16α-Bromoeipiandrosterone Restores T Helper Cell Type 1 Activity and Accelerates Chemotherapy-Induced Bacterial Clearance in a Model of Progressive Pulmonary Tuberculosis

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BALB/c mice with pulmonary tuberculosis develop a T helper cell type 1 response that peaks at 3 weeks, temporarily controlling bacterial growth. Then bacterial proliferation recommences, accompanied by increasing interleukin (IL)–4 levels and decreasing interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and inducible nitric oxide synthase (iNOS) levels. These changes mimic those in the human disease. In a previous study, administration of dehydroepiandrosterone (DHEA) beginning on day 60 after infection reversed these changes and protected the mice. However, DHEA is suboptimal for human use, partly because it is readily metabolized into sex steroids. 16α-Bromoepiandrosterone (EpiBr; 16α-bromo-5α-androstan-3β-ol-17-one) is a synthetic adrenal steroid derivative that does not enter sex steroid pathways. In the present study, when tuberculous BALB/c mice were treated with EpiBr 3 times/week beginning on day 60, inhibition of bacterial proliferation and increased expression of TNF-α, IFN-γ, and iNOS were observed, although decreased expression of IL-4 was also observed. Moreover, when given as an adjunct to conventional chemotherapy, EpiBr enhanced bacterial clearance. Trials for the use of EpiBr in the treatment of human tuberculosis are now justified.

In mice, immunity to tuberculosis is crucially dependent on Th1 cytokines and tumor necrosis factor (TNF)–α [1–4]. The same cytokines are known to be crucial in humans, because tuberculosis is readily activated when patients are treated with neutralizing antibodies to TNF-α [5], whereas children with defective receptors for interferon (IFN)–γ are very susceptible to mycobacterial disease [6]. Despite this need for Th1 responses, patients with tuberculosis exhibit interleukin (IL)–4–dependent phenomena, such as IgE antibody to Mycobacterium tuberculosis [7], expression of dendritic cell–specific intracellular adhesion molecule 3–grabbing nonintegrin [8], and antibody to cardiolipin [9]. Moreover, it has been proved by use of several different techniques that expression of IL-4 (and IL-13) is increased during human pulmonary tuberculosis [10–14] and correlates with cavitation and immunopathological tissue damage [10, 12]. The cytokine profile returns to Th1 after successful treatment [13]. Patients with tuberculosis also have circulating T cells that release IL-4 in response to tuberculosis antigen [13]. This observation, together with the presence of specific IgE antibody [7], indicates that the increased expression of IL-4 results from a specific response to tuberculosis antigen; the past failure of some authors to detect IL-4 during human tuberculosis [15] must be attributed to
low IL-4 levels, low mRNA copy numbers and half-life [16], and the presence of a splice variant of IL-4 (IL-4Δ2) [10, 11].

Because the increased expression of IL-4 during human tuberculosis is no longer a matter of dispute, the relevant animal models are those in which, as in humans, IL-4 expression is observed during the progressive phase of the disease [17]. This is observed during pulmonary infection in the BALB/c mouse [18, 19]; however, in some mouse strains, IL-4 seems to play no detectable role [20]. Importantly, in mouse strains that do exhibit the switch to a Th2-dominant response during the progressive phase of the disease, preimmunization—so that there is a Th2 response before infection—greatly exacerbates the disease and causes increased fibrosis, weight loss, and toxicity of TNF-α, all of which are components of the human disease [21–23]. Such results indicate that the IL-4 response is not merely a bystander consequence of late disease; rather, it is an important factor in determining susceptibility. Moreover, IL-4 gene disruption alleviates tuberculosis in the BALB/c mouse [24]. This issue is reviewed in detail elsewhere [25].

