Anti-endothelial cell antibodies: Detection and characterization in sera from patients with autoimmune hypoparathyroidism
(parathyroid gland/cell culture)

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ABSTRACT In a previous report, we described antibodies in autoimmune hypoparathyroidism (AHP) that are cytotoxic for cultured bovine parathyroid cells. In the present study, we show that sera from six AHP patients, but not from 26 patients with other autoimmune diseases or from 7 healthy subjects, react with bovine endothelial cells in culture (by flow cytometry and fluorescence microscopy) and in tissue sections (by immunohistology). We found uniformly that the immunoglobulin class reacting is IgM. Adsorption experiments showed that the antigenic determinants reacting with AHP sera were similar on bovine cultured endothelial cell membranes and in tissue sections of bovine parathyroid glands. The AHP sera also reacted with endothelial cells cultured from bovine adrenal medulla and pulmonary artery. Immunoblot analysis showed antibody binding to two major bands of 200 and 130 kDa solubilized from the membrane fraction of bovine parathyroid endothelial cells. Only one AHP serum consistently recognized endothelium-related structures on frozen sections of three different human parathyroid adenomas; two other sera reacted with one adenoma each; and three did not react with human adenomas. This indicates that human material is less suitable than bovine in detecting endothelium-related immune phenomena in AHP sera. The anti-endothelium IgM antibodies appear to be disease-specific but are not organ- or species-specific. The identification of endothelial cells as the target for antibodies in AHP raises the possibility that the endothelium suberves an important local function for endocrine epithelium.

In an earlier study, we found that antibodies in the sera of patients with autoimmune hypoparathyroidism (AHP) (1–5) reacted with and were cytotoxic for bovine parathyroid cells in culture (6). We have now utilized fluorescence flow cytometry and immunohistology with class-specific antisera to study the nature of antibodies reacting with antigen(s) on human as well as bovine cells. We found that it is primarily endothelial cells of parathyroid tissue that react with antibodies from patients with AHP.

MATERIALS AND METHODS

Patient Groups. Subjects were studied under approved protocols at the National Institutes of Health. Serum samples were obtained from 42 patients with the following diagnoses: 6, AHP7; 7, autoimmune thyroid disease; 4, Addison disease; 3, idiopathic pulmonary fibrosis; 4, primary biliary cirrhosis; 3, rheumatoid arthritis; 8, systemic lupus erythematosus; and 7, normal subjects.

Cell Culture. Bovine (PT-b) and cloned rat (PT-r) parathyroid cells were prepared and cultured as previously described (7, 8). In this study, we used PT-b cells from the same primary culture between the 3rd and the 15th passage. Endothelial cells from bovine parathyroid tissue (BPE) were cloned by limiting dilutions (M.L.B., K.S., and G.D.A., unpublished results) and cultured in Coo’s modified Ham’s F12 medium that contained antibiotics, 10% Nu-Serum (Collaborative Research, Waltham, MA), 2% Ultraser-G (IBF, Savage, MD), 200 µg/ml of d-galactose (Sigma), 1.0 mM Ca2+, and 0.5 mM Mg2+. Bovine adrenal medullary endothelial (BAME) cells (obtained from P. Lelkes, National Institutes of Health) were grown in minimal essential medium supplemented with antibiotics and 10% (vol/vol) heat-inactivated fetal calf serum (FCS) (Biofluids, Rockville, MD) as described (9). Bovine pulmonary artery endothelial (BPAE) cells (ATCC no. CCL 209) were maintained in minimal essential medium containing antibiotics and 20% FCS. Cloned human MCF-7 mammary cancer cells (obtained from M. Lippman, National Institutes of Health) and normal human skin fibroblasts (HSF) (ATCC no. CRL 1229) were maintained in Dulbecco’s modified Eagle’s medium supplemented with antibiotics and 20% FCS.

