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# Regulation of Mycobacterial Growth by the Hypothalamus-Pituitary-Adrenal Axis: Differential Responses of *Mycobacterium bovis* BCG-Resistant and -Susceptible Mice

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**The role of the hypothalamus-pituitary-adrenal (HPA) axis in regulating the growth of *Mycobacterium avium* in *Mycobacterium bovis* BCG-resistant and -susceptible congenic mice was evaluated. Restraint was used to activate the HPA axis, which resulted in an increase in the level of corticosterone in the plasma. Activation of the HPA axis increased the susceptibility of BALB/c.*Bcg*$^s$ mice to the growth of *M. avium*. In contrast, the growth of *M. avium* was not altered in BALB/c.*Bcg*$^r$ mice as a result of HPA activation. Adrenalectomy abolished the effect of HPA activation on mycobacterial growth, as did treatment of the mice with a glucocorticoid receptor antagonist, RU 486. Activation of the HPA axis also resulted in the increased susceptibility of splenic macrophages from *Bcg*$^s$ mice but not from *Bcg*$^r$ mice to *M. avium* growth in vitro. The production of tumor necrosis factor alpha and of reactive nitrogen intermediates by splenic macrophages from both strains of mice was suppressed as a result of HPA activation. The implications of these findings for resistance controlled by *Bcg* and for susceptibility to mycobacterial growth are discussed.**

The incidence of mycobacterial diseases has increased dramatically in the United States during the past 10 years (6, 28). An increase in *Mycobacterium avium* infections has occurred largely because of infection by the human immunodeficiency virus. While approximately 50% of the increased incidence in *Mycobacterium tuberculosis* can also be attributed to human immunodeficiency virus infection, infection of individuals who are not infected with human immunodeficiency virus has also increased significantly. The susceptibility of humans to mycobacterial disease is determined, in part, by genetic differences (13, 47, 51, 52) but is also affected by their environment. Homelessness and malnutrition (7, 26) are two cofactors that can contribute to the likelihood of mycobacterial infection. Additional factors such as ageing, chronic alcoholism, and stress have also been cited as being associated with activation of mycobacterial disease (11, 16, 22, 24, 33, 35, 37, 39, 58).

In mice, the genetic resistance to the in vivo growth of mycobacteria has been shown to be controlled by a gene, termed *Bcg*, which maps to chromosome 1 (48). A syntenic group of genes maps to human chromosome 2q in humans (47). We have reported that macrophages from congenic *Bcg*$^r$ and *Bcg*$^s$ mice express major histocompatibility complex (MHC) class II glycoproteins differently. Thus, macrophages from *Bcg*$^r$ mice can be induced to persistently express I-A, while macrophages from *Bcg*$^s$ mice will only transiently express I-A (25, 63). In subsequent studies, we reported that I-A expression by macrophages from the *Bcg*$^s$ mice was suppressed by activation of the hypothalamic-pituitary-adrenal (HPA) axis as a result of a stressor, physical restraint (60, 62). In contrast, I-A expression by macrophages from the *Bcg*$^r$ mice was not affected by HPA axis activation. Together, these observations suggested that mycobacterial growth that is controlled by *Bcg* may also be differentially affected by HPA axis activation.

The purpose of this investigation was to explore the role of the HPA axis in regulating the growth of *M. avium* in congenic *Bcg*$^r$ and *Bcg*$^s$ mice. We found that activation of the HPA axis by restraint increased the susceptibility of *Bcg*$^s$ mice to mycobacterial growth but did not affect the ability of *Bcg*$^r$ mice to limit the growth of the mycobacteria. The suppressive effects of HPA axis activation also resulted in an increased susceptibility to mycobacterial growth within macrophages from the *Bcg*$^s$ mice. The effect of HPA axis activation on the in vivo growth of the mycobacteria was abrogated by adrenalectomy and by treatment of mice with the glucocorticoid receptor antagonist RU 486. Activation of the HPA axis resulted in a suppression of tumor necrosis factor alpha (TNF-α) and reactive nitrogen intermediates produced by macrophages from both *Bcg*$^r$ and *Bcg*$^s$ mice.

## MATERIALS AND METHODS

**Animals.** Male BALB/c.*Bcg*$^s$ mice were obtained at 6 weeks of age from Harlan-Sprague-Dawley (Indianapolis, Ind.). The mice were housed in groups of five in PCS-80 laminar flow isolation cages (Lab Products, Inc., Maywood, N.J.). The mice were given food and water ad libitum and acclimated to their housing environment prior to the initiation of all experiments. Adrenalectomized and sham adrenalectomized mice were also obtained from Harlan-Sprague-Dawley. Adrenalectomized mice were then maintained on 1% saline in drinking water. BALB/c.*Bcg*$^r$ mice were provided by Michael Potter (National Cancer Institute [NCI]) (38) and bred in our animal facility. Male mice were used at 6 to 8 weeks of age.