In light of the pathological role of the Th2 response during tuberculosis, there might be therapeutic potential in treatments that turn off IL-4 release. The BALB/c mouse is an appropriate model in which to investigate this possibility. We have previously shown that treatment with a low dose of the adrenal androgen dehydroepiandrosterone (DHEA) (5-androsten-3β,17β-diol-17-one) or of its 3β,17β-dihydroxy derivative (5-androstene-3β,17β-diol) will partially block the switch to a Th2-dominant response in BALB/c mice with pulmonary tuberculosis [26–28]. This effect is thought to be attributable to the conversion of DHEA in mice into a series of immunomodulatory steroids (including 5-androstene-3β,17β-diol itself and 5-androstene-3β,7β,17β-triol) [29]. However, DHEA is metabolized differently in humans and rodents, with the proportion of highly oxidized metabolites being significantly greater in rodents than in humans. DHEA is also metabolized into sex steroids in peripheral tissues, and this limits its use and confuses interpretation. On the other hand, the synthetic adrenal steroid derivative 16α-bromoepiandrosterone (EpiBr; 16α-bromo-5α-androstan-3β-ol-17-one) is rapidly and completely metabolized into a series of naturally occurring polyhydroxylated metabolites by human and rodent cells. This reduces the issues of differing metabolisms that are associated with DHEA. No sex steroids have been identified as metabolites of EpiBr.

The present study used BALB/c mice to investigate the therapeutic potential of EpiBr for the treatment of progressive pulmonary tuberculosis. We show that EpiBr, when administered to BALB/c mice beginning on day 60 after infection, is as effective as DHEA in restoring Th1 activity; enhancing TNF-α, IFN-γ, and inducible nitric oxide synthase (iNOS) expression; and suppressing IL-4 expression. Moreover, treatment with EpiBr in addition to standard chemotherapy accelerates clearance of cultivable bacteria from the lungs.

MATERIALS AND METHODS

Experimental model of tuberculosis infection in mice. All work with animals was performed in conformity with the guidelines of the local ethics committee for experimentation in animals in Mexico. The tuberculosis model is described in detail elsewhere [19, 23, 30]. Male BALB/c mice were obtained from Jackson Laboratories and were used at 6–8 weeks of age. Virulent M. tuberculosis H37Rv was cultured in Younan’s modification of the medium of Proskauer and Beck. Colonies were harvested after 4 weeks and were suspended in PBS containing 0.05% Tween 80 (Sigma) by shaking for 10 min with glass beads. The suspension was centrifuged for 1 min at 350 g to remove large clumps of bacilli. A preliminary bacterial count was achieved by smearing the supernatant at a known ratio of volume to area and counting 10 random fields after staining by use of the Ziehl-Neelsen technique. The suspension was then diluted to a final concentration of 1×108 bacteria/100 µL of PBS and was aliquoted at −70°C. Before use, bacteria were recounted and viability was checked, as described elsewhere [31].

To achieve intratracheal infection, mice were anesthetized intraperitoneally with 56 mg/kg pentothal (Sigma). The trachea was exposed via a small midline incision, and 1×106 viable bacteria in 100 µL of PBS were injected; the incision was then sutured with sterile silk, and the mice were maintained vertically until the effects of the anesthetic had worn off. The infected mice were housed in groups of 5, in cages fitted with microisolators.

Drug administration. Except where specifically stated otherwise, on day 60, surviving mice were randomly allocated to the appropriate treatment groups. Thus, treatment was started 60 days after infection, and some mice from each group were then killed at 15-day intervals (i.e., on days 75, 90, 105, and 120). All data are reported as the mean ± SD of measurements from 3–8 mice. DHEA was obtained from Sigma. Selection of the appropriate doses is described in detail elsewhere [27]. Briefly, the chosen dose optimally inhibits thymic involution caused by a large dose (1.6 mg) of corticosterone [27]. Doses were dissolved in ultrapure olive oil (Sigma), and each dose contained 50 µg in a volume of 0.1 mL. EpiBr (HE2000) was supplied by Hollis-Eden Pharmaceuticals as a suspension in an aqueous medium with a nonionic detergent (to prevent drug-particle agglomeration), polysorbate-80, saline, and a viscosity-imparting agent, sodium carboxymethyl cellulose (2% polysorbate-80, 0.9% NaCl, 0.1% carboxymethylcellulose sodium, 0.05% phenol, and EpiBr vehicle). EpiBr was administered subcutaneously (sc) 3 times/week (on Monday, Wednesday, and Friday) in doses of either 0.02, 0.2, or 2.0 mg in an injected volume of 0.1 mL until the end of the experiments.
Assessment of colony-forming units in infected lungs. One lung from each mouse was used for counting colony-forming units, and the other lung was used for studying other parameters. Lungs were disrupted in a Polytom homogenizer (Kinematica) in sterile 50-mL tubes containing 3 mL of isotonic saline. Four dilutions of each homogenate were spread onto duplicate plates (Productos Hemobiologicos de Mexico) containing Bacto Middlebrook 7H10 agar enriched with oleic acid dextrose complex (both from Difco Laboratories). Plates were incubated for 21 days. For assessment of colony-forming units, 4 mice were killed at each time point in 2 different experiments; therefore, the data are reported as the mean ± SD of measurements from 8 mice.