Immunofluorescence in Cultured Cells. Sera were tested in an indirect immunofluorescence assay against a series of cultured cells using flow cytometry and/or fluorescence microscopy. For flow cytometry experiments, cells were released from culture plates using 0.05% trypsin in phosphate-buffered saline (PBS)/2 mM EDTA and resuspended in cold PBS (without Ca2+ or Mg2+)/2% FCS. Cells (5 × 105 in 1 ml) were incubated for 45 min in an ice bath with a 1:20 dilution of serum from AHP patients or normal patients. After repeated washing with PBS, cells were incubated for an additional 45 min in an ice bath with a 1:40 dilution of a monospecific, affinity-purified, fluorescein-conjugated goat anti-human IgG, IgM, or IgA (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The cells were washed again with PBS and used for flow cytometry (FACS 440, Becton Dickinson; operating with an argon laser at 488 nm and 200 mV) (10). Ethidium bromide (50 µg/ml) was used to gate out

Abbreviations: AHP, autoimmune hypoparathyroidism; PT-b cells, bovine parathyroid cells; BPE cells, bovine parathyroid endothelial cells; BAME cells, bovine adrenal medullary endothelial cells; PT-r cells, rat parathyroid cells; HSF cells, human skin fibroblasts; BPAE cells, bovine pulmonary artery endothelial cells; FCS, fetal calf serum.

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†Patients 1–6 represent patients 2–7, respectively, from a previous report (6). As previously described (6), sera from all AHP subjects mediated specific lysis of PT-b cells as determined by a 51Cr release assay. Patients 1–6 were clinically hypoparathyroid, patients 3–6 expressed mucocutaneous candidiasis, patients 5 and 6 were alopecic, and patients 4 and 6 displayed adrenal insufficiency. Patients 1 and 2 are twin sisters; patients 3–5 are siblings in another family. There were no other affected members in the family of patient 6.
dead cells from final counts (11). Quantitative data from flow cytometry are reported as the mean peak of fluorescence expressed in units of channel number. Comparable preparations were also taken for fluorescence microscopy (6).

**Immunohistologic Staining.** Immunofluorescence was performed as described (12) on cold methanol/acetone-fixed frozen sections obtained from several bovine and rat tissues, including parathyroid, adrenal, pituitary, kidney, and liver. Three human parathyroid adenomas were also used. Sections, 6–8 μm thick, were incubated with fluorescein-conjugated goat anti-human IgG, IgM, or IgA (1:20 in PBS). Double fluorescence staining was performed in human parathyroid adenomas to compare the distribution of vascular endothelium and antigen(s) recognized by AHP serum. Factor VIII-related antigen was chosen as a marker for endothelium (13). Sections were sequentially incubated with AHP serum and with a 1:30 dilution of rabbit antisera for factor VIII-related antigen (Calbiochem–Behring). After washing with PBS, sections were incubated with a mixture of fluorescein-conjugated goat anti-human IgM and fluorescein-or rhodamine-conjugated goat anti-rabbit IgG (1:30) (Accurate Chemicals, Westbury, NY), washed, and scanned by fluorescence microscopy.

**Adsorption Studies.** Cells (10 × 10⁶/ml) were resuspended in PBS supplemented with 2% FCS and were incubated with AHP serum (1:10 final dilution) for 4 hr at 4°C. Suspensions were then centrifuged, and the supernatant was used for immunofluorescence studies. Adsorption was also carried out using 10-fold concentrations of conditioned medium from PT-b, BPE, BAME, PT-r, and HSF cell cultures. The conditioned medium was concentrated by centrifugation on Centricon filters (Amicon). Concentrated medium was incubated with AHP serum (1:10 final dilution) for 4 hr at 4°C. This mixture was then immediately used for immunofluorescence analysis.

**Statistical Evaluation.** The significance of intergroup differences was evaluated by the nonparametric Wilcoxon rank sum test for independent samples (14).