**HPA activation via restraint stress.** To activate the HPA axis, the mice were restrained as described by Zwilling et al. (60). Briefly, the mice were placed individually into 50-ml conical centrifuge tubes which were punctured to allow for

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ventilation and to prevent hyperthermia. The tubes allow for limited forward and backward movement. The mice were maintained horizontally in tubes for single or multiple 18-h restraint cycles. Mice were restrained prior to the initiation of the dark (6-p.m.) phase of the 12-h day-night cycle and removed the following morning at the conclusion of the 18-h stress period. Following one or five 18-h restraint cycles, the mice were returned to their standard housing and then sacrificed 12 days after the initiation of the experiment. The mice that received 10 restraint cycles were restrained initially for 5 consecutive cycles, returned to their standard housing for 2 days, and then restrained again for another 5 consecutive cycles. The mice were then sacrificed immediately after the last restraint on day 12. Control transported mice were housed in groups of five and deprived of both food and water during periods in which the experimental mice were being restrained. Conventionally housed mice served as an additional control.

**Administration of RU 486 (Mifepristone).** The glucocorticoid receptor antagonist RU 486 (36) was kindly provided by Roussel-Uclaf (Romainville, France). RU 486 was dissolved in polyethylene glycol (molecular weight, 400) and injected subcutaneously at a dose of 25 mg/kg of body weight daily, beginning at 2 days prior to the initiation of restraint and continuing throughout the entire restraint period. Control mice were injected with equal volumes of the vehicle.

**Hormone replacement therapy.** Time release pellets (Innovative Research of America, Toledo, Ohio) were aseptically inserted through 5-mm incisions on the backs of the animals. The incisions were treated with topical antibiotics and sutured. To achieve basal hormone levels, pellets containing either 0.001 mg of epinephrine, 5 μg of *d*-aldosterone, and 0.5 mg of corticosterone were implanted (19). To achieve corticosterone levels consistent with those found following HPA axis activation, pellets containing 15 mg of corticosterone were implanted together with those containing basal epinephrine and *d*-aldosterone. Placebo pellets of cellulose were implanted in control mice. Mice were allowed to recover for 48 h before infection with *M. avium*.

**Determination of levels of corticosterone and ACTH in plasma.** Plasma samples were obtained at the time of sacrifice from blood anticoagulated with 5% EDTA. The concentrations of corticosterone and adrenocorticotropin (ACTH) were determined on fresh plasma by radioimmunoassays with an assay kit obtained from ICN Biomedical (Irvine, Calif.) as per the manufacturer's instructions. The amount of corticosterone or ACTH was calculated from a standard curve and expressed as nanograms (for corticosterone) or picograms (for ACTH) per milliliter.

**Assessment of in vivo mycobacterial growth.** A clinical isolate of *M. avium* (62) was initially grown on Lowenstein Jensen medium and transferred to Middlebrook 7H9 broth seed cultures. The mycobacteria were grown to a density of $5.5 \times 10^8$ CFU/ml of Middlebrook 7H9 broth and stored frozen at −70°C in 1-ml vials until use. The mycobacteria were diluted to $5 \times 10^4$ CFU in 0.2 ml of sterile pyrogen-free saline and injected intravenously via the tail veins. To determine the number of CFU contained within the spleens and lungs of mice, the organs were aseptically removed and homogenized with tissue sieves (Sigma Chemical Co., St. Louis, Mo.). Suspensions were serially diluted into sterile pyrogen-free saline and plated onto mycobacteria 7H11 agar supplemented with Middlebrook OADC Enrichment (Difco Laboratories, Detroit, Mich.). The plates were incubated for 2 weeks at 37°C in an atmosphere containing 10% $CO_2$ in air. CFU were determined and expressed as CFU per gram of tissue per gram of body weight.

**In vitro antimycobacterial activity.** The antimycobacterial activity of macrophages was assessed as described by Flesch and Kaufman (18). Splenic macrophages were obtained from pooled spleens, passed through tissue seives into Hanks' balanced salt solution (GIBCO, Grand Island, N.Y.) supplemented with 20% defined fetal bovine serum (Hyclone Laboratories, Logan, Utah), and subsequently passed through sterile needles with successively decreasing bore sizes in order to achieve a single-cell suspension. The cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) (GIBCO-BRL, Grand Island, N.Y.) supplemented with 10% defined fetal bovine serum and glutamine but without antibiotics. The splenic macrophages from this suspension were then enriched by adherence onto tissue culture dishes (100 by 20 mm; Becton Dickinson Labware, Lincoln Park, N.J.) by culturing the cells at 37°C overnight in an atmosphere containing 10% $CO_2$. Nonadherent cells were removed by gentle washing with Hanks' balanced salt solution, and adherent cells were removed by scraping with a rubber policeman. The cells were cultured again at a concentration of $10^5$ macrophages per well in a 96-well microtiter plate (Becton Dickinson Labware), and the plates were incubated overnight. The cultures were again washed with Hanks' balanced salt solution to remove remaining nonadherent cells. Purified macrophage cultures were then infected with $4 \times 10^5$ CFU of *M. avium* (bacteria-to-macrophage ratio of 4:1) suspended in 0.2 ml of IMDM without antibiotics and incubated overnight (16 h) at 37°C to allow for phagocytosis. Following incubation, the cultures were washed with fresh IMDM to remove any uningested bacteria. The infected macrophage cultures were then incubated in IMDM without serum for a period of 5 days to allow intracellular growth of the ingested bacteria. At the end of this incubation period, the cultures were lysed and pulsed with media containing a mixture (1:1) of 7H9 medium and IMDM with [$^3$H]uracil (5 μCi/ml) (Amersham, Chicago, Ill.; specific activity, 40 to 60 Ci/mmol) and 0.1% saponin. The bacteria were incubated overnight and were harvested onto glass fiber filter strips with a PHD Cell Harvester (Cambridge Technology, Inc., Watertown, Mass.). Radioactivity incorporated by the released bacteria was quantitated by liquid scintillation spectrometry.