Preparation of tissue for histological study and morphometry. For histological study, lungs were perfused via the trachea with 100% ethanol and were immersed in the same fixative for 24 h. Parasagittal sections were taken through the hilus, and these were dehydrated and embedded in paraffin, sectioned (5 μ), and stained with hematoxylin-eosin. The area of granuloma and the percentage of lung affected by pneumonia were measured by use of a Zidas image-analysis system (Zeiss). Measurements were taken blind, and data are reported as the mean ± SD of measurements from 3–4 mice.

Reverse-transcription (RT) polymerase chain reaction (PCR) analysis of cytokines in lung homogenates. After the mice were killed, lungs were removed, hilar lymph nodes and thymus were eliminated, and the tissue was immediately frozen, by immersion in liquid nitrogen. Three lungs, right or left, from different mice were used to isolate mRNA from each group at each time point, and the cDNA from the 3 mice was analyzed separately. mRNA was isolated by use of Trizol (Gibco BRL); cDNA was synthesized by use of Maloney murine leukemia virus reverse transcriptase (Gibco BRL) and priming with oligo dT. Expression of mRNA that encode TNF-α, iNOS, IFN-γ, and IL-4 was determined by RT-PCR 15, 30, 45, and 60 days after initiation of drug administration (which commenced 60 days after infection), such that determinations were made on days 75, 90, 105, and 120 after infection. In addition, data are shown for iNOS mRNA expression on days 1, 3, 7, 14, 21, 28, and 60 after infection; these data are from experiments in which administration of EpiBr was initiated 1 day before infection. The PCR products were electrophoresed on 6% polyacrylamide gels, and molecular-weight standards with known DNA mass concentrations (low DNA mass ladder; Gibco BRL) were run; the PCR products were then analyzed by use of an image-analysis densitometer linked to a computer program (ID image-analysis software; Kodak Digital Science). To determine, in nanograms, the quantity of PCR product for each cytokine, the computer program compared the optical densities from the experimental samples with the molecular-marker bands, of which the DNA content was provided by the manufacturer. To correct for errors in the quantity of starting material, the densitometer reading of the glyceraldehyde-3-phosphate dehydrogenase PCR product was used.

Quantification of cytokines in lung homogenates by ELISA. The lung homogenates used for RNA purification were also used to quantify IL-2, IL-4, and IFN-γ by ELISA. After homogenization and centrifugation, the protein phase was extensively dialyzed in sodium dodecylsulfate and was quantified by use of Bradford reagent (Bio-Rad) and a standard curve of bovine serum albumin. The ELISA method used to quantify cytokines in these extracts is described elsewhere [18, 23, 30].

EpiBr as a supplement to conventional chemotherapy. In preliminary experiments, 0.2 mg of EpiBr (3 times/week) was found to be the optimal dose for treating advanced experimental pulmonary tuberculosis. Thus, we used this dose of EpiBr to supplement conventional chemotherapy, to discover whether it might shorten the required duration of chemotherapy. On day 60, surviving mice were randomly allocated to 3 experimental groups. The first group of 20 tuberculous mice was treated with conventional chemotherapy—rifampicin (10 mg/kg), isoniazid (10 mg/kg), and pyrazinamide (30 mg/kg) administered daily by intragastric cannula—plus 0.2 mg of EpiBr administered sc 3 times/week. The second group of 20 tuberculous mice was treated with chemotherapy only, and the third group (control) of 20 tuberculous mice received the diluent. Five mice per group were killed by exsanguination under terminal anaesthesia after 1, 2, 4, and 6 weeks of treatment. Determination of numbers of colony-forming units in lungs was made as described above.

