Role of Angiotensin-converting Enzyme in Bacille Calmette-Guérin-induced Granulomatous Inflammation

INCREASED ANGIOTENSIN-CONVERTING ENZYME LEVELS IN LUNG LAVAGE AND SUPPRESSION OF INFLAMMATION WITH CAPTOPRIL

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ABSTRACT Lung lavage levels of angiotensin-converting enzyme (ACE)-like activity were increased in C57BL/6 mice with Bacille Calmette-Guérin (BCG)-induced chronic granulomatous pulmonary inflammation and splenomegaly. Contrariwise, ACE activity was not increased in lung lavage fluids of CBA mice that developed only minimal pulmonary inflammation in response to BCG. ACE-like activity correlated with the intensity of inflammation and Captopril, a specific competitive inhibitor of ACE activity, markedly suppressed the induction and maintenance of the BCG-induced inflammatory response in both lungs and spleen. It was necessary, however, to provide sustained treatment with large doses of Captopril in order to reduce the inflammatory response. After a single intraperitoneal injection of Captopril, ACE levels in lung lavage of BCG-injected mice were reduced but returned to preinjection levels or greater within 24 h. The highest dose of Captopril was more effective in reducing the lung fluid level of ACE in BCG-inflamed lungs. This suggests that sustained daily injections of Captopril were necessary to maintain reduced ACE levels. In vitro studies indicated that high concentrations of Captopril did not affect macrophage mobility or chemotactic activity for macrophages. Thus, ACE may act as a molecular mediator of BCG-induced granulomatous inflammation in the lung.

This study represents partial fulfillment for a Ph.D. in Pathology for Dr. Schrier. His present address is Department of Pathology, University of Michigan School of Medicine, Ann Arbor, Mich. 48109. Dr. Katzenstein's present address is Division of Surgical Pathology, Barnes Hospital, St. Louis, Mo. 63110. Address reprint requests to Dr. Moore.

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INTRODUCTION

Angiotensin-converting enzyme (dipeptidyl carboxypeptidase; peptidyl-dipeptide hydrolase; EC 3.4.15.1) (ACE)[1] is a glycoprotein that catalyzes the conversion of angiotensin I to angiotensin II and the inactivation of bradykinin (1). In several chronic human diseases, it is considered an important diagnostic and management tool (2–6). In addition to being elevated in the serum of patients with Gaucher's disease (6) and leprosy (7), ACE is increased in the serum and granulomatous lesions of sarcoidosis and murine schistosomiasis (4, 7, 8). Since ACE could be demonstrated only in peripheral epithelioid macrophages adjacent to lymphocytes within sarcoid granulomas, it was postulated that its production may be a lymphocyte-mediated immunological phenomenon (9). It is unknown whether ACE has any direct physiologic significance in chronic inflammatory processes.

We have developed a murine model of chronic pulmonary inflammation (CPI) and splenomegaly induced by killed Bacille Calmette-Guérin (BCG). In the

[1] *Abbreviations used in this paper*: ACE, angiotensin-converting enzyme; B6, C57BL/6 mouse; CPI, chronic pulmonary inflammation.

lung, the inflammation is characterized by diffuse, interstitial, nonnecrotizing granulomas, histologically similar to those observed in sarcoidosis (10). The inflammatory response to BCG is T cell-dependent (11) and strain-specific, i.e., C57BL/6 (B6) mice respond to BCG by developing a granulomatous inflammatory response whereas CBA mice form only a minimal, transient response. Nonspecific immunologic suppression of antigen- and mitogen-induced proliferation (12), as well as suppression of delayed hypersensitivity (13), are associated with the chronic response in B6 mice. Because of the histologic similarity of the response to BCG in B6 mice and that found in sarcoidosis, we measured serum and lung lavage ACE-like activity in B6 (high responder) mice, and compared them with levels found in CBA (low responder) and normal B6 mice. We also investigated the role of ACE in the pathogenesis of CPI and splenomegaly by using Captopril, a specific competitive ACE inhibitor.

