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Pleiotropic effects of the *Bcg* gene: III. Respiratory burst in *Bcg*-congenic macrophages

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SUMMARY

Macrophage respiratory burst, as assessed by H_2O_2 and O_2^- production, and HMS and chemiluminescence activity was investigated in a variety of conditions in macrophages from *Bcg*-congenic mice. Measurement of HMS and chemiluminescence in splenic macrophages challenged *in vitro* with BCG showed that the Bcg^r cells were more stimulated by the challenge than their Bcg^s counterparts. H_2O_2 production was measured in Bcg^r and Bcg^s splenic macrophages. PMA-triggering and LK-triggering were shown to stimulate a similar degree of H_2O_2 production Bcg^r and Bcg^s macrophages. In contrast, *in vitro* phagocytosis of BCG was shown to trigger superior production of H_2O_2 and O_2^- in the Bcg^r splenic macrophages as compared to their Bcg^s congenics. Finally, following the *in vivo* infection with BCG Montreal, Bcg^r splenic macrophages were superior producers of H_2O_2 (both spontaneous and PMA-triggered) in the early phase of infection.

Keywords *Bcg* gene macrophages H_2O_2 production HMS activity

INTRODUCTION

The resistance to infection with small doses of BCG Montreal is controlled by a chromosome 1 *Bcg* gene (Gros, Skamene & Forget, 1981) which is phenotypically expressed as an intrinsic function of the macrophage (Stach *et al.*, 1984).

Recent reports from this laboratory have been concerned with a systematic examination of macrophage functions in resistant (Bcg^r) and susceptible (Bcg^s) mice. We have demonstrated that Bcg^r macrophages are superior to their Bcg^s congenic counterparts in Ia expression, in antigen presentation and in the development of non-specific bactericidal activity (Denis *et al.*, 1988). These phenomena were interpreted as being indicative of an enhanced ability of resistant macrophages to become activated.

The magnitude of a respiratory burst is dependent upon the state of macrophage activation (Nathan and Root, 1977; Johnston, Godzik & Cohn, 1978; Jackett *et al.*, 1981). The data presented in this study show that Bcg^r macrophages are also superior in their oxidative metabolism compared to their Bcg^s congenics, thus suggesting a link between respiratory burst activity (as a marker of macrophage activation) and a natural resistance to BCG.

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MATERIALS AND METHODS

Mice

B10.ASgSn (B10.A) mice were bred in our laboratory from breeding pairs originally obtained from Jackson Laboratories, Bar Harbor, Maine. Inbred BALB/c mice were obtained from Canadian Breeding Farm, Montreal. C.D2 congenic mice were bred from the breeding pairs of the CD2-Idh-1^b-Pep-3^b line (Potter *et al.*, 1981). They are labelled as BALB/c.Bcg^r in this publication. The B10.A.Bcg^r congenic strain was constructed in this laboratory by the backcross NX system using resistance to BCG Montreal as a selective agent. The Bcg^r allele came from the A/J strain. Mice of either sex, 8–10 weeks old, were used for all experiments.

Bacterial strains and infection of mice

The BCG Montreal inoculum was prepared as described previously (Forget *et al.*, 1981). Mice were inoculated in the caudal tail vein with the dose of approximately $2{\cdot}5 \times 10^4$ colony-forming units (CFU). Growth curves of this strain in Bcg^r and Bcg^s mice have been shown elsewhere (Gros, Skamene & Forget, 1981).

Splenic adherent cells (SAC)

Mice were killed by cervical dislocation and their spleens were removed. A single cell suspension free of red blood cells was prepared using standard methods. Following centrifugation at 4°C, the cell pellet was resuspended in Dulbecco's Modified

Eagle's Medium (DMEM) with 15% fetal calf serum (FCS) at 5×10^7 cells/ml, plated in plastic dishes (Becton-Dickinson, 3001, Oxnard, Ca.) in one ml aliquots and then washed with warm PBS to remove non-adherent cells. One ml of DMEM (Gibco, Grand Island, NY) with 15% FCS and 3mM glutamine (Gibco) was added and the cells incubated until they were processed.