**Immunoblot Studies.** Serum IgM from AHP patient 6 and from one healthy donor was purified by boric acid precipitation and Sephadex G-200 column chromatography (15). Membranes were prepared according to Thom and colleagues (16). Cells were lysed in hypotonic borate buffer, filtered through nylon gauze, and centrifuged at 450 × g for 10 min to separate large particles. The supernatant was centrifuged at 12,000 × g for 30 min, and the pellet was resuspended in PBS, layered above a 5% sucrose cushion in PBS, and centrifuged at 24,000 × g for 1 hr. Purified membranes were collected at the sucrose/PBS interface. Membrane proteins were prepared for electrophoresis and fractionated on a 6% NaDodSO₄/polyacrylamide gel according to Laemmli (17). Electrophoretic transfer was carried out as described (18). The nitrocellulose membrane was blocked with a buffer containing 10% nonfat milk (Carnation, Los Angeles), 20 mM Tris (pH 7.5), and 500 mM NaCl. Blots were incubated overnight at 4°C with purified IgM from AHP patient 6. After extensive washing, blots were sequentially incubated at 4°C with affinity-purified goat anti-human IgM (Kirkegaard and Perry Laboratories), affinity-purified rabbit anti-goat IgG (Kirkegaard and Perry Laboratories), and 125I-labeled affinity-purified goat anti-rabbit IgG (2 × 10⁶ cpm/ml) (New England Nuclear). After extensive washing, blots were dried and submitted to radioautography.

**RESULTS**

**Indirect Immunofluorescence in Cultured Cells.** Immunofluorescence analysis on tryps inized resuspended PT-b, BPE, or BAME cells revealed that sera from AHP patients reacted with the cell membrane (Fig. 1A). No detectable staining was observed with normal sera (Fig. 1B) or with sera from 28 patients displaying autoimmune diseases other than AHP. We found only IgM antibodies reacting with the bovine cells. PT-r, MCF7, and HSF cells were completely nonreactive (data not shown). Flow cytometry analysis confirmed the fluorescence microscopy observations and showed a single peak of fluorescence with PT-b, BPE, BAME, and BPAE cells (Fig. 2A). The intensity of fluorescence varied somewhat among the bovine cell lines (data not shown) and among patients, probably reflecting different degrees of antigenic expression and different antibody titer, respectively (Table 1). Statistical analysis indicated that the fluorescence intensity in the bovine cell lines was significantly higher (P < 0.01) than in the other cell types tested (Table 1 and Fig. 2B). Among the non-AHP patients, serum from only one, an Addisonian, was reactive (Table 1). The reactivity in all cases was ascribable solely to IgM antibodies. The intensity of fluorescence obtained in bovine cells with the sera from other autoimmune disease patients and healthy donors was significantly (P < 0.01) lower than that obtained with sera from AHP patients. As illustrated in Fig. 3, adsorption of AHP serum with BAME cells completely abolished reactivity on BPE cells. Comparable results were obtained by preincubating AHP sera with PT-b and BPAE cells; adsorption with cell types other than bovine was without effect (data not shown).

A detectable reduction in fluorescence intensity was observed after incubating AHP sera with conditioned medium from PT-b, BPE, and BAME cells. In this instance, the reduction in specific fluorescence intensity ranged between 50% and 75%, as calculated by the shift in the mean peak of fluorescence toward the control value. Conditioned medium from PT-r or HSF cells were inert (data not shown).

**Immunohistochemistry.** Results of immunohistological studies on frozen sections of tissue were reasonably consistent with the immunofluorescent findings on cultured cells. All sera from AHP patients contained IgM antibodies that reacted with determinants in bovine parathyroid tissue closely related to the vascular endothelium and in close apposition to the epithelial cell membrane in the region of vascular cells (Table 2 and Fig. 4A and B). The non-AHP Addisonian patient displaying anti-endothelial antibodies also showed positive reactivity in the immunohistochemical analysis. A similar endothelium-related pattern was also observed in bovine adrenal, pituitary, and kidney, but there was much lower