In this study, ACE activity in mouse lung lavage correlated with the intensity of pulmonary granulomatous inflammation, and in vivo treatment with Captopril markedly suppressed the inflammatory response. Additional studies suggested that the doses of Captopril used were necessary to maintain sustained depression of ACE activity. Moreover, in vitro studies indicated that Captopril, even at high doses, did not reduce the mobility of or inhibit chemotactic activity for mouse macrophages. Thus, in addition to its value as an enzyme associated with granulomatous inflammation, ACE may also be involved in the pathogenesis of BCG-induced pulmonary granulomatous inflammation.

METHODS

Mice. Female C57BL/6N and CBA/J mice were obtained from Harlan Industries, Madison, Wis. They were maintained in the animal quarters of the Research Service, Wood Veterans Administration Medical Center and used between 6 and 19 wk of age. These animal quarters are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Induction of splenomegaly and chronic granulomatous pulmonary inflammation with killed BCG. This technique has been previously described by Allen et al. (10). Briefly, mice were injected intravenously with 300 μg of killed BCG in 0.2 ml of an oil-in-saline emulsion. Mice were killed at 7-d intervals and body, spleen, and lung weights obtained. Lung and spleen indices were calculated as follows:

$$\text{Lung or spleen index} = \frac{\text{organ wt/body wt BCG-injected mouse}}{\text{organ wt/body wt normal syngeneic mouse}}.$$

Lung lavage. Mice were bled from the retro-orbital plexus to obtain serum. They were then killed and lung lavaged with sterile Hanks' balanced salt solution (Difco Laboratories, Detroit, Mich.). Approximately 1 ml of lung lavage was obtained from each mouse by cannulation of the trachea with a syringe attached to PE-60 polyethylene tubing (Clay-Adams Div., Becton, Dickinson & Co., Parsippany, N. J.). The lavage was centrifuged at 450 *g* for 10 min and stored at −20°C until used.

Captopril treatment. Captopril (4 D-3-mercapto-2-methylpropranoyl-L-proline) (SQ 14,225) was a generous gift from Dr. Z. P. Horovitz, E. R. Squibb & Sons, Inc., Princeton, N. J. For injection, it was dissolved fresh in sterile saline at a concentration of either 5 or 15 mg/ml. Mice received an intraperitoneal dose of Captopril at dosages and intervals outlined in Figs. 4 and 6. Similar doses of sterile saline were administered as control.

ACE assay. ACE assays were performed on the lung lavage and serum using a fluorometric method described by Friedland and Silverstein (14). The method uses hippuryl-L-histidyl-L-leucine as substrate. Units of activity represent nanomoles of L-histidyl-L-leucine formed per minute per milliliter lung lavage or serum. In some instances, ACE activity is expressed as units of ACE per milligram of protein. All data are expressed as mean±standard error. When necessary, *t* tests were performed to compare means.

Total protein. This was determined by the Coomassie Blue technique (15) using bovine serum albumin (Miles Laboratories, Inc., Ames Div., Elkhart, Ind.) as standard.

Cell migration studies. To assess the effect of Captopril on the migration of macrophages, normal peritoneal macrophages were obtained from B6 mice and incubated with various concentrations of the drug in 10 vol of RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) that contained 15% heat-inactivated fetal bovine serum, 1% L-glutamine, 25 mM HEPES buffer, and 0.5% gentamicin. The cell suspension was inoculated into 100-ml capillary tubes that were sealed at one end and centrifuged at 75 *g* for 5 min. The tubes were cut at the cell-liquid interface and placed into Mackaness-type chambers. The chambers were filled with the same tissue culture medium containing the appropriate concentration of Captopril and incubated at 37°C for 48 h. The area of migration was magnified ~10 times, traced onto paper, and measured with a planimeter.