Collection of adherent cells

Adherent cells were dislodged from the plastic surfaces 48 h after plating whole spleen cells. Before collection from plastic dishes, splenic adherent cells were shown to be more than 97% macrophages by the acridine orange staining method (Hertel-Wulff, 1979). For detachment of these cells, one ml of 15 mM EDTA (1:5000 Versene, Gibco) was added to each dish. Following incubation at 37°C for 30 min the dishes were swirled vigorously to detach adherent cells. Cells were counted using the trypan blue, washed three times and adjusted to 5×10^6 cells/ml in PBS. Cell viability (98%) and the proportion of macrophages in the total population of adherent cells (> 97%) were similar in macrophage preparations of both allelic types. There was no significant difference in the protein content of Bcg^s and Bcg^r preparations.

Isotopes

D-[1-^{14}C] glucose and D-[6-^{14}C] glucose (specific activity of 60 mCi/mmol) were purchased from New England Nuclear Corporation (Boston, Mass). Isotope solutions at a concentration of 20 μCi/ml were divided in 10 μl aliquots and stored frozen at −20°C until used.

Phagocytic stimulus

BCG bacilli were grown as previously described (Forget *et al.*, 1981). Before use as a phagocytic stimulus, BCG was dialysed 48 h vs PBS and was suspended in PBS at a concentration of 5×10^7 CFU/ml.

Serum

Pooled serum from B10.A mice (10% concentration) was used in the hexose monophosphate shunt (HMS) assays. Serum was stored at −70°C in small aliquots until used.

Measurement of hexose monophosphate shunt (HMS) activity

Measurement of HMS activity in SAC was made after two days of culture according to the method of Dechatelet and Pierce (1981). The reactions were performed in 50 ml Erlenmeyer flasks in a total volume of 3 ml. Cells were suspended in PBS at a concentration of 5×10^6/ml. Reaction was initiated by the addition of cells, 5×10^7 BCG in 1 ml or PBS only in the case where resting HMS was measured, and by the addition of 0·7 ml containing 0·2μCi of the appropriate isotope in F12 medium without glucose (Gibco) and 0·3 ml of pooled serum. A vial with 0·5 ml hyamine hydroxide (Aldrich Chemicals, Milwaukee, WI) was fitted in the centre of the flask. Flasks were capped firmly and transferred to a 37°C shaking water bath. Reactions were started at timed intervals and stopped in the same sequence. At least four assays were performed for each set of conditions. Following 60 min of incubation or as otherwise indicated, reactions were stopped by adding 1·0 ml of 10% trichloracetic acid (Aldrich Chemicals, Milwaukee, WI), followed by further incubation for 30 min. Three millilitres of scintillation fluid

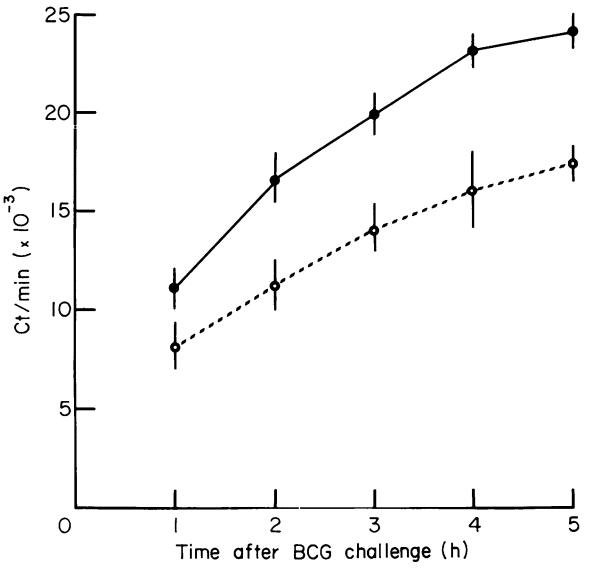

Fig. 1. Time course of HMS activity in spleen macrophages of B10.A (○) and B10.A.Bcg^r (●) strains. Results expressed as ct/min ± s.d. (standard deviation) of at least six determinations. Similar results were obtained in two additional experiments. $P < 0{\cdot}05$ at all time points.