**Production of TNF-α and reactive nitrogen intermediates.** Purified splenic macrophages at a concentration of $10^5$ macrophages per well in 96-well microtiter plates were stimulated with 100 ng of lipopolysaccharide (LPS; *Escherichia coli* O111:B4; Sigma) and 100 U of recombinant gamma interferon (rIFN-γ; GIBCO-BRL) for 72 h at 37°C. Cell-free supernatants were then used to determine the amounts of TNF-α and reactive nitrogen intermediates. TNF-α production was determined by enzyme-linked immunosorbent assay (ELISA) (Endogen Inc., Boston, Mass.), and the amount of reactive nitrogen intermediates (nitric oxide as indicated by the presence of the stable intermediate [$NO_2^-$] nitrite) was determined by using the Griess reagent (20). The amounts of TNF-α and nitric oxide (nitrite) were calculated with a standard curve and expressed as picograms per milliliter (for TNF-α) or micromolar (nitric oxide).

**Statistical analysis.** Each observation of the mycobacterial counts in the in vivo experiments was made for an independent animal, allowing the data to be analyzed with analysis of variance models (generally two-factor models). The analysis was done on a log scale in order to better satisfy the normality and homoscedasticity assumptions, and an exam-

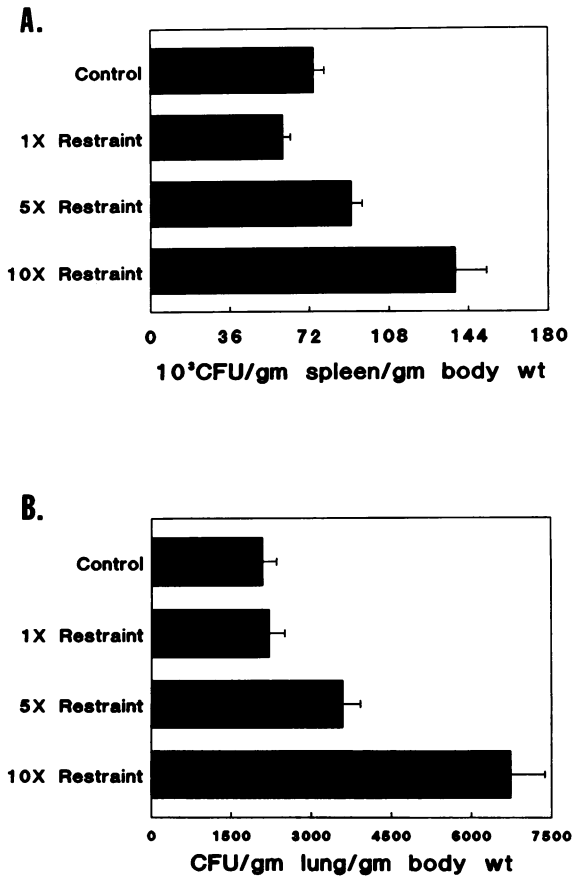


FIG. 1. Susceptibility to mycobacterial growth is increased as a result of HPA axis activation. Mice were restrained for 1, 5, or 10 18-h cycles after infection with $5 \times 10^4$ CFU of *M. avium*. The numbers of CFU in the spleens (A) and lungs (B) were determined 12 days after injection of the mycobacteria. The numbers of CFU isolated from the spleens and lungs prior to restraint were 13,519 CFU/g of spleen per g of body weight and 584 CFU/g of lung per g of body weight, respectively. The data are the means ± standard deviations (SD) for seven animals per group. The differences between 5- and 10-cycle restraint and control mice were significant by analysis of variance ($P < 0.001$).

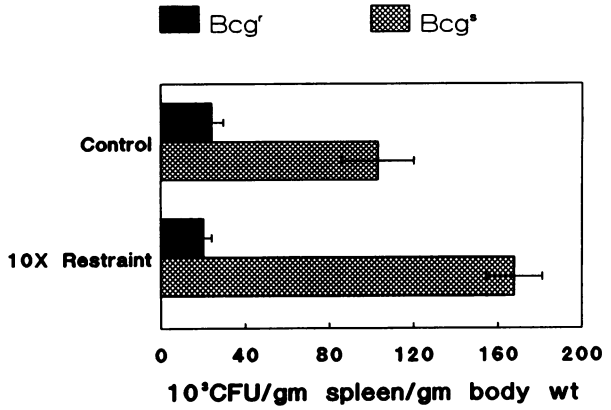


FIG. 2. Differential effect of HPA axis activation on mycobacterial resistance of BALB/c.*Bcg*$^r$ and BALB/c.*Bcg*$^s$ mice. Mice were restrained for 10 18-h cycles, and the numbers of CFU of *M. avium* in the spleens were determined 12 days after the infection. The numbers of CFU isolated from the spleens of *Bcg*$^r$ or *Bcg*$^s$ mice prior to restraint were 19,462 CFU/g of spleen per g of body weight and 18,083 CFU/g of spleen per g of body weight, respectively. The data are the means ± SD for seven animals per group. The difference between the values for the restraint and control groups of *Bcg*$^s$ mice was significant ($P < 0.001$). The difference between the growth of the mycobacteria in the spleens of *Bcg*$^r$ mice versus growth in those of *Bcg*$^s$ mice was also significant ($P < 0.001$). Similar observations were made concerning differences in the effect of HPA activation on growth of the mycobacteria in the lungs (data not shown).

ination of residual plots did not contradict the validity of the methods used. Finally, reports of the pairwise comparison of treatment groups were made by the least significant difference post-hoc test. Levels of ACTH in plasma in the in vivo experiments, as well as the nitric oxide and TNF-α measurements on the independent replications in the in vitro experiments, were similarly analyzed, with the exception that these analyses were on the absolute scale. Levels of corticosterone in the plasma of the adrenalectomized mice with placebo or with low-dose corticosterone implants were all lower than the assay limit of 25 ng/ml. These data were conservatively analyzed by using the worse-case value (i.e., at worst, our declarations of significance are understated).

