

Human Multiple Organ-reactive Monoclonal Autoantibody Recognizes Growth Hormone and a 35,000-Molecular Weight Protein

Jo Satoh, Karim Essani, Patrick R. McClintock, and Abner Louis Notkins

Laboratory of Oral Medicine, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205

Abstract. By fusing peripheral leukocytes from a patient with insulin-dependent diabetes with mouse myeloma cells, a heterohybridoma was isolated that, for over one year, has secreted a human monoclonal autoantibody, designated MOR-h1 (multiple organ-reactive human 1). This antibody reacts with antigens in several endocrine organs including the pituitary, thyroid, stomach, and pancreas. By double immunofluorescence, MOR-h1 was found to react specifically with growth hormone (GH)-containing cells in the anterior pituitary and, by enzyme-linked immunosorbent assay, MOR-h1 was shown to react with both natural and biosynthetic GH. Absorption experiments revealed that GH could remove the capacity of MOR-h1 to react not only with cells in the anterior pituitary, but also with cells in the thyroid, stomach, and pancreas. The demonstration with hyperimmune serum that these organs do not contain GH indicated that MOR-h1 was reacting with a different molecule(s) in these organs. By passing extracts of pituitary, thyroid, and stomach through an MOR-h1 affinity column and analyzing the eluted antigens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a 35,000-mol wt polypeptide was isolated from each of these organs. In addition, a 21,500-mol wt polypeptide with an electrophoretic mobility identical to purified human GH was isolated from the pituitary, but not the other organs. It is concluded that MOR-h1 reacts with a 35,000-mol wt polypeptide present in the pituitary, thyroid, and stomach and that this antibody also recognizes a determinant on GH.

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Introduction

In many autoimmune diseases, sera from patients contain antibodies that react with antigens in multiple organs (1-3). Because of the low titer of these antibodies and the polyclonality of sera, it has been difficult to determine whether different autoantibodies or the same autoantibody reacts with each of the organs. By hybridoma technology we isolated a number of monoclonal autoantibodies from mice (4, 5). Many of these antibodies were found to recognize antigens in multiple organs (5). Recently, we succeeded in preparing human monoclonal autoantibodies and found that many of them also were of the multiple organ-reactive (MOR)¹ type (6). One of these, MOR-h1, reacts with cells in the pituitary, thyroid, stomach, and pancreas. The present investigation was initiated to isolate and compare the antigen(s) in the different organs with which MOR-h1 reacts.

Methods

Monoclonal autoantibody. Peripheral blood lymphocytes from a patient with insulin-dependent diabetes mellitus were fused with mouse myeloma cells (Sp-1), and the hybridoma producing MOR-h1 monoclonal antibody was isolated as previously reported (6). This hybridoma has been secreting >20 µg/ml of human monoclonal IgM(κ) in RPMI-1640 medium supplemented with 15% fetal bovine serum for over 1 yr. Unconcentrated supernatant fluid from the hybridoma culture or supernatant fluid concentrated with 50% saturated ammonium sulfate was used throughout this study, except where indicated.

Fluorescence stainings of human tissues. Paraffin-embedded human tissues fixed with Bouin's solution were used for indirect immunofluorescence (7). For double immunofluorescence (8), tissue sections of human pituitary were incubated with an appropriate dilution of

1. *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; MOR, multiple-organ-reactive; NP-40, Nonidet P-40; PRL, prolactin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel (electrophoresis); TRITC, tetramethylrhodamine isothiocyanate; TSH, thyroid-stimulating hormone.

concentrated MOR-h1 for 1 h at room temperature, washed four times with phosphate-buffered saline (PBS), then incubated with rabbit antisera to human growth hormone (GH), adrenocorticotrophic hormone (ACTH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), or thyroid-stimulating hormone (TSH) (provided by the National Pituitary Agency, Baltimore, MD). After washing four times with PBS, the sections were incubated overnight at 4°C with a mixture of appropriate dilutions of tetramethylrhodamine isothiocyanate (TRITC)-conjugated F(ab')₂ fragment of goat anti-human IgM and fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment of goat anti-rabbit IgG (both from Cappel Laboratories, West Chester, PA). The sections were again washed and then viewed with a Zeiss epifluorescence microscope. To determine the cell type with which MOR-h1 reacted, the tissue sections were observed with rhodamine and fluorescein filters. Monoclonal human IgM (~5 µg/ml) and normal rabbit serum that did not bind to tissues were used as negative controls. Other controls showed that TRITC-conjugated anti-human IgM and FITC-conjugated anti-rabbit IgG did not stain pituitary tissue sections that had been incubated with rabbit anti-hormone serum (IgG) or human MOR-h1 (IgM), respectively.

