Antibodies cytotoxic to bovine parathyroid cells in autoimmune hypoparathyroidism

**POLYGLANDULAR AUTOIMMUNE SYNDROME/AUTOANTIBODIES/HYPOADRENALISM**


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**ABSTRACT** We utilized a recently developed long-term serum-free culture system for bovine parathyroid cells to detect antibodies in seven patients with autoimmune hypoparathyroidism (AHP). Antibodies were tested by indirect immunofluorescence methods and by cytotoxicity utilizing the chromium (51Cr) release technique. Seven AHP sera caused specific lysis (57 ± 6% release of 51Cr vs. 5 ± 1% for 56 controls (15 normal subjects and 41 patients with diverse other conditions associated with immune dysfunction)). The least effect of any of the AHP sera on cell lysis exceeded the greatest effect of any of the control sera. Absorption of AHP sera (two cases) with bovine pituitary, thyroid, liver, or kidney cells did not affect lysis, but absorption with adrenal or parathyroid cells caused a marked decrease in specific lysis. Cytotoxicity determined by 51Cr release increased with antisera concentration and time of incubation. Cytotoxicity was dependent on complement. Replicating parathyroid cells provide a uniform reproducible detection method for anti-parathyroid antibodies in AHP. The autoantibodies in AHP appear to be specific for tissue (parathyroid and adrenal cortex) but not for species.

Autoimmune endocrine disease was initially recognized as atrophy of certain endocrine glands (1). Claude and Gougerot in 1908 described lymphocytic infiltration and fibrosis in several endocrine organs and coined the term "pluriglandular endocrine atrophy" (2). Roitt et al. proposed organ-specific antibodies as a cause of endocrine atrophy in 1956 (3). They found thyroglobulin autoantibodies in the sera of patients with lymphocytic thyroiditis, and this concept was extended to the adrenal cell by Anderson et al. (4) and Blizzard et al. (5). Other researchers identified autoantibodies directed against the thyroid, ovary, testicle, pancreatic islet, and pituitary cells (3, 6-9).

Coexistence of several autoimmune endocrinopathies is unusual and was described in a family by Sutphin et al. (10). This family displayed moniliasis and features of hypoparathyroidism.

Blizzard et al., utilizing the indirect Coombs technique, found antibodies to parathyroid tissue in sera of 38% of 74 patients with idiopathic hypoparathyroidism (11). The same research group later characterized polyglandular autoimmune disease (PGA) types I and II (12, 13). Type I PGA encompasses hypoparathyroidism with adrenocortical insufficiency and/or chronic mucocutaneous candidiasis and may include insulin-dependent diabetes mellitus, pernicious anemia, primary hypogonadism, chronic active hepatitis, malabsorption, alopecia, vitiligo, and autoimmune thyroid disease. PGA type II includes adrenocortical insufficiency and autoimmune thyroid disease and/or insulin-requiring diabetes mellitus. In the current report, we have studied seven patients with autoimmune hypoparathyroidism (AHP), each showing circulating antibodies against parathyroid cells. A recently developed long-term culture system for bovine parathyroid cells was utilized to evaluate parathyroid antibodies in these patients. Circulating antibodies from AHP patients bound to parathyroid cells and caused complement-dependent lysis.

**METHODS**

**Patients.** Subjects were studied under approved protocols at the National Institutes of Health. Informed consent was obtained from all patients. Patients 1-7 were hypoparathyroid. None of the patients had been given bovine parathyroid hormone or thyroid-stimulating hormone. Additional sera were obtained from patients in protocols for other disorders with possible immune dysfunction.

**Cell Culture.** Bovine parathyroid cells were prepared and cultured as recently described in detail (14). Cells were cultured in serum-free Coon's modified Ham's F12 medium containing epidermal growth factor (100 ng/ml), bovine pituitary extract (5 μg/ml), bovine hypothalamic extract (150 μg/ml), insulin (10 μg/ml), transferrin (5 μg/ml), selenious acid (5 ng/ml), hydrocortisone (3.5 ng/ml), triiodothyronine (25 pg/ml), retinoic acid (15 ng/ml), D-galactose (200 μg/ml), putrescine (30 μg/ml), 0.3 mM CaCl2, and 0.5 mM MgCl2.