## RESULTS

The susceptibility to mycobacterial growth of *Bcg*$^s$ mice by HPA axis activation by restraint correlated with its duration (Fig. 1). HPA axis activation for 5 or 10 daily 18-h cycles of restraint resulted in a significant ($P < 0.001$) increase in mycobacterial growth (Fig. 1A). The number of CFU isolated from the spleens of mice restrained for 10 cycles was 138,000 CFU/g of spleen per g of body weight. In contrast, only 72,000 CFU/g of spleen per g of body weight were isolated from control mice. Similar observations were made following enumeration of mycobacterial growth in the lungs (Fig. 1B).

**Differential effects of HPA axis activation on congenic BALB/c.*Bcg*$^r$ and BALB/c.*Bcg*$^s$ mice.** When we compared the effects of HPA axis activation on mycobacterial growth in *Bcg*$^s$ and *Bcg*$^r$ mice, we found that restraint resulted in a significant increase ($P < 0.001$) in the number of mycobacterial CFU isolated from the spleens of *Bcg*$^s$ mice but did not affect the growth of the microorganisms in the spleens of *Bcg*$^r$ mice (Fig. 2). Thus, the mycobacteria isolated from the spleens of *Bcg*$^s$ mice increased from 100,000 CFU in control mice to more then 175,000 CFU following restraint. In contrast, the number of CFU isolated from the spleens of *Bcg*$^r$ mice remained at about 40,000. The data in Fig. 2 also show that *Bcg*$^s$ mice were more permissive for mycobacterial growth than were *Bcg*$^r$ mice.

**The effect of adrenalectomy on mycobacterial growth in BALB/c.*Bcg*$^s$ mice.** To more directly evaluate the role of the HPA axis in the restraint-mediated increase in susceptibility to mycobacterial growth, adrenalectomized BALB/c.*Bcg*$^s$ mice were used. The results in Fig. 3 show that adrenalectomy abrogated the effect of restraint on the in vivo growth of the mycobacteria. An increased number of mycobacteria were isolated from the spleens and lungs of unoperated control and sham-adrenalectomized mice. The number of *M. avium* CFU isolated from the spleens and lungs of adrenalectomized mice (restrained mice) receiving basal levels of adrenal hormones or placebo was not greater than that isolated from the spleens of control mice. In contrast, increased numbers of *M. avium* CFU were isolated from the