Chemotactic studies. To test the effect of Captopril on chemotactic activity for macrophages, supernatants from BCG-induced isolated pulmonary granulomas were used as a source of chemotactic activity,[2] and normal B6 peritoneal macrophages were used as indicator cells. Peritoneal macrophages were suspended at a concentration of 2.3 × 10^6/ml in Gey's balanced salt solution containing 2% bovine serum albumin. Captopril was added at various concentrations to lung lavage fluid containing chemotactic activity. Chemotaxis was tested in a modified Boyden chamber using one 5-μm filter (Nuclepore Corp., Pleasanton, Calif.). Activity was quantified by counting the number of cells that migrated through the filter in 10 oil-immersion fields.

RESULTS

Kinetics of the induction of splenomegaly and CPI by killed BCG in B6 and CBA mice. The kinetics of splenomegaly and CPI in CBA and B6 mice are shown in Fig. 1. During the 7–14 d after BCG injection, the

[2] Ripani, L., D. Schrier, and V. L. Moore. Unpublished observations.

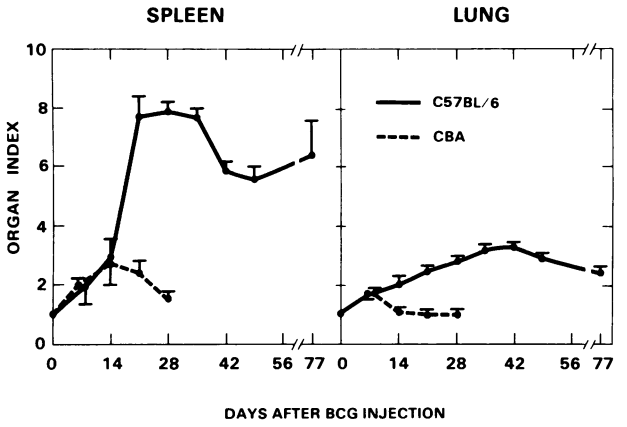

FIGURE 1 Spleen and lung indices of B6 and CBA mice injected with killed BCG in an oil-in-saline mixture. Mice were injected intravenously with 300 μg of BCG. Organ indices were obtained at various intervals. Each group consists of at least five mice. Expression of variation is mean±standard error.

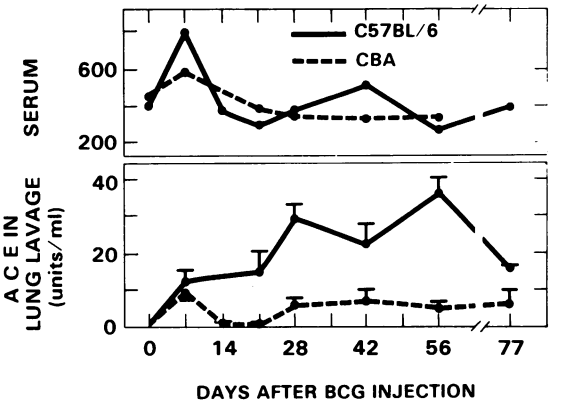

FIGURE 2 ACE activity in lung lavages and serum of mice injected with 300 μg of BCG. Values for serum represent the value for pools from five animals. Lung lavage values represent the mean±standard error from at least five mice.

intensity of responses produced by B6 and CBA mice were similar. In CBA mice, this initial period was followed by a rapid diminution in the inflammatory response shown to be controlled by cyclophosphamide-sensitive suppressor T cells (16). In contrast, B6 mice continued to display increasing lung and spleen indices through days 35–42 and declined gradually.

Since serum ACE levels are increased in several human diseases that are histologically similar to that found in BCG-induced inflammation, ACE activity was measured in the serum and lung lavage of mice.

