Characterization of Arterial Antigens Using Arterial Antigen-Reactive T Cell Clones From Spontaneously Hypertensive Rats

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We have previously demonstrated that arterial antigens derived from the aorta of spontaneously hypertensive rats (SHRs) stimulate arterial antigen-reactive T cell clones established from the spleens of SHR to proliferate and release cytokines. To identify immunogenic protein components associated with the arterial wall, arterial antigen-reactive T cell clones were tested against arterial antigens separated by SDS-PAGE and transferred to nitrocellulose. The greatest T cell reactivity was obtained with protein bands of molecular weight 66 kDa, 50 kDa, and 45 kDa. T cell clones reactive against the 50 and 45 kDa antigens from gels failed to respond to proteins of other molecular weight (M,) separated under reducing or nonreducing conditions, suggesting that these molecules are not subunits of larger proteins and may represent monomeric antigens polymerized into the arterial wall. These data suggest that certain epitopes of arterial wall antigens are immunogenic. T cells activated with these immunogenic epitopes could initiate or perpetuate vasculitis in the arteries of hypertensive rats. Am J Hypertens 1997;10:535–540 © 1997 American Journal of Hypertension, Ltd.

KEY WORDS: T cell clones, arterial antigen, autoimmunity, hypertension.

There is increasing evidence implicating immune system dysfunction in the pathogenesis of hypertension in the SHR. Various immunological disorders have been demonstrated in the SHR, including alteration of lymphocyte populations, abnormal mitogen proliferation and suppressor T cell function, decreased delayed-type hypersensitivity, and the production of thymocyte toxic autoantibodies. Such immunological dysfunction is commonly associated with autoimmune mechanisms, and it has been postulated that hypertension in the SHR may result, at least partially, from an autoimmune process. This hypothesis is supported by the fact that anti-rat thymocyte serum, thymic grafts, and chronic immunosuppression by cyclophosphamide treatments reduced blood pressure. Thus, hypertension in the SHR might involve delayed-type hypersensitivity and/or production of autoantibodies against components of the arterial wall, resulting in increased peripheral resistance and, therefore, elevation of blood pressure. Ebringer and Doyle suggested that elevated blood pressure chronically damages the vessel wall, which in turn exposes otherwise hidden components of the arterial wall and ultimately induces the production of autoantibodies. However,
no such antigenic epitopes have as yet been discovered. In the present study, we have used arterial antigen-reactive T cell clones in conjunction with SDS-PAGE immunoblotting for the investigation and analysis of the nature of immunoreactive protein components of the arterial walls that contribute to immunogenicity.

**METHODS**

**Animals** Animal care and use were approved by the Animal Care and Use Committee at the Masonic Medical Research Laboratory, Utica, NY. Male SHR rats aged 28 weeks were obtained from Taconic Farms (Germantown, NY). Rats were housed three to a cage and maintained on Purina (St. Louis, MO) rat chow and water ad libitum. The animal room was maintained at 22°C with automatic 12 h on and 12 h off light cycles and with 10 air changes/h.

**Isolation of Lymphocytes from Spleen** Spleens were aseptically removed and single cell suspensions were obtained by mincing spleens with scissors and gently pressing the fragments through a 100 μm stainless steel mesh. The resulting cell suspension was layered onto Ficoll-Paque gradient (Pharmacia LKB Biotechnology, Piscataway, NJ) at a ratio of 2 mL of cell suspension to 1 mL of the gradient and centrifuged at room temperature (25°C) for 20 min at 400 g. The lymphocytes were harvested from the Ficoll-medium interface, washed and resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 20 mmol/L HEPES, 2 mmol/L L-glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin and 5 × 10⁻⁵ mol/L 2-mercaptoethanol (2-ME). The resultant lymphocytes were counted and viability assessed by trypan blue dye exclusion. Routinely, viability is usually greater than 98%. The cell concentration was adjusted to 2 × 10⁶ for functional studies.