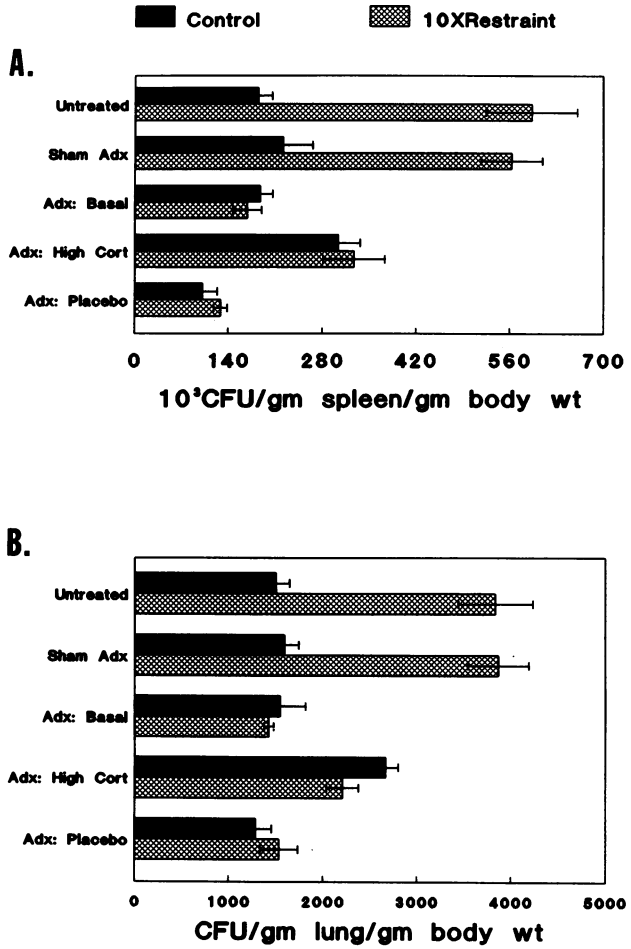


FIG. 3. Adrenalectomy abrogates the effect of HPA axis activation on mycobacterial susceptibility of *Bcg*$^s$ mice. Adrenalectomized (Adx) mice received time release pellets to provide basal levels of *d*-aldosterone and epinephrine and basal or activated levels of corticosterone (cort) or placebo pellets 2 days prior to initiation of the experiment. The mice were infected with $5 \times 10^4$ CFU of *M. avium* and were then restrained for 10 18-h cycles. The numbers of CFU in the spleens (A) and lungs (B) were determined 12 days after initiation of the experiment. The numbers of CFU isolated from the spleens of adrenalectomized or untreated mice prior to restraint were 32,081 CFU/g of spleen per g of body weight and 33,484 CFU/g of spleen per g of body weight, respectively. The number of CFU from the lungs of adrenalectomized mice was 782 CFU/g of lung per g of body weight, and the number of CFU from the lungs of untreated mice was 807 CFU/g of lung per g of body weight. The data are the means ± SD for seven animals per group. The effect of HPA activation was significant ($P < 0.001$). The effect of restraint in adrenalectomized mice was not significant, i.e., adrenalectomy abrogated the effect of restraint. The effect of implantation of time release pellets containing high doses of corticosterone on mycobacterial growth was significant ($P < 0.001$).

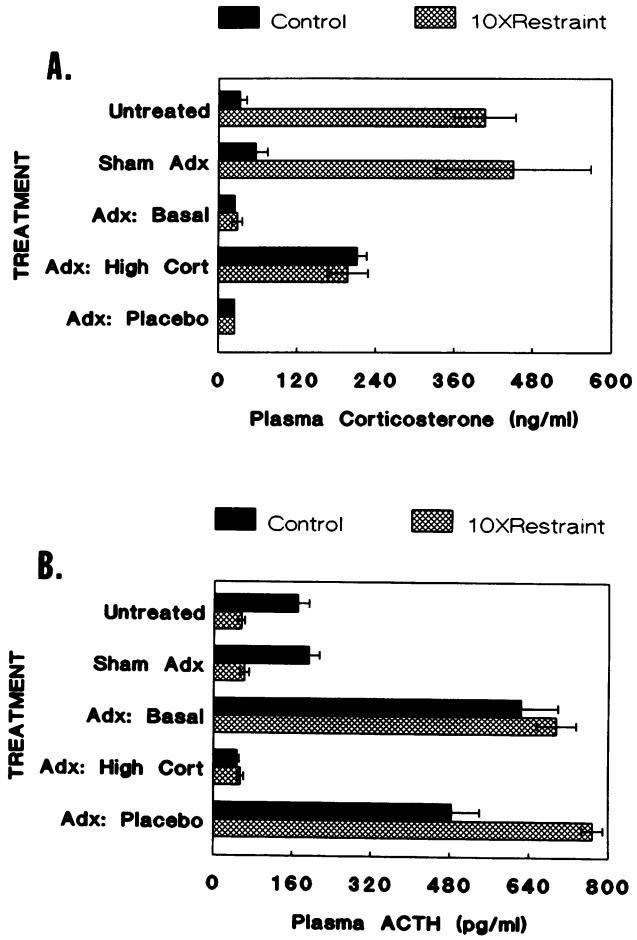


FIG. 4. Effect of adrenalectomy and restraint on levels of corticosterone (cort) and ACTH in plasma. Mice were treated as described in the legend to Fig. 3. Plasma samples were obtained from mice at the time of sacrifice, and the levels of corticosterone (A) and of ACTH (B) were determined by radioimmunoassay. Levels of corticosterone and ACTH in plasma are expressed as nanograms and picograms, respectively, per milliliter. Adx, adrenalectomized.

spleens of adrenalectomized mice that received basal-level replacement of *d*-aldosterone and epinephrine but that had elevated levels of corticosterone. The results in Fig. 4A show that adrenalectomy resulted in decreased levels of corticosterone in the plasma of restrained mice compared with those in control and sham-adrenalectomized mice. In contrast, the levels of ACTH in the mice with high levels of corticosterone were suppressed (Fig. 4B) and were elevated in the plasma of mice with low levels of corticosterone. The use of time release pellets that yielded a high level of corticosterone resulted in low levels of ACTH and increased the susceptibility to mycobacterial growth (Fig. 3). Implantation of time release pellets containing high levels of corticosterone into *Bcg*$^r$ mice failed to alter the growth pattern of the mycobacteria. Thus, despite the high levels of corticosterone in the plasma of these mice, no difference in the growth of the mycobacteria between mice receiving pellets containing high levels of corticosterone and those receiving placebo pellets was observed (data not shown).

**The glucocorticoid receptor antagonist RU 486 abrogates the effect of restraint-mediated HPA activation.** To directly implicate the high levels of corticosterone that resulted from activation of the HPA axis, mice were injected with the glucocorticoid receptor antagonist RU 486. The results in Fig. 5 show that RU 486 abrogated the increased susceptibility to mycobacterial growth that occurred as a result of HPA axis activation. Thus, while 158,626 CFU were isolated from the spleens of carrier-injected mice, only 76,456 CFU