Reactivity of MOR-h1 with hormones. Human pituitary hormones, including natural human GH (National Pituitary Agency) and biosynthetic human GH produced by recombinant DNA techniques (kindly provided by Genentech, South San Francisco, CA), human monocomponent insulin (Novo Research Institute, Copenhagen), thyroxine, bovine thyroglobulin, porcine intrinsic factor, and bovine serum albumin (BSA) (all from Sigma Chemical Co., St. Louis, MO), were used in an enzyme-linked immunosorbent assay (ELISA) or for absorption experiments.

For ELISA, Immulon II plates (Dynatech Laboratories, Alexandria, VA) were coated with 1 µg/well of antigens in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Before use, the plates were treated for 1 h with 10 µg/well of poly-L-glutamic acid (11,000 mol wt) to block residual protein-binding sites on the wells. After washing five times with PBS (pH 7.3) containing 0.05% Tween 20 (PBS-Tween), the plates were incubated for 2 h with MOR-h1 supernatant fluid serially diluted in PBS-Tween containing 0.1% gelatin, washed five times with PBS-Tween, then incubated with peroxidase-labeled goat anti-human IgM (Cappel Laboratories) for 2 h. All incubations were at room temperature. The optical density (OD) was read with a microplate reader (Flow Laboratories, McLean, VA). As a control, the capacity of immunoreactive antigens (e.g., GH, ACTH, LH, PRL, insulin) to coat microtiter plate wells was confirmed by using specific antibodies to these antigens in a separate ELISA. To measure the reactivity of MOR-h1 with autoantigens isolated from an MOR-h1 affinity column, microtiter wells were coated with these autoantigens and an ELISA was performed by methods already described.

For the absorption experiments, concentrated MOR-h1 (~50 µg/ml), rabbit anti-human GH serum, or guinea pig anti-porcine insulin serum (Miles Laboratories, Elkhart, IN) were incubated overnight at 4°C with an equal volume of natural or biosynthetic human GH (1 mg/ml), human insulin (1 mg/ml), or BSA (1 mg/ml) solution. The mixtures were incubated with tissue sections, which were then washed and stained with FITC-conjugated anti-human, anti-rabbit, or anti-guinea pig immunoglobulin.

Preparation of tissue extracts. Each human tissue (pituitary, thyroid, and stomach) was washed in Tris-saline (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.8) containing 2 mM phenylmethyl sulfonylfluoride and 1 trypsin inhibition unit per milliliter of aprotinin, and homogenized.

An equal volume of Tris-saline containing 1% Nonidet P-40 (NP-40) was added, the homogenate was placed on ice, and then centrifuged at 100,000 g for 1 h to remove the particulate materials. The supernatant thus obtained was filtered through nitrocellulose membrane (0.2 µm), sodium azide was added, and aliquots were stored at -20°C. About 10 g of frozen tissue was used to prepare each 100 ml of extract.