ACE activity in the serum and lung lavage of CBA and B6 mice injected with killed BCG. The ACE activity in lung lavage and serum is depicted in Figs. 2 and 3. Serum ACE was measured on pooled serum samples in both B6 and CBA mice and expressed as ACE units per milliliter of serum. While serum ACE values for both strains were increased above initial levels during several intervals, no sustained increases were noted. The ACE in lung lavage was expressed as either ACE units per milliliter of lung lavage (Fig. 2) or as ACE units per milligram of protein (Fig. 3). Using both techniques, there was only a transient increase in ACE activity in CBA mice peaking at day 7; levels returned to approximately initial values by day 14 and remained throughout the experimental period.[3] The ACE levels in B6 mice also demonstrated a transient increase in ACE activity peaking at day 7, but after day 14 ACE levels increased until day 42 when they reached a plateau. In both CBA and B6 mice, ACE activity roughly followed the kinetic curve of the organ indices, indicating that the level of ACE activity was correlated with the intensity of inflammation in this system.

The effect of Captopril on CPI and splenomegaly induced in mice. Since the preceding experiments indicated that levels of ACE activity in lung lavage correlated with the intensity of inflammation, experiments were performed to test the possibility that ACE is important in the inflammatory process. Captopril,

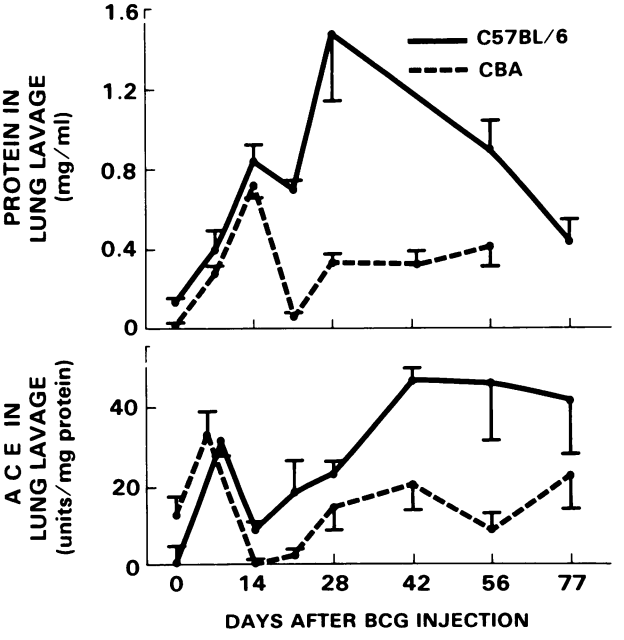

FIGURE 3 Total protein and ACE activity expressed as a fraction of protein. Each value represents the mean±standard error of at least five mice.

[3] In Fig. 3 (bottom), the ACE levels in lung lavage of CBA mice appear higher at day 0 because of the low value for protein in lung lavage of normal CBA mice.

a specific competitive inhibitor of ACE activity, was administered to BCG-treated B6 mice at doses and intervals shown in Fig. 4. When Captopril (50 mg/kg) was given 2 d before and continued daily through day 21 after BCG treatment, there was a significant reduction in splenomegaly but not in the lung index (group 1). However, a higher dose of Captopril (150 mg/kg) using the same treatment schedule resulted in a significant reduction in both the spleen and lung index (group 2). In addition to inhibiting the BCG-induced inflammation, Captopril also suppressed an ongoing inflammatory response (groups 3 and 4). In these experiments, Captopril treatment was not initiated until 21 d after BCG treatment. Data in group 5 (Fig. 4) indicate the necessity for continued Captopril treatment to reduce BCG-induced inflammation since BCG-injected mice treated with the drug from 21 through 42 d after BCG and killed at day 63 were not significantly suppressed. These data were confirmed by histologic examination. The lungs of animals receiving BCG and saline contained numerous well-formed noncaseating granulomas associated with an extensive interstitial mononuclear cell infiltration (Fig. 5). The inflammatory process in these mice involved >75% of the lung parenchyma. In contrast, the lungs of mice given Captopril (150 mg/kg) 2 d before and continuing through 21 d after BCG contained only scattered, tiny, noncaseating granulomas with minimal associated interstitial inflammation. This inflammatory reaction involved only 10% or less of the parenchyma. Spleens from the Captopril-treated mice showed a similar reduction in granulomatous inflammation compared with the saline-treated controls. Whereas spleens from saline-treated animals contained rare loosely-formed granulomas, such granulomas were absent from the spleens of Captopril-treated animals (data not shown).