**Sera.** Sera for antibodies were analyzed immediately or after storage at −20°C for up to 2 years. Samples of normal sera or pooled samples from normal subjects were used as controls. Complement was inactivated by incubating sera at 56°C for 30 min.

**Immunoglobulin Extraction.** Sera (1 ml) were stirred at 4°C and saturated ammonium sulfate solution was added dropwise to a final concentration of 50% saturation. After 2 hr of stirring, the mixture was centrifuged for 15 min at 27,000 x g at 4°C. The protein pellet was washed twice with 50% saturated ammonium sulfate, dissolved in 1 ml of phosphate-buffered saline (PBS; Biofluids, Rockville, MD), pH 7.2, and dialyzed against the same solution. Protein was determined spectrophotometrically at 280 nm.

**Cytotoxicity Assay.** Serum-mediated lysis was determined by release of 51Cr from cultured bovine parathyroid cells by the following method: Subconfluent cultures of bovine parathyroid cells were incubated with 300 μCi (1 Ci = 37 GBq) per 100-mm culture dish of Na251CrO4 (New England Nuclear) in Coon's modified Ham's F12 medium for 2 hr at 37°C in 5% CO2 atmosphere. Cells were washed twice with PBS and harvested with calcium- and magnesium-free PBS containing 5 mM EDTA. Cells (2 x 105) in a final volume of 100 μl were

**Abbreviations:** PGA, polyglandular autoimmune [disease]; AHP, autoimmune hypoparathyroidism.
distributed into Eppendorf microcentrifuge tubes and incubated in triplicate at 37°C with diluted serum (1:5, 1:10) or immunoglobulin for 4 hr unless otherwise indicated. Maximal release of 51Cr was determined by lysis of cells with 100 μl of 1% Triton X-100. Spontaneous release was assessed in cells incubated without AHP serum. Cells were centrifuged and the amount of 51Cr in the supernatant was assayed by liquid scintillation spectrometry. Cytotoxicity was expressed as previously described (15, 16). Guinea pig serum was added where indicated as a source of complement to heat-inactivated serum.

**Immunofluorescence Detection of Antibodies.** Cultured bovine parathyroid cells were dispersed in calcium- and magnesium-free PBS containing 5 mM EDTA. Cells (4 × 10⁶ per ml) were resuspended in PBS with 1% fetal calf serum for 45 min with normal serum or heat-inactivated AHP serum at 1:20 dilution. Cells were washed twice at 4°C in PBS supplemented with 1% fetal calf serum and resuspended with fluoresceinated F(ab')₂ fragments of goat antiserum to human IgG plus IgM plus IgA (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 45 min. After two further washes, cells were plated and examined in duplicate with a Leitz Ortholux fluorescence microscope with BG38 and 2KP 490 excitation filters and a S525 blocking filter.

**Absorption Studies.** Dispersed cells were prepared from bovine parathyroid, pituitary, thyroid, kidney, or liver cells by collagenase digestion. Cells (10⁶ per ml) from each of these tissues were incubated with sera for 2 hr at 37°C. The supernatant was collected and used in the chromium release assay.

**RESULTS**

**Patients.** Pertinent clinical features of the patients are listed in Fig. 1. All patients were clinically hypoparathyroid. Patients 5 and 7 display adenyl insufficiency and four patients suffer chronic mucocutaneous candidiasis. Patient 1 was the mother of twin daughters, patients 2 and 3. Patient 3 displayed seropositive rheumatoid arthritis. Patient 2 showed borderline-detachable adenyl antibodies (measured courtesy of George Bright, Medical Univ. of South Carolina) and patient 1 displayed positive partial cell antibodies (measured courtesy of Williams Reilly, Univ. of Florida). Patients 4–6 are siblings in another family; there were no other affected members in the family of patient 7.

**Indirect Immunofluorescence.** All seven subjects showed circulating antibodies reactive against bovine parathyroid cells as detected by indirect immunofluorescence. Antibodies were detectable after storage at −20°C for as long as 2 years. Pooled normal sera (n = 15) and sera from patients with surgical hypoparathyroidism (n = 3) produced no detectable immunofluorescence.