(Aquafluon, NEN) were then added to each vial. Vials were capped and radioactivity determined in a liquid scintillation counter (Packard, Scarborough, Ontario). Since live organisms were used for challenge, controls were included in which macrophages were omitted to account for glucose oxidation of the organism itself. Data are thus expressed as Δct/min, reflecting directly the HMS activity (ct/min from [1-^{14}C] glucose-ct/min from [6-^{14}C] glucose).

Release of O_2^-

Macrophages were assayed for O_2^- production over an appropriate period of time by monitoring the superoxide dismutase-inhibitable reduction of ferricytochrome C (Sigma, St. Louis, Mo.) (Johnston, Godzik & Cohn, 1978), 30 h after plating whole spleen cells. In selected experiments, O_2^- release following phagocytosis of various mycobacteria was measured; the assay mixtures were cleared of bacteria by filtration before reading absorbance.

Determination of chemiluminescence

Measurement of SAC chemiluminescence was performed 2 days after plating. Adherent cells were detached from plastic surfaces, counted with trypan blue and distributed in scintillation vials (1×10^6 cells per vial in 1 ml of Hank's balanced salt solution (HBSS) plus 10% FCS). Luminol reagent was a saturated solution of luminol (5-amino 2,3 dihydro-1,4 phtalazinedione (Sigma) in heat inactivated FCS (Allen, 1977), at 10^{-4}M in FCS in 1 ml. Samples were kept for 1 h in the dark. Triplicate samples were measured for 10 min before the addition of bacteria to determine background activity. A suspension of approximately 10^7 bacteria in 1 ml. HBSS (Gibco) was then added (final volume 3 ml) and measurements continued. Measurements of the chemiluminescence induced by the interaction of bacteria with splenic macrophages was carried out in an out of coincidence mode (Allen, 1977). Control cells

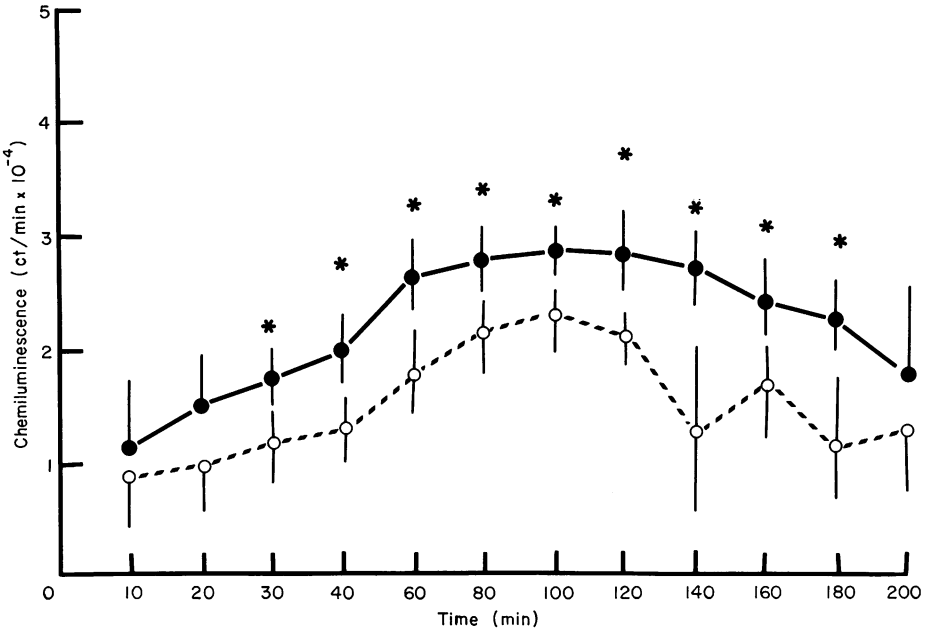

Fig. 2. Chemiluminescence response of splenic macrophages from B10.A.*Bcg*^{r} (●) and B10.A (○) mice to BCG. Each point indicates the mean value for five determinations ± s.d. Similar results were obtained in two additional experiments. *$P < 0.05$.