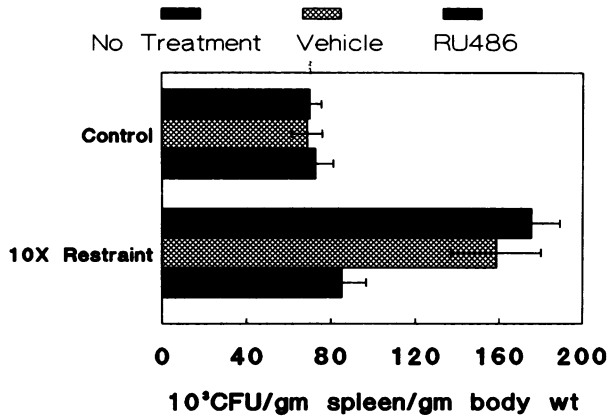


FIG. 5. The glucocorticoid receptor antagonist RU 486 abolishes the suppressive effect of HPA axis activation. Mice were treated with 25 mg of RU 486 per kg in polyethylene glycol (molecular weight, 400) or with carrier only for 2 days prior to HPA axis activation and daily for the duration of the experiment. The mice were infected with $5 \times 10^4$ CFU of *M. avium* and were restrained for 10 18-h cycles. The numbers of CFU in the spleens were determined 12 days after the initiation of the experiment. The CFU in the spleen prior to HPA axis activation was 20,792 CFU/g of spleen per g of body weight. Data are the means ± SD for seven animals per group. The effect of RU 486 was significant ($P < 0.001$).

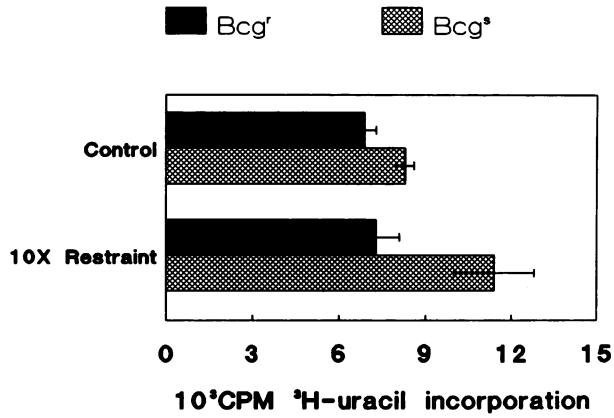


FIG. 6. Differential effect of HPA axis activation on antimycobacterial activity of macrophages from BALB/c.*Bcg*$^r$ and BALB/c.*Bcg*$^s$ mice. Splenic macrophages were isolated from mice and infected with *M. avium*. The growth of the mycobacteria was determined by pulsing cultures with [$^3$H]uracil after lysis of the macrophages with saponin. Infected cultures were pulsed immediately following a period of phagocytosis in order to determine the numbers of microorganisms taken up initially. Replicate cultures were pulsed after 5 days of growth within macrophages. The amount of [$^3$H]uracil taken up by the *M. avium* cells following release from the *Bcg*$^s$ macrophages immediately after phagocytosis was 574 cpm, while that taken up by *M. avium* cells following release from *Bcg*$^r$ macrophages was 523 cpm and did not differ as a result of HPA axis activation. The *M. avium* cells released from macrophages immediately after phagocytosis incorporated 45,334 cpm of [$^3$H]uracil when pulsed after 5 days of growth, and this amount of label incorporated did not differ between bacteria released from macrophages from *Bcg*$^r$ and *Bcg*$^s$ mice. The data are the counts per minute of [$^3$H]uracil taken up by the bacteria released from macrophages after 5 days of in vitro culture. The data are from a representative experiment. The effect of HPA activation is significant ($P < 0.001$).

were isolated from the spleens of RU 486-treated mice. This value did not differ from that obtained from the spleens of control mice.

**HPA axis activation increases susceptibility of splenic macrophages to mycobacterial growth.** The macrophage is the major effector cell that controls the growth of the mycobacteria. HPA activation also resulted in a differential effect on macrophages from *Bcg*$^r$ and *Bcg*$^s$ mice. The results in Fig. 6 show that activation of the HPA axis by restraint resulted in a significant increase ($P < 0.001$) in mycobacterial growth in macrophages from BALB/c.*Bcg*$^s$ mice. In contrast, the growth of *M. avium* in macrophages from BALB/c.*Bcg*$^r$ mice was not affected by HPA axis activation.

**Macrophage TNF-α and NO production is suppressed by HPA activation.** An antimicrobial effector pathway that has been shown to be important in the control of mycobacterial growth in mice is the IFN-γ-dependent, TNF-α-induced production of reactive nitrogen intermediates (9, 18). Activation of the HPA axis by restraint resulted in a suppression of TNF-α production following stimulation of splenic macrophages from restrained *Bcg*$^r$ and *Bcg*$^s$ mice with rIFN-γ and LPS (Fig. 7). The amount of TNF-α was reduced from 1,798 pg/ml produced by macrophages from *Bcg*$^s$ control mice to 1,035 pg/ml produced by the splenic macrophages following activation of the HPA axis ($P < 0.002$). The effect of HPA axis activation on TNF-α production by macrophages from *Bcg*$^r$ mice was less than that observed for macrophages from *Bcg*$^s$ mice. NO production was also reduced from 40 to 16 μM following restraint (Fig. 8). HPA axis activation also resulted in a decrease in the production of TNF-α and of reactive nitrogen intermediates by macrophages from the *Bcg*$^r$ mice.

## DISCUSSION

The results of this investigation show that activation of the HPA axis can increase the susceptibility of mice to mycobacterial growth. This effect was limited to the *Mycobacterium bovis* BCG-susceptible population. The increase in the susceptibility of genetically susceptible mice was directly proportional to the duration of HPA axis activation. Thus, multiple restraint experiences were required to increase the susceptibility of the *Bcg*$^s$ mice. In contrast, we have previously reported that a single restraint experience was sufficient to suppress MHC class II expression by macrophages from the *Bcg*$^s$ mice (60).