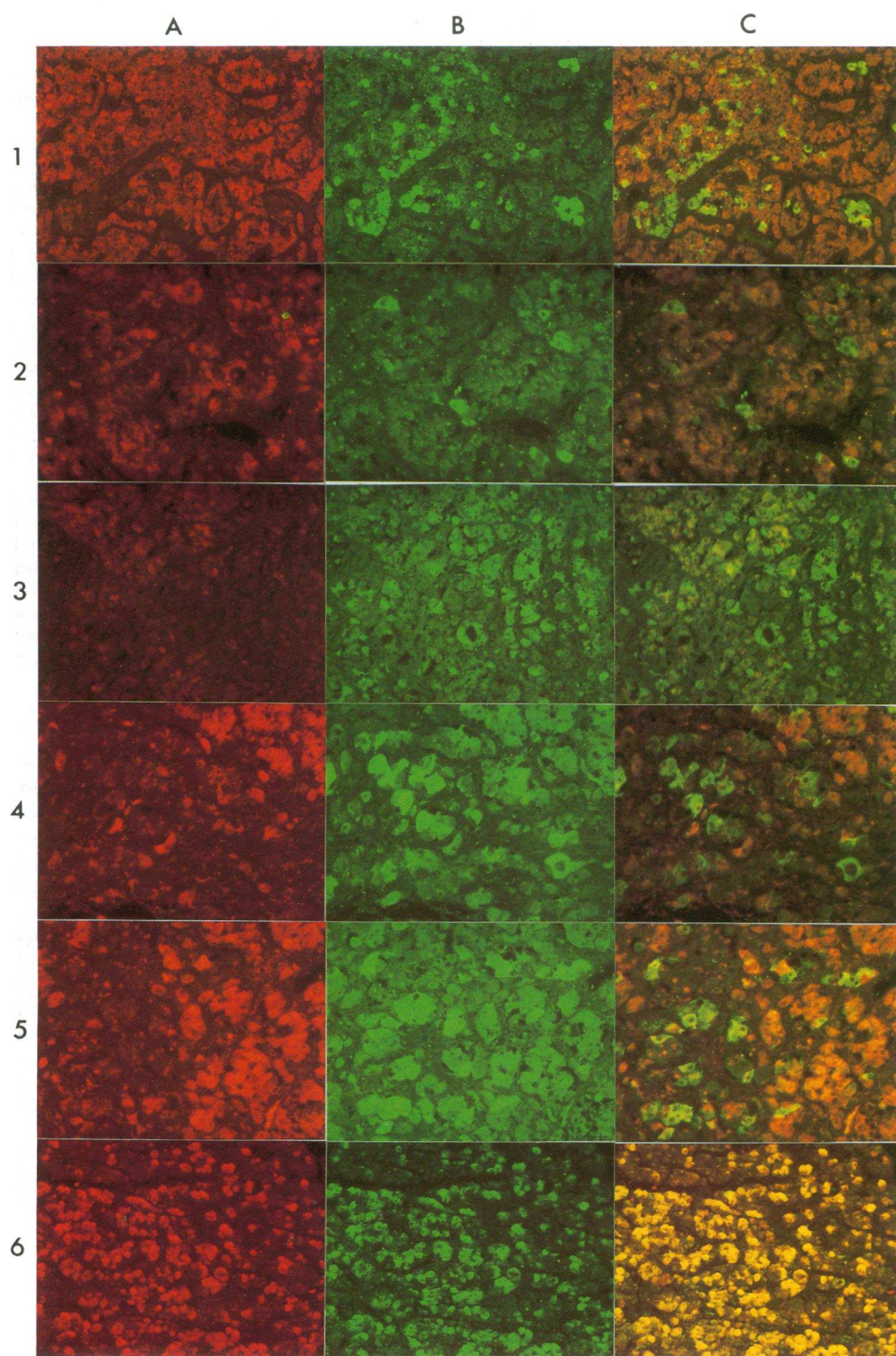
Purification of MOR-h1. MOR-h1 was purified by affinity chromatography. Briefly, 1.0 g of freeze-dried CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) powder was swollen and washed in 1 mM HCl followed by washing with coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3). Approximately 15 mg IgG from sheep anti-human IgM serum (Cappel Laboratories) was added and the mixture was subjected to end-over-end mixing for 2 h at room temperature. The remaining active groups on the Sepharose beads were blocked by incubation with 1 M ethanolamine (pH 8.0) for 2 h at room temperature and the mixture was poured into a column. The column was washed several times to remove unconjugated sheep IgG. Hybridoma supernatant fluid containing MOR-h1 was then passed through the column at a rate of 15–20 ml/h. After washing with Tris-saline, the bound MOR-h1 was eluted with 0.1 M glycine-HCl (pH 2.5). The eluate was adjusted to pH 7.5, dialyzed against deionized water at 4°C, lyophilized, and stored at 4°C.

Purification of autoantigens. Immuno-affinity columns were prepared by conjugating affinity purified MOR-h1 to CNBr-activated Sepharose 4B as described above. Tissue extracts were passed through the column and after extensive washing, the absorbed antigens were eluted using 0.1 M glycine-HCl (pH 2.5). The eluate was adjusted to pH 7.5, dialyzed, lyophilized, redissolved in Tris-saline, and recycled through the affinity column. The recycled eluate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli (9).