Statistical analysis. Student’s t test (2-tailed) for unpaired data and the nonparametric Mann-Whitney U test were used as appropriate. In addition, data from the ELISAs and PCR assays were analyzed as follows. For each subject, the dependent variable represented the appropriate cytokine (INF-γ, IL-4, IL-2, or TNF-α). Resultant sequences were submitted to general linear-model analysis. Results that were significant overall were submitted to pairwise comparisons by use of the Scheffe procedure. Data from the experiments assaying for additive or synergistic effects of conventional chemotherapy plus EpiBr were analyzed as follows. For each subject, the dependent variable represented the number of colony-forming units. Resultant sequences were submitted to general linear-model analysis. Results that were significant overall were submitted to pairwise comparisons by use of the Scheffe procedure. Two-sided P<.05 was considered to be significant. Because of the small sample sizes, a set of nonparametric tests was performed. Statistical analyses were performed by use of SAS software (version 9.1).

RESULTS

Effect of EpiBr on colony-forming units in the lungs. A previous study has shown that DHEA administered 3 times/week,
beginning 1 day before infection, reduces the number of colony-forming units in the lungs [27]. This effect was reproduced with DHEA and with the 0.2- and 2.0-mg doses of EpiBr, although the lowest dose of EpiBr (0.02 mg) was less effective (data not shown). Of much greater clinical importance is the observation that the 0.2- and 2.0-mg doses of EpiBr administered 3 times/week beginning on day 60 (when the disease is already well advanced) significantly reduced the number of colony-forming units by day 75, 15 days after initiation of treatment \( (P < .025) \). The number of colony-forming units increased progressively in the control (untreated) mice (○). Data are the mean ± SD of measurements from 8 mice/time point.

**Effect of EpiBr on the histological appearance of the lungs when administered beginning on day 60.** In this model, granuloma formation correlates with resistance, whereas pneumonia indicates progression and is associated with increased expression of IL-4 [18, 30]. The 0.2-mg dose of EpiBr resulted in a lower percentage of the lungs being involved in pneumonia from day 90 onwards, but no significant increase in granuloma was found, except on day 120 \( \left( P < .025 \right) \) (data not shown).

**Effect of EpiBr on expression of cytokines in the lungs when administered beginning on day 60.** Figures 2 and 3 show data for expression of cytokines and \( P \) values for pairwise comparisons of means at individual time points, adjusted for multiple comparisons by use of the Scheffe procedure. These calculations were preceded by general linear-model analysis of time trends, for which the \( P \) values are shown in the text below but not in the figures. Among control mice, expression of IL-2, IL-4, and TNF-\( \alpha \) remained rather stable between days 75 and 120 (i.e., there were no significant time trends), whereas the ELISA data for expression of IFN-\( \gamma \) showed a significant downward time trend \( (P < .003) \). In contrast, among the treated mice there were significant upward time trends for IFN-\( \gamma \) \( (P < .001, \) for both ELISA and RT-PCR data) and TNF-\( \alpha \).
control mice, expression of mRNA that encodes iNOS peaked on day 7 after infection, subsequently declined, and then stayed low until the experiment was terminated or the mice died. Treatment of mice beginning on day −1 with 0.2 mg of EpiBr resulted in a steady, high level of expression of iNOS mRNA that was apparent beginning on day 1 and that was significant at all time points, except on day 7, the day on which iNOS mRNA expression peaked among control mice. EpiBr eliminated the decrease in iNOS mRNA expression seen among control mice beginning on day 7 (figure 4A). Similarly, when treatment was initiated on day 60—that is, when the infected mice already had very low levels of iNOS mRNA—expression was increased by day 75, and, by day 105, there was complete restoration of iNOS mRNA to levels as high or higher than the 7-day peak observed among control mice (figure 4B).

**Effect of combined treatment with chemotherapy and EpiBr.** Figures 2, 3, and 4 indicate correction of the cytokine profile by EpiBr. The efficacy of chemotherapy against tuberculosis is dependent on synergy with the immune system. For example, treatment of bacille Calmette-Guérin infection is difficult in patients with faulty IFN-γ receptors [33]. Thus, correcting the cytokine profile might facilitate and accelerate the sterilizing effects of chemotherapy. To test this, mice in the progressive, Th2-biased phase of the disease (day 60) were treated either with conventional chemotherapy alone or with conventional chemotherapy in combination with 0.2-mg of EpiBr 3 times/week. Two separate experiments are shown in figure 5. In experiment 1, the means for the combined therapy group and the group receiving chemotherapy alone were significantly different at week 2 (P < .001), after adjustment for multiple comparisons by use of the Scheffe procedure. In experiment 2, the