**FIG. 1.** Staining patterns of BPE cells in suspension. Reaction of serum from AHP patient 6 (A) and a normal subject (B). Indirect immunofluorescence staining was obtained with fluorescein-conjugated anti-human IgM as the second antibody. The exposure times for the micrographs in A and B. (×300.)
intensity of immunohistochemical staining than was found in the parathyroid tissue (data not shown). In these other tissues, antigenic material was not as evenly distributed as in the parathyroid sections; it was detectable in certain areas but not in others, and it had no obvious relation to the abundance of vascular endothelium. Sera from other autoimmune disease patients and healthy subjects did not react with such endothelium-related structures in any tissue examined, although sera from lupus erythematosus and Graves disease patients did react with the expected nuclear or cytoplasmic determinants. Bovine liver and all rat tissues including parathyroid, adrenal, pituitary, kidney, and liver were uniformly unreactive (data not shown). Incubation of AHP serum with PT-b, BPE, BAME, or BPAE cells dramatically reduced or completely abolished the fluorescent staining on bovine parathyroid sections. Adsorption with PT-r or HSF cells was without effect (data not shown).

Less consistent results were obtained from testing sera from AHP patients on three human parathyroid adenomas. Only serum from patient 2 gave positive results with all three specimens. Two other sera were positive with one specimen each (Table 2 and Fig. 4C), and three sera were reactive with none of the adenomas. The immunofluorescence pattern closely resembled the one observed in bovine parathyroid tissue. In some areas, however, the immunohistochemical staining was also detectable on the surface or in the cytoplasm of parathyroid epithelial cells. Adsorption of positive

**Table 1. Reactivity of serum from subjects with AHP or other autoimmune diseases and from healthy subjects on cultured cells by flow cytometry**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>IgM fluorescence</th>
<th>Bovine cells*</th>
<th>Other cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHP 1</td>
<td>49–67</td>
<td>33–38</td>
<td></td>
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<tr>
<td>AHP 2</td>
<td>65–81</td>
<td>32–38</td>
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<tr>
<td>AHP 3</td>
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<td>31–35</td>
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<tr>
<td>AHP 4</td>
<td>51–58</td>
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<tr>
<td>AHP 5</td>
<td>49–55</td>
<td>28–33</td>
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</tr>
<tr>
<td>AHP 6</td>
<td>58–70</td>
<td>28–32</td>
<td></td>
</tr>
<tr>
<td>Other autoimmune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diseases (n = 28)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Addison disease (n = 1)</td>
<td>41–54</td>
<td>31–36</td>
<td></td>
</tr>
<tr>
<td>Normal (n = 7)</td>
<td>29–36</td>
<td>29–35</td>
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</tbody>
</table>

Reactivity was detected with fluorescein-conjugated anti-IgM. Values represent the range of intensity expressed as the mean peak of fluorescence in units of channel numbers. ND, not determined.

*PT-b, BPE, BAME, and BPAE cells.
†PT-r, MCF-7, and HSF cells.

**DISCUSSION**

The current studies elucidate further our earlier findings that antibodies circulating in AHP react with cultured PT-b cells (6). We now show that antigenic determinants reacting with such antibodies are found predominantly on endothelial cells and exist in human as well as in bovine tissue. The close similarity of antigen(s) in cell cultures and whole tissues is illustrated by the relationship between immunofluorescence intensity between cells in suspension and in tissue slices and by results of adsorption studies. This rules out the possibility that the expression of the determinants recognized by AHP sera with PT-b, BPE, BAME, or BPAE cells markedly reduced the intensity of fluorescence closely related to the vascular endothelium on human parathyroid adenomas, leaving unchanged the epithelial staining (data not shown). Adsorption with PT-r or HSF cells had no effect.

**Immunoblot Studies.** Immunoblot analysis of purified membrane preparations showed that serum from AHP patient 6 identified major bands of 200 and 130 kDa in BPE cell membranes (Fig. 5). The high background reactivity observed in this analysis is characteristic of immunoblots exposed to IgM antibodies. Membranes from HSF cells showed complete absence of these bands (Fig. 5).