Results

Identity of cells in the anterior pituitary with which MOR-h1 reacts. We previously reported that MOR-h1 reacts with cells in the anterior pituitary (6). To identify the cell type involved, sections of the anterior pituitary were incubated with human MOR-h1 and then with rabbit antisera to anterior pituitary hormones (i.e., anti-ACTH, -FSH, -LH, -PRL, -TSH, or -GH). The sections were subsequently stained with TRITC-conjugated anti-human IgM, which reacted with human MOR-h1, and with FITC-conjugated anti-rabbit IgG, which reacted with the rabbit anti-hormone antibodies (Fig. 1). When viewed with rhodamine filters, the cells reacting with MOR-h1 stained orange-red (column A), and when viewed with fluorescein filters, the cells reacting with anti-hormone antibodies stained yellow-green (column B). By double immunofluorescence, cells reacting with antibody to ACTH, FSH, LH, PRL, or TSH showed FITC-staining, whereas those reacting with MOR-h1 showed TRITC-staining and were distinguishable from the FITC-stained cells (column C, 1–5). In contrast, the cells reacting with antibody to GH could not be distinguished from those reacting with MOR-h1 (column C, 6). It is concluded that MOR-h1 reacts with cells containing GH, but not with cells containing ACTH, FSH, LH, PRL, or TSH.

Demonstration that MOR-h1 reacts with human GH. Evi-



dence that MOR-h1 reacts specifically with GH comes from ELISA in which microtiter plates were coated with hormones and then incubated with MOR-h1. As seen in Fig. 2, MOR-h1 reacted with GH, but not with a variety of other proteins (e.g., ACTH, LH, PRL, insulin, thyroxine, thyroglobulin, intrinsic factor, BSA).

Further evidence that MOR-h1 reacts with GH comes from experiments in which MOR-h1 was absorbed with GH and then the absorbed and unabsorbed samples were incubated with sections of anterior pituitary. As seen in Fig. 3 *A* and *B*, unabsorbed MOR-h1 brilliantly stained cells in the anterior pituitary, whereas the absorbed MOR-h1 failed to stain these cells. Natural GH and biosynthetic GH yielded similar results in both absorption experiments and ELISA.

Reactivity of MOR-h1 with other organs. Since MOR-h1 not only reacted with the anterior pituitary but also with cells in the thyroid, stomach, and pancreas, we wanted to see whether absorption with GH would reduce the capacity of MOR-h1 to react with these organs. The data in Fig. 3 and Table I show that unabsorbed MOR-h1 reacted with pituitary, thyroid, stomach, and pancreas, whereas absorbed MOR-h1 did not react with these organs. To be certain that the absorption with GH was specific, MOR-h1 was absorbed with insulin or BSA. Neither antigen significantly reduced the reactivity of MOR-h1 with the pituitary, thyroid, stomach or pancreas (Table I). In contrast, under identical conditions, insulin completely absorbed out the reactivity of a hyperimmune anti-insulin serum as monitored by the staining of pancreatic islet cells.

The demonstration that absorption of MOR-h1 with GH eliminated its capacity to react with pituitary, thyroid, stomach, and pancreas raised the formal possibility that GH molecules might be present in these organs. To rule out this possibility, sections of these various organs were incubated with a known hyperimmune serum to GH. As seen in Table I, only cells in the anterior pituitary reacted with the hyperimmune serum to GH and absorption with GH eliminated this reactivity. This indicates that MOR-h1 is not reacting with GH in the thyroid, stomach, and pancreas, but with a different molecule that may share a similar configuration or epitope with GH.

Isolation of MOR-h1 reactive antigens. To determine the nature of the antigen(s) with which MOR-h1 reacts, tissue extracts were passed through an MOR-h1 affinity column, the bound molecules were eluted, concentrated, and analyzed by SDS-PAGE. As seen in Fig. 4 (lanes A and B), thyroid and stomach extracts yielded one major polypeptide with an apparent molecular weight of 35,000. The 35,000-mol wt polypeptide also was detected in the pituitary (lane C). When tested by ELISA, the three 35,000-mol wt polypeptides reacted strongly with MOR-h1, but not with pooled human IgM (data