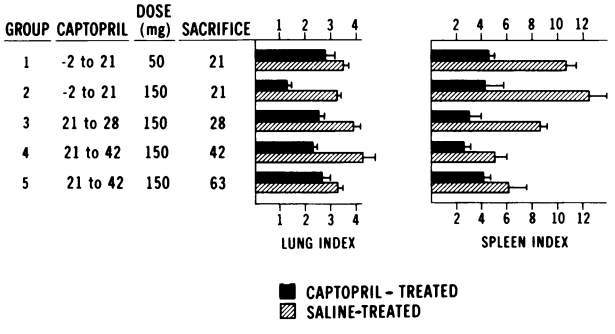

FIGURE 4 Effect of Captopril on BCG-induced pulmonary inflammation. B6 mice were injected intravenously with 300 μg of killed BCG in an oil-in-saline mixture. They were injected intraperitoneally with Captopril or saline at the intervals and doses shown. Numbers under Captopril refer to interval of Captopril treatment in relation to BCG, e.g., group 1 received the drug daily 2 d before and through 21 d after BCG. Numbers under sacrifice refer to days after BCG injection. Each group represents at least five mice (both Captopril—and saline-treated) and is expressed as the mean±standard error.

The effect of Captopril on blood and lung fluid levels of ACE in normal or BCG-injected B6 mice. Since it is possible that the effect of Captopril at the dose used was not restricted to its inhibitory activity on ACE, we performed additional experiments to determine the effect of a single intraperitoneal injection of either 50 or 150 mg/kg of Captopril on the blood and lung fluid levels of either normal B6 mice or mice that had been injected with 300 μg of BCG 28 d previously. Data in Fig. 6 show that Captopril at either 50 or 150 mg/kg reduced serum ACE in either normal or BCG-injected B6 mice, but that these values returned to preinjected levels or greater within 24 h (A and C). Most importantly, Captopril reduced ACE levels in lung fluids of mice previously treated with BCG (B). The dose of 150 mg/kg was more effective in maintaining a sustained depression of ACE activity. However, even with this higher dose, ACE activity in lung fluid began rising by 4 h and was ~50% of the preinjection level by 24 h. Normal lung fluids contained only minimal levels of ACE (D). These data suggest that repeated treatments with Captopril are necessary to attain sustained depressions of ACE activity in the blood and lung fluid of either normal or BCG-inflamed mice.

The effect of Captopril on the migration of mouse macrophages and on chemotactic activity. It is possible that Captopril modulated BCG-induced inflammation by some activity not related to its effect on ACE. We therefore tested the effect of the drug on the in vitro migration of mouse macrophages and on chemotactic activity produced by isolated granulomas from BCG-inflamed lungs. Doses as high as 625 μg/ml of Captopril did not significantly influence the migration of mouse peritoneal macrophages. If this is extrapolated to in vivo treatment (assuming even distribution of the drug in tissue), this dose is approximately four times greater than the highest dose given daily (150 mg/kg). In addition, doses as high as 500 μg/ml of Captopril did not affect the chemotactic activity produced by lung granulomas. Thus, these data suggest that the suppressive effect of Captopril on BCG-induced inflammation was not caused by an effect on the migration of macrophages or on chemotactic activity produced by pulmonary granulomas.

DISCUSSION

This study has demonstrated that levels of ACE in lung lavage fluid were closely correlated with the intensity