**Table 2. Reactivity of serum from subjects with AHP or other autoimmune diseases and from healthy subjects on parathyroid tissue of bovine and human origin**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Human adenoma</th>
<th>Bovine</th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tbody>
<tr>
<td>AHP 1</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AHP 2</td>
<td>++</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AHP 3</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>AHP 4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
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<tr>
<td>AHP 5</td>
<td>++</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AHP 6</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Other autoimmune disease (n = 20)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal (n = 6)</td>
<td>-</td>
<td>ND</td>
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</tbody>
</table>

Indirect immunofluorescence was developed with fluorescein-conjugated anti-human IgM as the second antibody. Each experiment was carried out at least twice with the same specimen. Reactivity was characterized as being negative/weak (-), moderate (+), strong (+++), or very strong (+++). The tissues were normal bovine parathyroid and three human parathyroid adenomas. ND, not determined.
antibodies on cultured cells represents an artifact due to nonphysiological in vitro conditions rather than an intrinsic endothelial cell characteristic. In flow cytometry experiments, PT-b cells gave a single peak of fluorescence with AHP patients' sera, indicating a uniform distribution of reacting antibodies on the cell population. PT-b cells are comprised of both epithelial and endothelial parathyroid cells (M.L.B., K.S., and G.D.A., unpublished data). The single peak of fluorescence in this mixed cell population may reflect transfer of this antigen from endothelial to epithelial cells. This possibility is supported by the observation that concentrated conditioned medium from such cultures can absorb the anti-bovine endothelium antibodies and that endothelium-related antigenic material in frozen parathyroid sections was found in close apposition to epithelial cells. In a previous paper we showed that sera from the same AHP patients caused complement-mediated lysis of PT-b cells (6). Analysis of earlier and current findings indicates that the magnitude of cytotoxic effect is proportional to the intensity of the immunofluorescent staining. In our previous report, we also observed that adsorbing AHP patients' sera with bovine organ extracts other than parathyroid decreased the cytotoxic activity on PT-b cells to a variable extent (6). Immunohistochemical data obtained here reveal that the distribution of antigenic material in certain bovine organs such as adrenal, kidney, and pituitary is not uniform. This suggests that tissues vary in their content of antigen(s) that react with AHP antibodies. The antibodies identified here reacting with endothelium are in the IgM class. We note that Haspel et al. (19), who studied monoclonal antibodies obtained from mice affected by autoimmune polyendocrine disease, also found such antibodies to be of the IgM class.

We found variable reactivity of AHP sera with human parathyroid adenoma tissue. Inconsistencies in detecting anti-parathyroid antibodies by using human substrata in idiopathic hypoparathyroidism have already been noted (1). Recently, it has been reported that human parathyroid adenomas may not be representative of normal expression of tissue antigens (5); variation in tumor tissue expression of antigens may account, at least in part, for the inconsistent reactivities that we observed in our series of patients and for the variable and low yield for detection of anti-parathyroid antibodies in AHP (1-5, 20-22). The age distribution of our patients was 12-24; in the study of Posillico et al. (20), it was 56-71.

**Fig. 5.** Immunoblots with extracts of BPE cell membranes and human fibroblast membranes reacted with IgM preparation from AHP patient 6. Lanes: A, human fibroblasts; B, BPE cells. The major bands of 200 and 130 kDa are indicated. A third band (in lane B), just below the 200-kDa marker, also was observed, but its significance is unclear because the background is also increased in that area. The positions of the molecular size standards (in kDa) are also indicated.