**Isolation of In Vivo-Activated Interleukin-2 (IL-2) Responsive T Cell Lines** IL-2 has been used to selectively expand a population of T lymphocytes activated in vivo at inflammatory sites of autoimmune diseases, and the cells have been shown to conserve their specificity and function. In an effort to isolate activated antigen-reactive, IL-2 responsive T cells that might be of pathogenetic importance in hypertension, spleen cells (2 × 10⁶/mL) were cultured in RPMI 1640 medium supplemented with recombinant human IL-2 (10 U/mL). After a 7-day expansion in culture, the proliferating T cells were layered on Ficoll-Paque density gradient to remove dead cells. The T cell lines were maintained by a regimen of 14-day stimulation with mitomycin C (50 μg/mL)-treated spleen cells and 100 ng/mL of anti-CD3 mAb, followed by IL-2 dependent expansion phase. Using this approach, the T cells could be expanded 20 times their number in the course of the 14-day cycle. For use in functional studies, the T cell lines were used after the second expansion phase, where adequate numbers of cells could be generated.

**Generation of IL-2 Dependent T Cell Clones** In order to carry out a more detailed analysis of the T cell responses to the arterial antigens (AA) of different molecular weights, T cell clones were generated from IL-2 dependent T cell lines by limiting dilution analysis (LDA) at 0.3 cells/well as previously described. The LDA was performed in 96-well, round bottomed microtiter plates in the presence of 10⁵ syngeneic mitomycin-C spleen cells as filler cells in RPMI 1640 medium containing 10 units/mL of rIL-2 and 100 ng/mL of anti-CD3 mAb. Control wells contained 10⁵ syngeneic mitomycin-C treated cells in 0.2 mL of RPMI 1640 medium alone. In all experiments, 24 replicate cultures were set up for each cell concentration. The cultures were fed on day 7 by replacing 0.1 mL of growth medium with fresh rIL-2 containing medium. After a further 7 days in culture, the microwells were microscopically scored for growth. The clones were expanded and maintained by addition of fresh IL-2 every 3 days, and filler cells together with 100 ng/mL of anti-CD3 mAb every 7 days. At no time were the clones exposed to specific antigens except in proliferation assays. Before use in proliferation assays, the T cell clones were rested, that is, not stimulated with feeder cells + anti-CD3 mAb for 14 days. The phenotype of the T cell clones was then determined by flow cytometry. Such an approach has successfully been used to generate stable long-term IL-2 dependent T cell clones from spontaneously hypertensive rats.

**Preparation of Arterial and Control Antigens** Aortas and livers were aseptically removed from adult (18 week old) SHR, minced with scissors, and suspended in 10 mL of phosphate-buffered saline (PBS), as previously described with some modifications. They were then mechanically homogenized with an electric homogenizer until no intact cells could be observed by phase microscopy. The homogenate was sonicated for 10 min at 140 W and then centrifuged for 60 min at 100,000 g in an ultracentrifuge (model L8-M; Beckman Instruments, Palo Alto, CA) at 4°C. After determining the protein concentration, the antigen was aliquoted at 100 μg/mL and lyophilized. A liver extract served as control antigen and was treated in a similar fashion. For use in T cell proliferation assay, an aliquot of the lyophilized material was reconstituted with 1 mL sterile PBS.

**Proteolytic Digestion of AA** Proteolytic digestion of AA was performed by incubating 1 mL (2 mg/mL) of AA with 1 mL of either the agarose alone or 1 mL (10 units) of proteinase K-agarose at 37°C for 4 h. The suspension was centrifuged at 20,000 g for 20 min and...
the supernatant was collected. After determining the protein concentration, the supernatant was aliquoted at 100 μg/mL and lyophilized.