Table 1. O_2^- release by splenic macrophages following activation by various stimuli

Source of splenic macrophages	Priming with LPS*	Stimulus	O_2^- release†
A) B10.A	—	BCG	79 ± 13
B) B10.A	+	BCG	336 ± 21
C) B10.A.*Bcg*^{r}	—	BCG	148 ± 26
D) B10.A.*Bcg*^{r}	+	BCG	431 ± 35

* Macrophages exposed overnight *in vitro* to LPS (10 ng/ml).

† Values expressed as nmol O_2/mg protein/3 h ± s.e.m of four independent experiments.

A vs C; B vs D; $P < 0.05$.

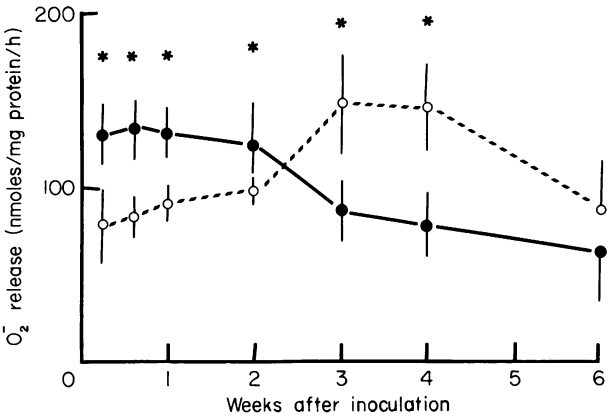

Fig. 3. PMA-stimulated O_2^- release by splenic macrophages of the B10.A strain (○) and the B10.A.*Bcg*^{r} (●) following i.v. inoculation with a low dose (1.8×10^4 CFU) of BCG Montreal. Values shown are mean ± s.d. of four independent experiments. * $P < 0.05$.

incubated in HBSS (no bacteria) produced equivalent amounts of background chemiluminescence (data not shown).

To prepare lymphokines (LK), a suspension of spleen cells (10^7/ml) was cultured in complete medium containing CON A (Sigma) (5μg/ml). After 3 days, culture supernatant was collected and dialysed against DMEM for 48 h. Supernatants were then filter-sterilized and aliquoted. LK-induced H_2O_2 production was induced by culturing SAC in 1 ml of LK supernatant or control supernatant diluted in complete medium to the desired concentration. Dishes were incubated for 72 h and medium in the dishes was replaced by fresh material of the same type at 24 h intervals. BCG-induced H_2O_2 production was measured following the phagocytosis of bacilli by macrophages at various BCG to cell ratios.

H_2O_2 measurement

H_2O_2 production was measured 30 h after plating whole spleen cells by the method of horseradish peroxidase (HRP) dependent oxidation of phenol red as described by Freund and Pick (1985). Culture supernatants were removed, monolayers were rinsed with phenol red-free HBSS and a solution containing phenol red (Sigma) 0·2 mg/ml, HRP (HRP, type 2, 190 purpurogallin U/mg, Sigma), 20 units/ml and in some cases phorbol myristate acetate (PMA) (Sigma 125 ng/ml) or the appropriate amounts of BCG (1 ml total in HBSS without phenol red) was added to each dish. Plates were incubated for appropriate periods of time at 37°C. Supernatants were removed and centrifuged at 1,200 *g* for 10 min and the oxidized phenol red in the supernatants was measured by absorbance at 610 nm. Mean absorbance values were calculated and results expressed as nmol H_2O_2/mg protein/h using standard curves generated with H_2O_2 solutions of known concentrations. Protein determinations were made on homoge-