**Cytotoxicity Assessed By Chromium Release.** Cytotoxicity was assessed with 1:5 dilutions of serum samples from each of the 7 AHP patients, using the 51Cr release technique in 4-hr incubations (Fig. 2). Specific lysis with AHP samples was 57.8 ± 6.4% and was significantly higher than in 15 normal controls (NL) (8.1 ± 1.2%). Cytotoxic activity did not appear to be related to age or duration of disease. Sera from patients with other immune-related diseases showed little specific lysis (Fig. 2). Serum from patients with either surgical hypoparathyroidism (n = 3) or idiopathic hypoparathyroidism (n = 2) did not cause significant lysis (data not shown).

Sera from patients 2 and 7 were absorbed with freshly dispersed bovine pituitary cells, bovine thyroid cells, bovine liver cells, or bovine kidney cells without any significant change in specific lysis. Lysis, however, was decreased after absorption with bovine adrenal cells or bovine parathyroid cells (Table 1).

Normal serum caused no increase in lysis at 1:5 or 1:10 dilutions for up to 5 hr. Lysis produced with serum from patient 7 was observed as early as 30 min and increased with concentration of serum (Fig. 3). Cell lysis was evaluated with serial dilutions of serum from patient 7 (Fig. 3 Inset). Significant lysis (P < 0.01) was detected at 1:20 dilution.

**Complement Dependence of Cell Cytotoxicity.** AHP serum alone produced specific lysis of bovine parathyroid cells as measured by 51Cr release, but heat-inactivated serum, gammaglobulin, or guinea pig serum alone did not cause significant lysis. The addition of guinea pig serum to heat-inactivated serum or AHP gammaglobulin restored the cytotoxic effect (Fig. 4).

**DISCUSSION**

In the current study we characterized and consistently detected antibodies in AHP. We utilized a recently developed

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Fig. 1. Clinical features of cases 1–7. Patient 1 is the mother of twin daughters, patients 2 and 3; patient 3 displays seropositive rheumatoid arthritis and patient 2, borderline adrenal antibodies. Patients 4–6 are siblings in another kindred. Patient 7 shows premature ovarian failure and other members of her family are not known to be affected.

Fig. 2. Serum-mediated specific lysis of bovine parathyroid cells expressed as 51Cr release. In 7 AHP samples, specific lysis was significantly higher than for 15 normal controls (NL). Sera from patients with nonparathyroid autoimmune disease included the following: 3 with autoimmune hemolytic anemia (AIHA), 5 with hereditary angioneurotic edema (HANE), 3 with insulin-dependent diabetes mellitus (IDDM), 4 with idiopathic pulmonary fibrosis (IPF), 4 with chronic hepatitis B (CHB), 6 with rheumatoid arthritis (RA), 4 with systemic lupus erythematosus (SLE), and 7 with autoimmune thyroid disease (ATD). Analyses were performed in triplicate; the coefficient of variation did not exceed 10%.

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[1] Percent specific lysis = [(ER − SR)/(MR − SR)] × 100, where ER is release in experimental cells, SR is spontaneous release in medium alone, and MR is maximal release after cell lysis by 1% Triton X-100.
long-term culture system for bovine parathyroid cells to identify antibodies. With this system anti-parathyroid antibodies can be detected by immunofluorescence or by $^{31}$Cr release. Antibodies were identified in all of seven hypoparathyroid subjects regardless of disease status.

Blizzard et al. (11), using pathological human parathyroid tissue as the test material, found positive antibodies by immunofluorescence in 38% of 74 patients with idiopathic hypoparathyroidism. Positive sera in that study included samples from patients with hypoparathyroidism alone or hypoparathyroidism in association with an autoimmune phenomenon. The use of heterogenous test material, parathyroid adenomas (obtained at surgery) or normal parathyroid tissue (obtained at autopsy), apparently produces inconsistent results. Fifty percent of the adenomas were unsatisfactory for use due to apparent inhibition of fluorescence (11). Irvine and Scarth (17) describe IgG antibodies to parathyroid oxyphil cells in only 1 of 9 patients with idiopathic hypoparathyroidism. That patient also expressed parietal cell antibodies, but patients with multiple autoimmune features were not tested.

The chromium release assay has been used previously to analyze for antigen-specific cytology in lymphocytes in patients with insulin-dependent diabetes mellitus (8). It also was applied successfully to determine complement-dependent cytotoxic activity in sera of patients with pernicious anemia (16). We have adapted this technique to analyze for cytotoxic antibodies in cultured bovine parathyroid cells. Sera from 7 patients with AHP caused specific lysis of parathyroid cells (57.8 ± 6.4% compared to 8.1 ± 1.2% for normal controls). The effect of AHP antiserum on cell lysis was a function of serum dilution and time of incubation.