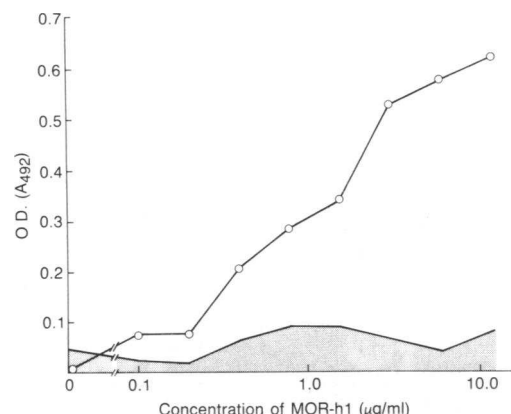


Figure 2. Reactivity of MOR-h1 with GH as measured by ELISA. Microtiter plates were coated with GH or a variety of other antigens (ACTH, LH, PRL, insulin, thyroxine, thyroglobulin, intrinsic factor, BSA). The open circles (—○—) show the reactivity of MOR-h1 with GH, while the shaded area represents the reactivity of MOR-h1 with other antigens. Concentration of MOR-h1 is expressed in micrograms of IgM per milliliter.

not shown). Moreover, when the gels were overloaded, a second polypeptide with a molecular weight approximating that of human GH (21,500 mol wt) was also detected, but only with the pituitary extract (lane C). Identical bands were seen with natural GH (lane D) and biosynthetic GH (lane E). A band of ~15,000 mol wt was sometimes found in pituitary extracts and natural GH, but not biosynthetic GH (lanes C, D, and E, respectively). In other experiments (not shown), natural GH, biosynthetic GH, BSA, or HeLa cell extracts were passed through an MOR-h1 affinity column, but only GH was retained as evaluated by SDS-PAGE.

Discussion

We recently suggested that MOR antibodies may be a partial explanation for multiple organ autoimmunity seen in some of the human autoimmune diseases (5, 6). MOR antibodies could act in several different ways. First, an autoantibody elicited against a protein in one organ could react with all organs containing that protein. Second, such an antibody could react with different proteins in various organs so long as those proteins shared common epitopes. Third, under certain circumstances, the binding site of a low affinity antibody might accommodate structurally unrelated epitopes on different proteins (10, 11). Such an antibody would be truly polyreactive. The first possibility is relatively easy to distinguish from the second and third, but the third is difficult to distinguish from the second.

Figure 1. Reactivity of MOR-h1 with GH-containing cells in anterior pituitary. Sections of anterior pituitary were incubated with MOR-h1 and then the same sections were incubated with rabbit antibody to (1) ACTH, (2) FSH, (3) LH, (4) PRL, (5) TSH, or (6) GH. The

sections were stained with TRITC-conjugated anti-human IgM and FITC-conjugated anti-rabbit IgG. (Column A) viewed with rhodamine filter. (Column B) viewed with fluorescein filter. (Column C) viewed with rhodamine and fluorescein filters.