Table 2. H_2O_2 production induced by different stimuli in *Bcg*^{r} and *Bcg*^{s} splenic macrophages

Strain	Priming signal	Triggering signal	H_2O_2 production (nmoles/mg protein/h)
A) BALB/c	—	PMA	89±18*
B) BALB/c.*Bcg*^{r}	—	PMA	98±21
C) BALB/c	—	LK	98±15
D) BALB/c.*Bcg*^{r}	—	LK[3]	101±12
E) BALB/c	LK	PMA	172±31
F) BALB/c.*Bcg*^{r}	LK	PMA	189±18
G) BALB/c	—	BCG (25:1)	51±17
H) BALB/c.*Bcg*^{r}	—	BCG (25:1)	89±11
I) BALB/c	—	BCG (50:1)	63±26
J) BALB/c.*Bcg*^{r}	—	BCG (50:1)	126±17

* Results expressed as means±s.d. of three independent experiments for each stimulus.

G vs H and I vs J significant at $P<0.01$.

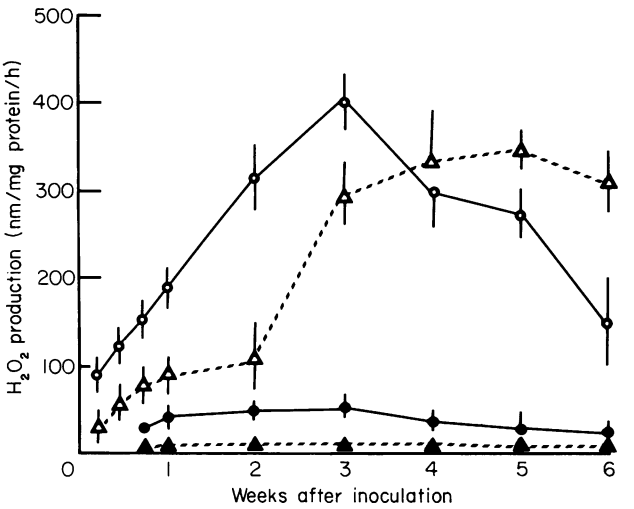

Fig. 4. H_2O_2 production in BALB/c.*Bcg*^{r} and BALB/c strain spleen macrophages following i.v. infection with a low dispersed dose of BCG Montreal. BALB/c.*Bcg*^{r} spontaneous (●) and PMA-induced (○) and BALB/c spontaneous (▲) and PMA-induced (△) H_2O_2 production was measured at various times following infection. Results of three independent experiments. Spontaneous H_2O_2 production: significantly ($P<0.05$) higher production in BALB/c-*Bcg*^{r} SAC at 5 days 1, 2, 3 and 4 weeks after infection. PMA-induced H_2O_2 production: significantly ($P<0.05$) higher production in BALB/c.*Bcg*^{r} SAC from 24 hours to 3 weeks. Significantly ($P<0.05$) higher production in BALB/c SAC at 5 and 6 weeks.

nates prepared by incubating adherent cells with 1 ml NaOH (1 M) for 24 h at 37°C, by the method of Lowry *et al.*, (1951) with bovine serum albumin (BSA) as standards.

Endotoxin contamination

Reagents were endotoxin-free, as determined using the E-toxate kit purchased from Sigma.

Statistical analysis

Statistical significance of the data was analysed using Student's *t*-test.

RESULTS

Stimulation of the macrophage hexose-monophosphate shunt by BCG infection

Splenic macrophages from *Bcg*-congenic mice were examined for their HMS activity following BCG challenge. *Bcg*^{r} macrophages were significantly superior ($P<0.05$) to their *Bcg*^{s} counterparts in the stimulation of HMS activity over the whole period of experiment (Fig. 1).

Chemiluminescence of Bcg^r *and* Bcg^s *splenic macrophages following phagocytosis of mycobacteria*

The chemiluminescence reaction of splenic macrophages from *Bcg*^{r} and *Bcg*^{s} mice following phagocytosis of BCG is shown in Fig. 2. Macrophages from *Bcg*^{r} mice were higher responders, starting at 30 min following the addition of bacteria and still being significantly different at 180 min after challenge with BCG.