![Figure 3. Effect of 16α-bromoepiandrosterone (EpiBr; 16α-bromo-5α-androstan-3β-ol-17-one) on expression of mRNA that encodes cytokines in the lungs of mice when administered beginning on day 60 after infection with Mycobacterium tuberculosis, as measured by reverse-transcription polymerase chain reaction. Shown are expression of mRNA that encodes interleukin (IL)-2, IL-4, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α between days 75 and 120 among control (untreated) mice (○) and among mice treated with 0.2 mg of EpiBr 3 times/week (■).](image)

![Figure 4. Effect of 16α-bromoepiandrosterone (EpiBr; 16α-bromo-5α-androstan-3β-ol-17-one) on expression of inducible nitric oxide synthase (iNOS) in the lungs of mice infected with Mycobacterium tuberculosis. Mice were treated with 0.2 mg of EpiBr 3 times/week (■) beginning on day −1 (A) or day 60 (B) after infection. In the latter experiment, by day 105 there was complete restoration of iNOS mRNA (P < .005) (B) to levels as high or higher than the day 7 peak seen among control (untreated) mice (○) (A).](image)
DISCUSSION

The purpose of the present study was to determine whether a novel synthetic immune-regulating hormone, EpiBr, could improve the course of M. tuberculosis infection in mice. The results presented here indicate that EpiBr can reduce the bacterial load associated with progressive tuberculosis and, from an immunological perspective, restore the Th1 bias and down-regulate IL-4 expression associated with M. tuberculosis infection. This was observed both when the compound was administered beginning 1 day before intratracheal infection with M. tuberculosis and, more important from a therapeutic point of view, when it was administered beginning on day 60 after infection during late progressive disease, with or without antibiotic therapy.

The mechanism of action of EpiBr on the immune system is unknown. Because EpiBr and DHEA produce similar results, they are probably acting via the same mechanism. Some authors have reported the presence of specific receptors for DHEA in lymphocytes [34], and it may be that lymphocytes become susceptible to regulation by DHEA during the process of signal-induced activation [35]. DHEA may also interact with the nuclear pregnane X receptor [36], but there is currently no evidence that any of these receptors mediate the effects seen. DHEA and molecules of similar structure can also act as peroxisome proliferators in rodents, via their capacity to activate peroxisome proliferator–activated receptor α (PPARα) [37]; therefore, PPARα activation and some of its physiologically relevant downstream effects on gene expression (transactivation or transrepression) could be involved. However, in toxicology studies, hepatic peroxisome proliferation in rats was not observed after 28 consecutive days of sc administration of 500 mg/kg EpiBr. Interestingly, both DHEA and EpiBr can modulate the DNA binding of transcription factor activator protein–1 (AP-1) [38]. This is a rather general property of nuclear receptors, including the glucocorticoid receptor [39]. The effects seen in the present study could be explained by antiglucocorticoid activity, and interference with AP-1 could play a role, but this would require evidence that the effects of EpiBr and of cortisol on this factor were different and opposite.

Treatment with EpiBr shifted the cytokine profile of infected mice toward a Th1-dominant response, suggesting that EpiBr might act by directly altering the balance or expression of cytokines elicited in response to infection. During the late progressive stage of infection, the immune response has already switched toward a Th2 response, and IFN-γ, TNF-α, and iNOS are severely down-regulated [25]. Thus, the striking increase in expression of iNOS after administration of EpiBr could be an indirect or direct consequence of the ability of EpiBr to reverse the Th2 bias, because IL-4 down-regulates expression of iNOS, whereas IFN-γ and TNF-α up-regulate its expression [32]. This shift away from a Th2-dominant response has also been observed in a cohort of HIV-infected subjects treated with EpiBr [40].

Alternatively, this compound might directly accelerate bactericidal effects of the infected cells. Preliminary in vitro data have suggested that EpiBr might increase the killing of M. tuberculosis within macrophages. We are currently expanding these studies, as well as ones directed at understanding the fundamental immunological changes that are brought about by EpiBr.

A major problem in the control of tuberculosis is the lack of compliance caused by the need for 6-month treatment regimens. The evidence reported here that EpiBr can speed up bacterial clearance when used as an adjunct to chemotherapy is particularly exciting. Such a combined therapy could not only accelerate the effect of chemotherapeutic regimens currently used to control M. tuberculosis but also provide the immunological support needed to limit recurrence of infection.
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