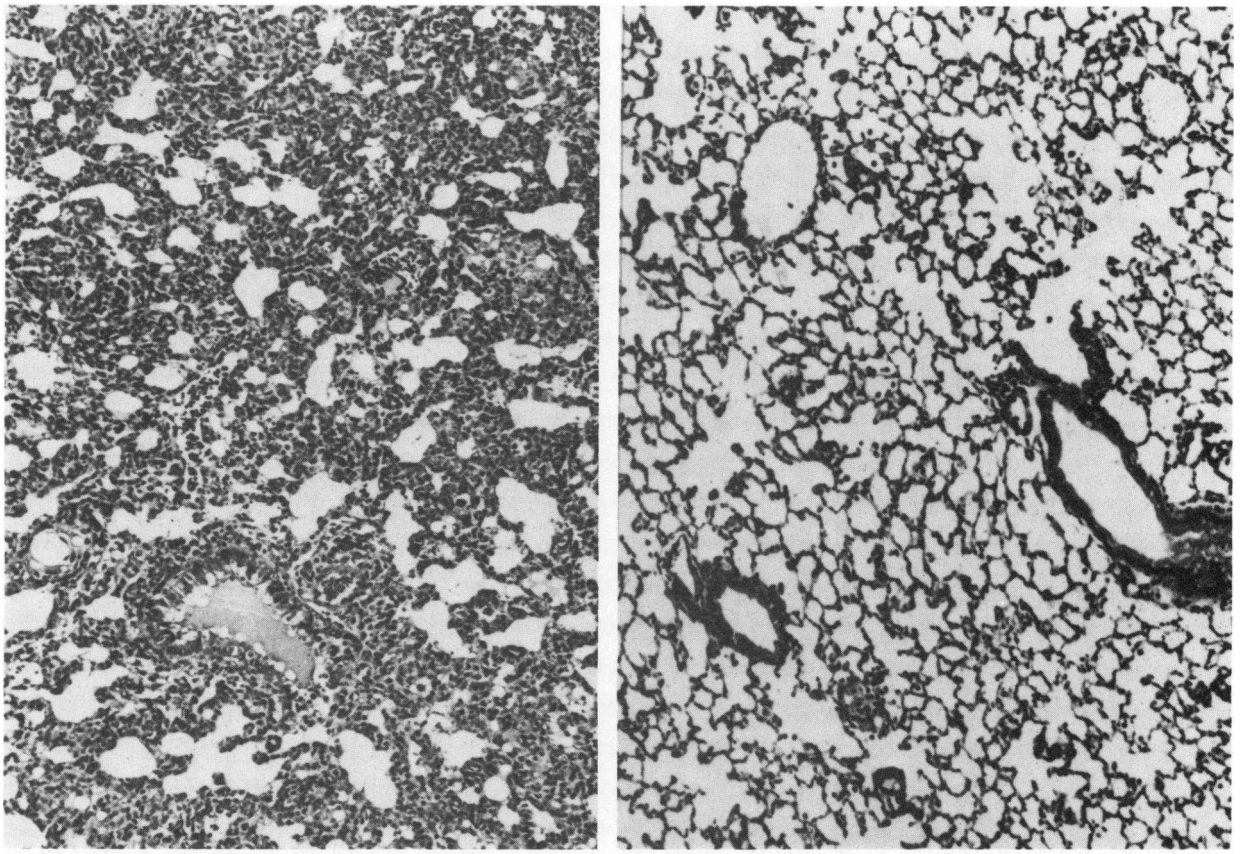

FIGURE 5 Histopathology of B6 mouse lungs (left) given BCG and saline, and (right) given BCG and Captopril. These animals correspond to group 2, Fig. 4. Mice given BCG and saline developed intense granulomatous pulmonary inflammation, whereas those given BCG and Captopril had markedly reduced inflammation (hematoxylin and eosin stain, ×40).

of chronic granulomatous inflammation in mice. In this system, the development of inflammation in high responder mice is dependent upon T lymphocytes (11) for its full expression, whereas low responsiveness is due, at least partially, to a population of cyclophosphamide-sensitive suppressor T cells (16). In addition, inflammation can be quantified by measuring increases in lung and spleen weight. The impetus for this study in mice was several reports indicating that serum levels of ACE are useful diagnostically and possibly prognostically as markers of granulomatous lung disease (2–6).