**Fig. 4.** Section of bovine parathyroid tissue stained with AHP serum (patient 6) using fluorescein-conjugated anti-human IgM as the second antibody (A) or with rabbit anti-human factor VIII-related antigen using fluorescein-conjugated anti-rabbit IgG as the second antibody (B). The highest concentration of the antigenic material recognized by serum from AHP patients was found distributed in regions corresponding to interstitium or vascular endothelium (arrows in A). However, the reaction product was not restricted to the endothelium but was diffusely distributed throughout the whole gland giving the appearance of an external matrix in close apposition to the epithelial cell membrane (arrowheads and Inset in A). The distribution of endothelium is outlined by the reactivity with factor VIII-related antigens in B (arrows). Illustrations have been taken from comparable sections of the same parathyroid tissue. The intense yellowish auto-fluorescence of intracytoplasmatic granules is an intrinsic property of the unstained tissue. (A) (×420) (Inset) (×630) (B) (×315) (C) Section of human parathyroid adenoma doubly stained using AHP serum (from patient 2) plus fluorescein-conjugated anti-human IgM anti-serum and also factor VIII-related antigen and rhodamine-conjugated anti-rabbit IgG antiserum. Two consecutive exposures from the same microscopic field were made on the same frame using filter sets for fluorescein or rhodamine. Fluorescence (green and red) codistributes in most areas (arrows) and sometimes appears as yellow staining due to superimposition of green and red fluorochromes. Deposits of IgM only are also visible. (×630)
Immunofluorescent deposits, similar, but not identical, in pattern, were found in bovine and human parathyroids. This suggests that the antigenic determinants in the two kinds of tissues do not completely overlap. Adsorption experiments confirmed this view by showing that AHP serum preincubated with bovine endothelial cells still gave detectable reactions with human parathyroid adenoma epithelium.

The significance of the anti-endothelium antibodies in the pathogenesis of AHP cannot be conclusively established. The reactivity with the outer cell membrane in culture and with accessible determinants in whole tissues fulfills one of the essential prerequisites for a pathogenic antibody (5). The anti-endothelial antibodies do not appear to be organ-specific since reactivity is found in nonparathyroid tissues. The lack of reactivity with bovine liver and all rat tissues might reflect low concentrations of antigen that are undetectable with our methods, lack of the antigen in those tissues, or existence of related antigens distinct in immuno-reactivity. Although not organ-specific, the anti-endothelium antibodies appear to be disease-specific; in only one subject without clinical evidence of parathyroid dysfunction were characteristic IgM antibodies detectable. This patient displayed overt Addison disease. Adrenocortical insufficiency is an extremely frequent finding in AHP patients with type I polyclonal autoimmune disease (21, 22). A 20% incidence of parathyroid antibodies in patients with isolated idiopathic adrenocortical insufficiency has been reported (22). In addition, experimental immune hypoparathyroidism in dogs is associated with immunopathological features of the adrenal cortex (23). As envisioned by others (24), the possibility exists that in some cases damage to the parathyroid glands may be subclinical or that circulating antibodies appear before glandular dysfunction becomes fully developed.

We have used immunoblotting techniques to gain further insight into the nature of the antigen(s) recognized by AHP patients' serum. These studies identified major bands of 200 and 130 kDa in BPE cell membranes that reacted with AHP serum. Although more AHP patients clearly need to be investigated to allow final conclusions, the possibility arises that these structures represent the major target for AHP antibodies.

Recently, Posisillico et al. (20) described three cases of idiopathic hypoparathyroidism displaying antibodies that inhibit secretion of parathyroid hormone from dispersed cells. They also reported that these sera reacted with components on tissue sections of human parathyroid adenomas. However, one could not determine whether endothelial or epithelial cells contained the major reactive component. Complement-fixing, non-species-specific, anti-endothelial cell antibodies have been detected in a number of autoimmune diseases and are often reported to be reactive with diverse organs (25–29). Major differences exist, however, between those findings and our results. With few exceptions, previously described anti-endothelium antibodies were primarily IgG or, more rarely, IgA. Most important, all reports known to us deal with non-organ-specific autoimmune diseases, such as systemic lupus erythematosus, scleroderma, and rheumatoid arthritis, and/or with long-term chronic infectious disorders.

By using immunofluorescence techniques, we have detected, in the sera of AHP patients, antibodies of the IgM class directed against antigen(s) found on bovine endothelial cells. The exact significance of these antibodies in regard to the pathophysiology of the disease remains to be clarified and correlated with clinical findings in AHP and other patients. Forsberg et al. (30) have suggested that endothocrine cells, by releasing ATP to interact with purinergic receptors, can regulate endothelial cells. Control of blood flow might be one function influenced thereby (30). Specific antibodies against endothelial cells might explain the involvement of several organs in AHP. Do endothelial cells, the entity bearing the target antigen for antibodies of the type studied here, provide a key function required for normal endocrine epithelial cell physiology?

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