O_2^- release by normal Bcg^r *and* Bcg^s *splenic macrophages following* in vitro *stimulation*

Bcg^r and *Bcg*^{s} splenic macrophages were challenged *in vitro* at a ratio of approximately 25 bacteria: cell and the O_2^- release was monitored. In selected experiments, macrophages were primed by overnight exposure to LPS (10 ng/ml) prior to stimulation with mycobacteria. Results are shown in Table 1. With or without priming by exposure to LPS, *Bcg*^{r} splenic macrophages were superior producers of O_2^- than their *Bcg*^{s} counterparts upon challenge with BCG Montreal.

O_2^- release by splenic macrophages following infection with a small dose of BCG Montreal

In the next set of experiments O_2^- release by splenic macrophages of *Bcg*^{r} and *Bcg*^{s} mice was measured following intravenous inoculation of a small dose of BCG Montreal. Results are shown in Fig. 3. *Bcg*^{r} splenic macrophages were superior producers of PMA-induced O_2^- release in the early phase of infection (4 days to 2 weeks) than their *Bcg*^{s} counterparts.

H_2O_2 production by Bcg^r *and* Bcg^s *macrophages from normal mice*

In the first series of experiments the capacity of macrophages to produce H_2O_2 following triggering with PMA was investigated. SAC of both strains released a copious amount of H_2O_2 but no significant differences could be demonstrated between *Bcg*^{r} and *Bcg*^{s} mice. The effect of lymphokine treatment of macrophages on the H_2O_2 production was examined next. Enhancement of H_2O_2 production was observed in LK-treated SAC of both strains, H_2O_2 production rose progressively in LK-treated cells and reached a peak at 3 days without any significant differences between *Bcg*^{r} and *Bcg*^{s} cells. In a separate set of experiments, splenic macrophages were treated with LK for 3 days after which they were triggered for H_2O_2 production with PMA. This led to equivalent H_2O_2 production in both *Bcg*^{r} and *Bcg*^{s} macrophages.

Finally, the capacity of SAC from normal *Bcg*^{s} and *Bcg*^{r} mice to produce H_2O_2 following BCG phagocytosis *in vitro* was studied (Table 2). BCG-triggering of H_2O_2 release by SAC was

efficient in both strains. However, significantly higher production of H_2O_2 by Bcg^r SAC was seen in comparison with that of Bcg^s cells.

Macrophage H_2O_2 production in BCG infected mice

The spontaneous and PMA-triggered H_2O_2 production by Bcg^r and Bcg^s SAC was measured following i.v. infection with 2×10^4 BCG.

SAC from Bcg^r mice released significant though small amounts of H_2O_2, notably at 4 days, and at 1, 2, 3, and 4 weeks after infection, whereas SAC from Bcg^s mice were very inefficient producers of spontaneous H_2O_2. PMA-triggered H_2O_2 production by macrophages from BCG-infected mice was also studied. The Bcg^r SAC were superior H_2O_2 producers in the early part of infection (from 4 days to 3 weeks after infection). After 3 weeks, PMA-induced H_2O_2 release subsided and diminished progressively until 6 weeks after infection. The H_2O_2 production by SAC of infected Bcg^s mice increased slowly following infection but increased sharply at 3 weeks after infection. Four weeks after infection, similar H_2O_2 release was measured in both strains. However, in Bcg^s mice PMA-induced H_2O_2 release remained undiminished after 4 weeks and at 5 weeks and 6 weeks after infection. Superior release of H_2O_2 was measured in the Bcg^s macrophages when compared with their Bcg^r counterparts (Fig. 4).