**Preparation of ImmunobLOTS** Proteins in arterial antigen samples were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) and by immunoblotting. For SDS-PAGE analysis, 100 μg of AA was boiled for 4 min in sample buffer comprising of 500 mmol/L Tris-HCl (pH 6.8), 100 mmol/L dithiothreitol (DTT), and 0.05% of bromophenol blue containing 0.02% SDS. The AA preparation was then electrophoresed in 10% polyacrylamide gels and stained with Coomassie blue. For immunoblotting, the separated proteins were electrotransferred for 1 h at 15 volts onto nitrocellulose filters (Bio-Rad Labs., Richmond, CA) using a Bio-Rad trans-blot semidry apparatus. The precipitate was washed three times with PBS and resuspended in 1 mL of plain RPMI 1640. The suspension was divided into 100 μL aliquots

**Reactivity of IL-2 Dependent T Cell Clones to Nitrocellulose-Bound AA** To determine whether T cells in spleens of SHR have been activated by AA in vivo during the hypertensive process, we expanded T cells obtained from SHR spleens in the presence of IL-2, in the absence of added AA, in order to select for T cells activated in situ to express IL-2 receptors. Of the 24 T cell lines tested, four T cell lines proliferated in response to AA supplied either as soluble antigen or in the form of antigen bound to nitrocellulose. Table 1 shows a representative experiment demonstrating proliferation of a T cell line derived from SHR spleen. A higher concentration of nitrocellulose-bound AA was required to stimulate an equivalent response to that obtained with soluble AA. The T cell line failed to respond when tested with an irrelevant antigen myelin basic protein (MBP), which confirmed the specificity of the T cell lines from the spleens of SHR (Table 1). No proliferation was obtained in the absence of APC, and the nitrocellulose devoid of AA was not mitogenic.

**Reactivity of IL-2 Dependent T Cell Clones to Nitrocellulose-Bound AA** In order to identify the molecular weight (Mₚ) of the AA component stimulating the proliferative response of the T cell clones, AA was subjected to SDS-PAGE and, following transfer to nitrocellulose filters, the different antigenic fractions were tested in proliferation assay. Figure 1 shows the response of eight T cell clones to six antigenic fractions of AA. The results are expressed as the average ± standard error of the mean (SEM) of the proliferative responses of eight T cell clones tested against each of the six antigenic fractions. The results are presented this way because of considerable overlapping values. All the clones proliferated in response to soluble AA. When the individual nitrocellulose-bound antigen fractions were tested, only three antigenic fractions corresponding to 66 kDa, 55 kDa, and 45 kDa induced a significant proliferation in the clones, with the most potent stimulatory activity residing in the 66 kDa antigen. Neither of the other antigenic fractions nor the nitrocellulose filter alone exhibited such a stimulatory effect. No proliferation was observed in the absence of APC. Positive results (proliferation > 1000 counts/min) were obtained with the AA-reactive T cell clones. Of the eight AA-
TABLE 1. REACTIVITY OF IL-2 DEPENDENT T CELL LINE TO NITROCELLULOSE-BOUND AA

<table>
<thead>
<tr>
<th>T Cell Line</th>
<th>APC</th>
<th>Antigen</th>
<th>Proliferation (×10^3 count/min ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>AA 0.2 µg/mL</td>
<td>7.9 ± 0.4</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+2.0 µg/mL</td>
<td>15.2 ± 0.2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+20 µg/mL</td>
<td>22.3 ± 3.6</td>
</tr>
<tr>
<td>+**</td>
<td>+</td>
<td>+20 µg/mL + nitro</td>
<td>—</td>
</tr>
<tr>
<td>+</td>
<td>—</td>
<td>MBP 20 µg/mL</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>+</td>
<td>—</td>
<td>IL-2</td>
<td>34.9 ± 4.1</td>
</tr>
</tbody>
</table>

AA-reactive T cell line (2 × 10^4/mL) was stimulated with AA or control antigen myelin basic protein (MBP) as soluble antigen or nitrocellulose-bound as dot-blot antigen in the presence of mitomycin C-treated syngeneic spleen cells antigen-presenting cells. A positive control of the response of the T cell line to IL-2 was included in the assay. Proliferation as monitored by [³H]-TdR incorporation was determined at 72 h. Results are expressed as mean counts per minute (c.p.m.) ± standard error of mean (SEM) of triplicate cultures. Control responses of the T cell line to (*) medium, (†) APCs without antigen and (**) soluble antigen in the presence of nitrocellulose were also included in the assay.