Serum from patients with other autoimmune diseases (autoimmune thyroid disease, systemic lupus erythematos, insulin-dependent diabetes mellitus, autoimmune hemolytic anemia, rheumatoid arthritis, hereditary angioedema, idiopathic pulmonary fibrosis, and chronic hepatitis) did not cause significant parathyroid cell toxicity as assessed by $^{31}$Cr release. Sera from patients with surgical hypoparathyroidism also were inert in this assay.

Species specificity has not been addressed in previous studies of parathyroid antibodies. Monoclonal antibodies from the type I reovirus-infected mouse also exhibit organ-specific reactivity across species (18). The antiparathyroid antibody clearly reacts strongly with cells of bovine origin. This is fortuitous in that it allows utilization of a defined and uniform test system, replicating parathyroid cells in culture. Test material used in earlier studies (13) was derived from sources (human surgical tissue) that were variable and nonhomogeneous.

Absorption with intact parathyroid as well as adrenal cells removed the factor causing cell lysis. This might be expected in view of the well-known association between hypoparathyroidism and adrenal insufficiency in PGA type I. Earlier studies showed a 20% incidence of antiparathyroid antibodies in patients with isolated idiopathic adenocortical insufficiency (13). In addition, induced isomune hypoparathyroidism in dogs is associated with lymphocytic infiltration and sclerosis of the adrenal cortex (19).

Table 1. Absorption of antiparathyroid sera with diverse bovine cells

<table>
<thead>
<tr>
<th>Cells used for absorption</th>
<th>Serum no. 2</th>
<th>Serum no. 7</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>49 ± 7</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>Adrenal cortex</td>
<td>19 ± 1*</td>
<td>16 ± 4*</td>
</tr>
<tr>
<td>Pituitary</td>
<td>45 ± 4</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>Thyroid</td>
<td>47 ± 1</td>
<td>49 ± 6</td>
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<tr>
<td>Kidney</td>
<td>52 ± 1</td>
<td>61 ± 4</td>
</tr>
<tr>
<td>Liver</td>
<td>53 ± 3</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>Parathyroid</td>
<td>5 ± 1†</td>
<td>8 ± 1†</td>
</tr>
</tbody>
</table>

Dispersed bovine cells (10⁶ per ml) were prepared by collagenase digestion from each tissue and incubated with sera from patients 2 and 7 for 2 hr at 37°C. The sera were then used in the $^{31}$Cr release assay at 1:10 dilution, and cytotoxicity is expressed as percentage specific lysis. Spontaneous release was assessed in cells incubated without sera, and maximal release of $^{31}$Cr was determined after cell lysis with 1% Triton X-100. Cytotoxicity was expressed as previously described (15, 16). Results are expressed as the mean ± SD of 10 replicate determinations.

*P < 0.01 vs. unabsorbed control.
†P < 0.001 vs. unabsorbed control.

FIG. 3. Cytotoxicity as a function of serum concentration. Cytotoxicity was assessed by $^{31}$Cr release for pooled normal sera at 1:5 (●) and 1:10 (○) dilutions and serum from patient 7 at 1:5 (▲) and 1:10 (△) dilutions. Data represent the mean ± SD of triplicate experiments. (Inset) Cell lysis as a function of antiserum dilution. Serum from patient 7 was diluted as indicated and cytotoxicity was assessed after 4-hr incubations. Cell lysis was significant at 1:20 dilution (30%) and increased to almost 70% at 1:5 dilution. Data represent the mean ± SD of triplicate analyses.

FIG. 4. Complement and cytotoxicity. Serum from patient 7 was diluted 1:10 and specific lysis was determined. Heat-inactivated serum, gammaglobulin, and guinea pig serum at 1:10 dilution did not cause significant lysis. Addition of guinea pig serum as a source of complement to heat-inactivated AHP serum or to gammaglobulin fraction of patient 7 restored the cytotoxic effect.
The long-term bovine parathyroid cell culture system and the studies initiated here should make it possible to characterize further the nature of cell-specific antibodies in AHP and perhaps to identify the antigen(s) involved.

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