DISCUSSION

Innate resistance to infection with BCG and with a variety of other mycobacteria (Forget *et al.*, 1981; Denis *et al.*, 1986) has been shown to be controlled by a locus situated on chromosome 1 which also controls the resistance to *Salmonella typhimurium* and *Leishmania donovani*. Several laboratories documented that the macrophage expresses this gene by the demonstration of enhanced microbiostatic or microbicidal activity *in vitro* exhibited by macrophages explanted from Ity^r mice (Lissner, Swanson and O'Brien, 1984).

Descriptive studies dealing with the characteristics of macrophage populations in genetically-resistant and susceptible strains suggest the myriad pleiotropic effects of this gene. We have recently demonstrated that the *Bcg* gene regulates the degree of macrophage ability to present various unrelated antigens to T cells (Denis *et al.*, 1988). This observation, taken together with other findings, namely that splenic macrophages from Bcg^r mice contain higher proportion of Ia^+ cells than their Bcg^s counterparts, that the Bcg^r peritoneal macrophages (in contrast to Bcg^s macrophages) support continuous Ia^+ antigens expression *in vitro* (Johnson and Zwilling, 1985) and that the Bcg^r macrophages demonstrate enhanced microbicidal activity against a variety of microbial agents (Lissner, Weinstein and O'Brien, 1985) suggest that the *Bcg* gene might modulate the acquisition of macrophage competence for activation.

One of the objective markers for characterizing the stage of macrophage activation has been the magnitude of respiratory burst (Johnston, Adams and Hamilton, 1986). The H_2O_2 release as well as increased superoxide production by macrophages have been shown to depend on the degree of sequential activation and triggering by the number of immunologic and nonimmunologic signals (Nathan and Root, 1977; Johnston, Godzik and Cohn, 1978). Similarly, the stimulation of HMS activity and an increase in glucose oxidation is considered to be a reliable marker of macrophage activation following challenge with a variety of immunomodulating substances (Myrvik and Evans, 1967; De Chatelet *et al.*, 1982).

This paper demonstrates that all criteria of induced respiratory burst were significantly greater in Bcg^r splenic macrophages exposed to BCG *in vitro* or *in vivo* compared with Bcg^s macrophages.

Our data thus suggest the quantitative link between the ability of the splenic macrophages to become activated by exposure to BCG and the resistance to infection *in vivo*. It should be pointed out that such a link has already been alluded to in the system of natural resistance to *Leishmania donovani* (Crocker, Blackwell and Bradley, 1984). In that particular study, only the animals genetically resistant to *Leishmania* infection responded favourably to treatment with macrophage activating substances *in vivo* by the enhancement of resistance.

The question raised by our studies is whether the enhanced activity of oxidative burst induced by exposure to BCG in the macrophages of genetically resistant animals represents just another facet of the pleiotropic effect of the *Bcg* gene, or whether it is in fact related to the antibacterial resistance *in vivo*. It has been demonstrated that the killing of mycobacteria by macrophages requires macrophage production of oxygen metabolites, notably H_2O_2 (Jackett, Aber & Lowrie 1978a; Jackett, Aber & Lowrie 1978b). In addition to this, macrophage killing of mycobacteria can be inhibited by catalase (Walker and Lowrie, 1981). Many factors have been shown to enhance release of oxygen metabolites: infection (Nathan and Root, 1977: Lepay *et al.*, 1985), phagocytosis of mycobacteria (Gangadharam and Edwards, 1984) and priming of macrophages by bacterial products (Pabst and Johnston, 1980; Murray, 1982). However, recent evidence suggests that mycobacterial growth inhibition is not dependent upon release of reactive oxygen intermediates (Flesh & Kaufmann, 1987).

For the moment, the only definite interpretation that our data allow is that the Bcg^r and Bcg^s macrophages are, for genetic reasons, in a different state of responsiveness. It appears that the Bcg^r macrophages have already been primed for activation more effectively than the Bcg^s macrophages. Consequently, the phagocytosis of mycobacteria leads to the quantitatively different expression of many markers of macrophage activation in the Bcg^r and Bcg^s cells, the respiratory burst described in this study being but one of them.

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