AA, arterial antigens.

reactive T cell clones tested, four (A1, B3, B8, and D5) reacted with a protein of Mₚ 66 kDa and two clones (A4, C9) reacted with a protein of Mₚ 50 kDa. The other two clones (D7, E10) recognized antigens with Mₚ 45 kDa and 30 kDa, respectively. The higher molecular weight fractions 70 to 90 kDa failed to stimulate the T cell clones. Visual inspection of the polyacrylamide gels stained for total protein (Figure 2) did not indicate a marked difference in the concentration of proteins in the molecular weight regions of the AA proteins.

T Cell Recognition of AA Is Retained During Preparation of AA Because blood vessel wall antigens may be immunodominant in cell-mediated immune response in hypertension, it was important to characterize the nature and stability of the inherent epitopes recognized by the AA-reactive T cell lines. As shown in Table 2, digestion of proteins in AA by proteinase K-agarose (Sigma) treatment resulted in loss of antigen capable of activating the AA-reactive T cell lines in vitro. These results suggest that AA and epitopes recognized by AA-reactive T cell lines are protein in nature.

**DISCUSSION**

We have recently reported the existence of AA-reactive T cell clones in SHR. However, very little is known about the components of the arterial wall that trigger cell-mediated immune responses in SHR. Ebringer and Doyle suggested that the elevated blood pressure chronically damages the vessel wall, which in turn exposes otherwise hidden components of the arterial wall and ultimately induces the production of autoantibodies. However, no such antigenic epitopes have yet been discovered. This study was designed to characterize the antigenic epitopes associated with the arterial wall using arterial antigen-reactive T cell clones.

The known immunologic activities associated with the components of the arterial wall are fundamental to understanding the immune responses to AA, and may ultimately have relevance in the design of therapies for immune intervention. Our data clearly indicate that a small proportion of T cell lines and clones derived from spleens of adult SHR proliferate in vitro to soluble AA. Further, T cells derived from SHR spleens and expanded only by IL-2 and not selected in vitro by AA, responded strongly to AA. These data suggest that a proportion of T cells in the spleens of SHR have been activated by blood vessel wall antigens in situ. Although the target autoantigen in hyper-
onset of clinical hypertension, suggesting that arterial antigen reactive autoantibodies may directly contribute to vascular damage and to the development of hypertension in the SHR. Our data support the notion that proteins associated with the blood vessel walls are the immunodominant antigen in the acquisition of cell-mediated immunity. The vascular lesions in SHR are distributed in large and small arteries of the mesenteries, kidney, and lung. It is therefore reasonable to assume that, because these antigens occur in aorta, then they must exist in other arterial vessels. It is clear that the immunodominant blood vessel wall associated antigens are protein in nature, as treatment with proteinase K abolished recognition by the T cell clones. The SDS-PAGE immunoblotting technique has been shown to facilitate the identification of antigenic epitopes recognized by T cells and to analyze the contribution of antigens of different molecular weight to the overall T cell response to the antigen.18

In this method, proteins are separated by standard SDS-PAGE, and after transferring to nitrocellulose, individual molecular weight fractions can be tested for their ability to stimulate T cell clones. In the present study, we have applied this SDS-PAGE immunoblotting technique, by using AA-reactive T cell clones as reagents to ascertain the nature of the immunoreactive antigenic epitopes of the arterial wall that contribute to immunogenicity. The results indicated that arterial antigen reactive T cell clones proliferated against essential hypertension. In a previous study, we have demonstrated the presence of circulating antibodies reactive with arterial antigen in young SHR before the onset tension is unknown, it is presumably present on the arterial wall. This hypothesis is supported by increased T-cell reactivity against human arterial antigen in patients with essential hypertension. In a previous study, we have demonstrated the presence of circulating antibodies reactive with arterial antigen in young SHR before the onset of clinical hypertension. Our data support the notion that proteins associated with the blood vessel walls are the immunodominant antigen in the acquisition of cell-mediated immunity.