The failure of HPA axis activation to increase the susceptibility of *Bcg*$^r$ mice was not the result of an unresponsiveness of this strain of mice to HPA axis activation. Thus, activation of the HPA axis resulted in a similar increase in the levels of corticosterone in the plasma of both strains of mice (60). Additionally, HPA axis activation suppresses the induction of MHC class II expression by macrophages from *Bcg*$^r$ mice (50, 61). We also found that the HPA activation resulted in a suppressed capacity of macrophages from *Bcg*$^r$ and *Bcg*$^s$ mice to produce TNF-α and $NO_2^-$ following stimulation with rIFN-γ and LPS. This observation supports those that have shown that corticosteroids suppress both TNF-α production and the production of reactive nitrogen intermediates (3, 15, 59).

The suppression of TNF-α and $NO_2^-$ production by macrophages of both *Bcg*$^r$ and *Bcg*$^s$ strains of congenic mice as a result of HPA axis activation appears to indicate that the mechanism(s) of resistance that is controlled by *Bcg* may not be regulated by glucocorticoid hormones. Our results can also be interpreted as indicating that the production of TNF-α and of $NO_2^-$ may also be independent of *Bcg*

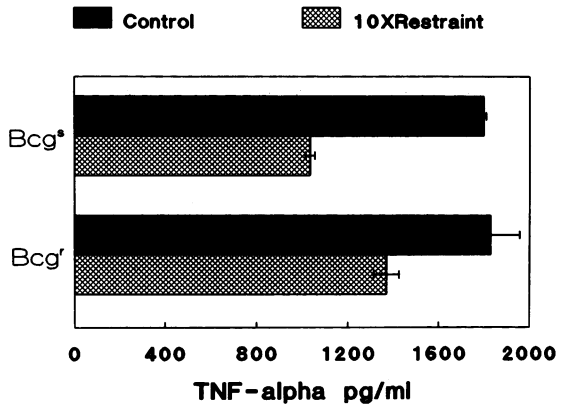


FIG. 7. HPA axis activation diminishes TNF-α production by macrophages from BALB/c.*Bcg*$^r$ and BALB/c.*Bcg*$^s$ mice. Splenic macrophages were obtained from mice following 10 18-h restraint cycles. The macrophages were stimulated with 100 U of rIFN-γ and 100 ng of LPS for 72 h. The amount of TNF-α produced by the macrophages was determined with cell-free supernatant by ELISA. Stimulation with LPS alone resulted in the production of 1,200 ± 74 pg of TNF-α per ml by macrophages from *Bcg*$^r$ mice and 1,105 ± 82 pg/ml by macrophages from *Bcg*$^s$ mice and was not significantly different. Activation of the HPA axis reduced TNF-α production to 405 ± 28 pg/ml and 325 ± 51 pg/ml. Treatment with rIFN-γ induced the production of 110 pg of TNF-α per ml, a value which did not differ between macrophages from *Bcg*$^r$ or *Bcg*$^s$ mice and which was not reduced as a result of HPA axis activation. The data are the means ± SD of three separate determinations. The effect of HPA activation is significant ($P < 0.002$).

control. Macrophages from both strains of mice produced similar quantities of the cytokine and of reactive nitrogen intermediates. Several reports have also shown that there are no apparent differences between the levels of TNF-α released by macrophages from resistant or susceptible mice

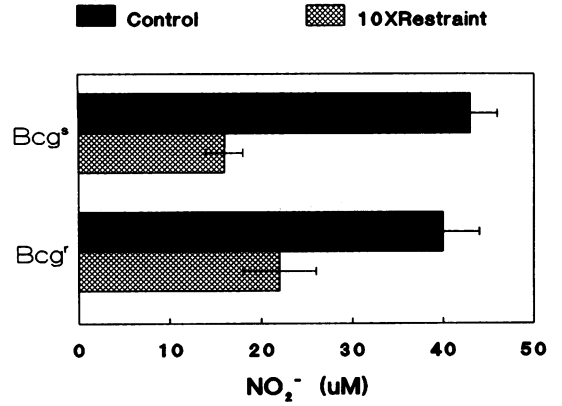


FIG. 8. HPA axis activation suppresses the production of reactive nitrogen intermediates by macrophages from BALB/c.*Bcg*$^r$ and BALB/c.*Bcg*$^s$ mice. Supernatants from cultures were obtained as described in the legend to Fig. 7, and the production of reactive nitrogen intermediates was determined with the Griess reagent (28). Stimulation of the cultures with LPS alone or rIFN-γ alone resulted in the production of less then 10 μM $NO_2^-$, which did not differ between macrophages from *Bcg*$^r$ and *Bcg*$^s$ mice. The addition of $N^G$-monomethyl-l-arginine to the cultures completely abolished the production of reactive nitrogen intermediates. The data are the means ± SD of five separate determinations. The effect of HPA activation was significant ($P < 0.001$).

