type of therapy are not unequivocally beneficial as far as tumor immunoprophylaxis or therapy is concerned; in some cases this mode of adjuvant therapy has been reported to have a positive effect on tumor growth (14, 17). One plausible explanation for such an effect is a greater stimulation of humoral immunity than of cell-mediated immunity, resulting in "blocking factors" that protect tumor cells against cytotoxic lymphocytes. Thus an ideal condition would be the one described in the present report, where humoral immunity may be suppressed while cell-mediated immunity is enhanced. The conditions that contribute to this split mode of immune modulation as a result of BCG challenge warrant further consideration and investigation.

Summary. Immune competence to sheep erythrocytes, skin allografts, and antigenic tumor transplants was studied in random-bred CFDH as well as inbred (syngeneic) strain-2 guinea pigs receiving repeated BCG scarification. Peripheral blood leucocyte (PBL) changes and histopathological alterations of the skin site and regional lymph nodes were also evaluated during the course of treatment. The results demonstrate that in this experimental model BCG scarification depresses humoral immune competence and augments cellular immunity. Elevation of the PBL counts was also indicative of an acute systemic effect resulting from repeated BCG treatment. Flaring of previous scarification sites, activation and enlargement of draining nodes, and progressive responsiveness of nodes draining sites of previous scarification were also observed.

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