**FIGURE 2.** Characterization of AA. One hundred micrograms of AA were dissolved in sodium dodecyl sulfate (SDS) gel sample buffer and then subjected to 10% SDS gel electrophoresis for 3 h at 200 V/36 mA under reducing conditions until the bromophenol blue tracking dye reached the bottom of the gel. The gel was then stained with 0.05% Coomassie blue in 10% acetic acid. Molecular weight standards used were myosin heavy chain, 200 kDa, β-galactosidase, 116 kDa, phosphorylase b, 97 kDa, bovine serum albumin, 66 kDa, and ovalbumin, 45 kDa.

**TABLE 2. T CELL REACTIVITY OF AA DURING PROTEIN PREPARATION**

<table>
<thead>
<tr>
<th>T Cell Clones</th>
<th>Medium</th>
<th>AA</th>
<th>AA + Proteinase K</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.6 ± 0.1</td>
<td>11.6 ± 0.1</td>
<td>0.8 ± 0.1*</td>
<td>35.1 ± 2.4</td>
</tr>
<tr>
<td>B3</td>
<td>0.8 ± 0.1</td>
<td>13.5 ± 0.4</td>
<td>0.7 ± 0.2*</td>
<td>30.7 ± 0.9</td>
</tr>
<tr>
<td>B8</td>
<td>0.9 ± 0.2</td>
<td>16.5 ± 0.5</td>
<td>1.4 ± 0.1*</td>
<td>45.1 ± 3.2</td>
</tr>
<tr>
<td>D5</td>
<td>0.7 ± 0.1</td>
<td>19.8 ± 1.0</td>
<td>1.5 ± 0.4*</td>
<td>47.2 ± 11.1</td>
</tr>
</tbody>
</table>

Cell proliferation studies were performed as described in Materials and Methods section. Medium consisted of T cell clones and antigen-presenting cells only. Values are expressed as means ± SEM.

* P < .001, versus AA, by Student’s t test.

AA, arterial antigens
products of larger precursors. Thus, we can conclude that antigens of 66 kDa, 50 kDa, and 45 kDa are the key immunoreactive epitopes of the arterial wall protein complex. Because the concentration of protein in each lane on the gel was not assayed, it cannot be determined whether the immunodominance demonstrated by the proliferative response is a reflection of the concentration of particular antigen or concentration of T cell clones with appropriate specificities. Visual inspection of gels stained for total protein does not indicate the presence of high concentration of proteins in sections of the gel stimulating maximum T cell proliferation. Thus, it appears that immunodominance is based on immune recognition rather than on relative antigen concentration. It was rather surprising that none of the large molecular weight antigens 70 to 97 kDa induced proliferation of the T cell clones. It is possible that these proteins are causing suppression of proliferation of the T cell clones. It is also conceivable that the detection of immunodominant antigens by immunoblotting may reflect other properties of the proteins, such as their strong binding to the nitrocellulose filter paper and the efficiency of processing and presenting the antigens from the nitrocellulose-bound antigen.

Despite these pitfalls, analysis by SDS-PAGE immunoblotting does offer the potential of obtaining more information than proliferation assays with unfraccionated antigens. This technique can be used to identify the gene coding for the immunodominant antigenic epitope of the arterial wall. This can be achieved by producing monoclonal antibodies to the purified proteins on the nitrocellulose filters, which can be used to screen recombinant DNA libraries. Alternatively, the purified proteins on the nitrocellulose filters can be microsequenced so that specific oligonucleotides can be synthesized and used to screen recombinant DNA libraries.

In summary, we have identified the nature of immunoreactive protein components associated with the arterial wall that contribute to immunogenicity in the SHR. Our data show that the 66 kDa protein, which induced the greatest T cell reactivity, might be the immunodominant epitope of AA, which could activate autoreactive T cells to initiate or perpetuate vasculitis in the arteries of hypertensive rats.

ACKNOWLEDGMENTS

We thank Mrs. Kathy Dupont for excellent secretarial assistance and Dr. Charles Antzelevitch for critical review of the manuscript.

REFERENCES