following stimulation of *Mycobacterium lepraemurium*-infected macrophages with LPS (21) or stimulation with lipoarabinomannan (10) or *Leishmania* species (30, 55). In contrast, Blackwell et al. (4) reported that *Lsh*$^r$ macrophages produced more TNF-α than did *Lsh*$^s$ macrophages. Our results regarding $NO_2^-$ production are similar to those reported by Appleberg and Sarmento (1) but are different from those found by Liew et al. (30). Others have reported that macrophages from BCG-susceptible mice produce more or less $NO_2^-$ than macrophages from BCG-resistant mice (42). The reasons for the differences reported by the different laboratories is not clear but may relate to the differences in natural killer (NK) cells associated with splenic macrophage preparations. Ramarathinam et al. have reported that macrophages from resistant mice produce a factor that regulates IFN-γ production by NK cells and suggests that a function of the *Ity*$^r$ gene may be to regulate IFN-γ production. Thus, NK cells from resistant mice are stimulated to produce more IFN-γ (40, 41). We have previously reported that an increased stimulus can attenuate the effect of HPA activation (61). It is possible, therefore, that the differences that we have observed in TNF-α and $NO_2^-$ production may be the result of differences in IFN-γ production. Similarly, stimulation of the cultures with rIFN-γ and LPS may have attenuated the effect of restraint on the macrophages from the *Bcg*$^r$ mice. These possibilities are currently being explored by using mycobacteria to stimulate TNF-α and $NO_2^-$ production and by comparing the levels of IFN-γ produced following stimulation of spleen cell cultures with antigen.

The role of corticosterone in mediating the effects of HPA axis activation was demonstrated in three ways. First, adrenalectomy abrogated the effect of HPA activation. Second, implantation of time release pellets, which released similar levels of corticosterone attained during HPA axis activation, resulted in an increase in the susceptibility of the *Bcg*$^s$ mice to mycobacterial growth. Finally, treatment of the *Bcg*$^s$ mice with the glucocorticoid receptor antagonist RU 486 abrogated the effects of HPA axis activation.

The effect of corticosteroids on macrophage function has been the subject of considerable investigation. Glucocorticoids have been shown to inhibit cytokine production by macrophages (29, 50) and to suppress MHC class II expression (50, 57) and tumoricidal activity (23). However, glucocorticoid-mediated effects can also potentiate some macrophage functions. This includes the potentiation of the uptake of opsonized erythrocytes by rIFN-γ-stimulated macrophages (57) and the expression of cytokine receptors (49, 54). Activation of macrophages also results in the up regulation of corticosteroid receptors in macrophages (44, 45). However, this appears to be similar in a macrophage cell line derived from BALB/c BCG-susceptible mice as well as in macrophages from BCG-resistant C3H/OuJ mice. The positive effects of glucocorticoids have been attributed to glucocorticoid response elements which positively regulate gene expression. In contrast, no consensus regulatory sequences that account for the negative regulatory effects of the glucocorticoids have been identified (2).

The role of HPA axis activation in controlling the growth of tuberculosis in humans has been the subject of some discussion (11, 24, 58). Several reports have shown that injection of glucocorticoids suppresses the antimicrobial activity of macrophages and exacerbates the growth of mycobacteria (12, 46, 53). Our investigation is the first that shows that HPA axis activation increases the susceptibility of mice to mycobacterial growth. Rook et al. (43) reported that dexamethasone increased the susceptibility of mono-

cytes from some human donors to mycobacterial growth but not of monocytes from other donors. This may be analogous to the observation that we have made for mice, namely, that an increase in corticosterone levels, which occurs as a result of HPA activation, increases the susceptibility of *Bcg*$^s$ mice but not of *Bcg*$^r$ mice to mycobacterial growth (60, 63). Recently, North and Izzo (34) have shown that weekly injections of SCID mice with hydrocortisone acetate increased the susceptibility of the mice to the growth of *M. tuberculosis*. In contrast, the resistance of isocongenic immunocompetent mice to mycobacterial growth was resistant to treatment with hydrocortisone, presumably because of the development of specific immunity. Several reports have suggested that the resistance of macrophages to *Listeria*, *Salmonella*, or *Toxoplasma* species induced by rIFN-γ is not affected by glucocorticoid treatment (31, 46). Thus, it is possible that the insensitivity of the mycobacterial resistance mechanism(s), controlled by *Bcg*, to HPA axis activation is insensitive to corticosteroid treatment. IFN-γ, perhaps produced by NK cells early after infection, or some other cytokine (32, 40, 41) may induce an antimycobacterial mechanism that is not sensitive to glucocorticoids. Our results also appear to rule out the IFN-γ-induced, TNF-α-dependent induction of the reactive nitrogen intermediate pathway of mycobacterial resistance (9) as the primary pathway of *Bcg*-mediated resistance.

Finally, our results suggest that activation of the HPA axis may account for the increase in susceptibility to mycobacterial growth. While we have used restraint to activate the HPA axis, it is possible that during the natural course of the disease, chronic inflammatory events that lead to macrophage activation and the production of interleukin-1, TNF-α, or interleukin-6 may result in stimulation of the hypothalamus that eventually results in increased levels of cortisol and suppression of glucocorticoid-sensitive resistance mechanisms (8, 14). This suppression may be particularly apparent in individuals that are innately susceptible to mycobacterial disease and may account for the increase in the incidence of active tuberculosis among susceptible populations. Other cofactors such as homelessness, malnutrition, and chronic alcoholism, as well as stressful life events (7, 11, 16, 22, 24, 26, 27, 33, 35, 37, 39, 58), may also result in HPA axis activation or activation of the sympathetic nervous system (5, 17, 56) and compound the effects of the disease processes.

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