Although we found close correlation between the intensity of inflammation and ACE activity in lung lavage fluid, serum ACE activity did not correspond to the intensity of the inflammatory response in the lungs or spleen. In contrast to this study, serum ACE is reported to be a good diagnostic marker for sarcoidosis, a disease associated with granulomatous inflammation (4, 7) and to be elevated in murine schistosomiasis (8). To our knowledge, parallel studies in humans on ACE activity in lung lavage and serum have not been reported.

Another important finding was that Captopril, a specific inhibitor of ACE, reduced the intensity of inflammation in both the spleen and lungs. Suppression occurred when the drug was given before and during the inflammatory response, as well as when administered after inflammation had already developed. However, it was necessary to continually treat with Captopril in order to reduce inflammation. Moreover, additional studies suggested that continued daily injections of Captopril were necessary to attain a sustained depression of ACE activity. Furthermore, the effect of the drug on BCG-induced inflammation was not explicable either because of an effect on macrophage mobility or on chemotactic activity. These data suggest that ACE plays a role in the mediation of BCG-induced chronic granulomatous inflammation, although we have not entirely ruled out the possibility that Captopril had other effects unrelated to its activity on ACE.

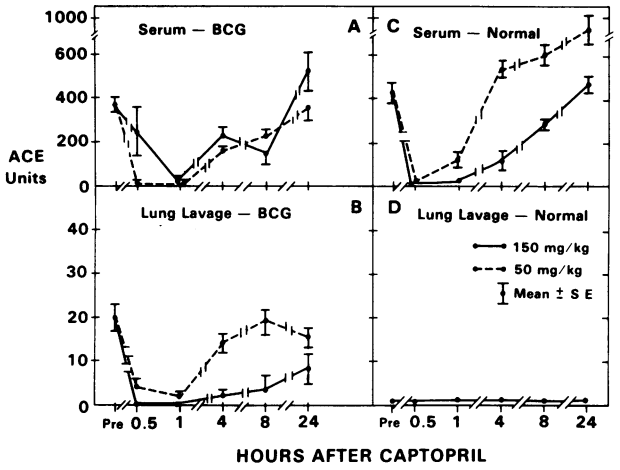

FIGURE 6 The effect of Captopril on blood and lung fluid levels of ACE in normal and BCG-injected B6 mice. One group of mice was injected intravenously with 300 μg killed BCG in oil-in-saline emulsion; the other group was normal B6 mice. Groups of normal or BCG-injected mice were bled or lung lavaged to obtain normal values of ACE. Other groups of mice were injected intraperitoneally with either 50 or 150 mg/kg of Captopril. Mice were killed at the intervals indicated, blood and lung lavage were processed, and ACE levels determined. A minimum of three mice was used for each interval.

In a recent study on murine schistosomiasis, Weinstock et al. (17) demonstrated that Captopril induced decreased ACE activity in liver granulomas in association with a reduction in granulomatous inflammation. Thus, ACE appears to play a role in two model systems of granulomatous inflammation. The mechanisms by which Captopril suppresses inflammation are not known at the present time but it may be through the kinin-prostaglandin pathway. In this regard, Schwartz et al. reported that the administration of Captopril to patients altered prostaglandin levels and suggested that these changes were associated with the hypotensive response (18).

The possible relationship of immunologic events to ACE synthesis is not clear. However, Silverstein et al. (9) reported that ACE could be localized in epithelioid macrophages and giant cells of sarcoidosis patients around the periphery of granulomas where lymphocytes usually coexist. Epithelioid macrophages in the center of sarcoid granulomas and control nonsarcoid granulomas did not exhibit ACE activity. They suggested that ACE synthesis may be dependent upon interaction between lymphocytes and epithelioid macrophages. In addition, we have observed that normally high responders BCG-injected B6 mice that are deprived of T lymphocytes have lower levels of ACE in their lung fluids (11). This suggests that T cells are necessary for the synthesis of ACE. This possibility is also suggested by in vitro studies reported by Friedland et al. (19).

Continued studies in this animal model system may provide a better understanding of the molecular mechanisms of granulomatous inflammation and the role of ACE in this process.

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