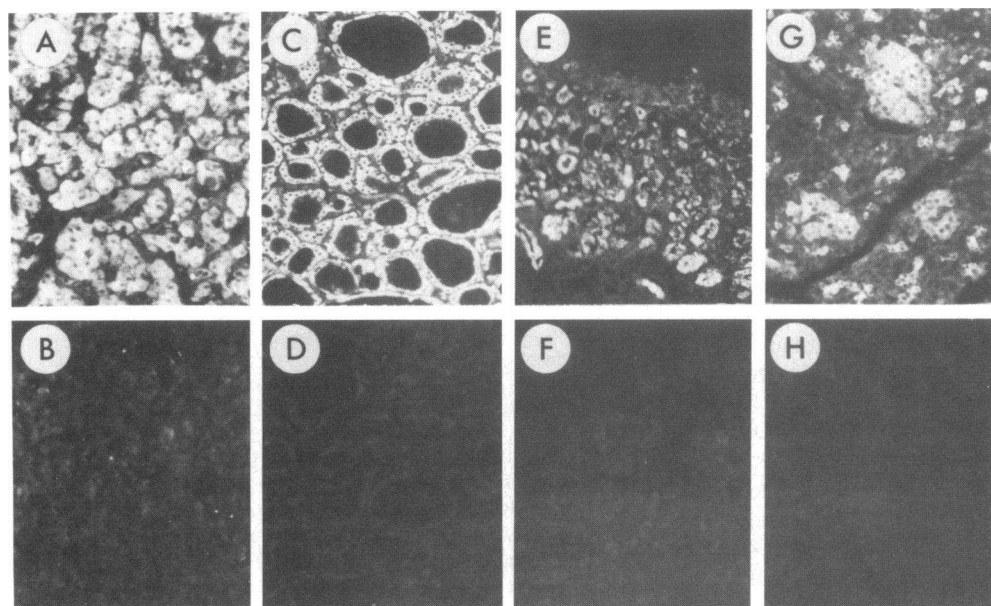


Figure 3. Reactivity of MOR-h1 before and after absorption with GH. Sections of the anterior pituitary (A, B), thyroid (C, D), stomach (E, F) and pancreas (G, H) were incubated with unabsorbed MOR-h1 (A, C, E, G) or MOR-h1 that had been absorbed with GH (B, D, F, H). The sections were then stained by indirect immunofluorescence. Absorbed MOR-h1 showed no reactivity.

In the present experiments we showed that MOR-h1 reacts with a 35,000-mol wt protein in the pituitary, thyroid, and stomach. Several proteins, including one of ~35,000 mol wt, also have been isolated from the pancreas with an MOR-h1 affinity column (Essani, K., and J. Satoh, unpublished observations). The demonstration of a 35,000 mol wt protein in three and perhaps four organs argues in favor of the first alternative that MOR-h1 recognizes the same protein in different organs. However, the demonstration that MOR-h1 reacts with a 21,500-mol wt protein (GH) isolated from the pituitary provides evidence in support of the second (and perhaps third) alternative that MOR-h1 can react with different proteins. Thus, our experiments show that both possibilities can coexist. Moreover, the demonstration by immunofluores-

cence that MOR-h1 stains GH-containing cells in the pituitary and the isolation of both a 35,000- and 21,500-mol wt protein from the pituitary argues that MOR-h1 may react with different proteins even within the same cell. Whether the 35,000-mol wt protein and GH share a common epitope or whether MOR-h1 binds, but weakly, to unrelated epitopes on these two proteins (i.e., alternative three) is not clear. In this context, preliminary experiments indicate that MOR-h1 reacts poorly in radioimmunoassays with ^{125}I -labeled GH. In part, this may be due to the low affinity of IgM antibodies (12). If, however, MOR-h1 is directed primarily against the 35,000-mol wt protein, it may show a higher affinity for that protein than for GH. In fact, preliminary experiments suggest that the affinity of MOR-h1 is greater for the 35,000-mol wt protein than for GH (Satoh, J., and K. Essani, unpublished observations). Isolation of a large quantity of the 35,000-mol wt protein is required for further characterization. The presence of the 35,000-mol wt protein in several different hormone-secreting cells raises the possibility that it may play a role in the secretory process.

By hybridoma technology monoclonal antibodies of the MOR type have now been obtained from many sources including virus-infected mice (5, 13), mice with autoimmune diseases (5, 14), and normal mice (15, 16). Similarly, human MOR monoclonal autoantibodies have been prepared from the lymphocytes of patients with autoimmune abnormalities (6, 17) and from normal individuals (18). Human-human hybridomas (6, 17), human-mouse heterohybridomas (6), and B lymphocytes transformed by Epstein-Barr virus (18) also have yielded such antibodies. The widespread presence of these autoantibodies and the ease with which they have been obtained suggest that MOR antibodies are a major component of the hosts' normal B cell repertoire. If some of these MOR

Table I. Reactivity of Absorbed and Unabsorbed MOR-h1

Antibody	Absorbed with*	Indirect immunofluorescence†			
		Anterior pituitary	Thyroid follicle	Gastric mucosa	Pancreatic islets
MOR-h1	PBS	++	++	++	+
MOR-h1	GH	—	—	—	—
MOR-h1	Insulin	++	++	++	+
MOR-h1	BSA	++	++	++	+
Anti-insulin	PBS	—	—	—	++
Anti-insulin	Insulin	—	—	—	—
Anti-GH	PBS	++	—	—	—
Anti-GH	GH	—	—	—	—

* Appropriate dilutions of antibody were absorbed overnight at 4°C with an equal volume of antigen (1 mg/ml).

† ++, strong fluorescence; +, weak fluorescence; —, no fluorescence.

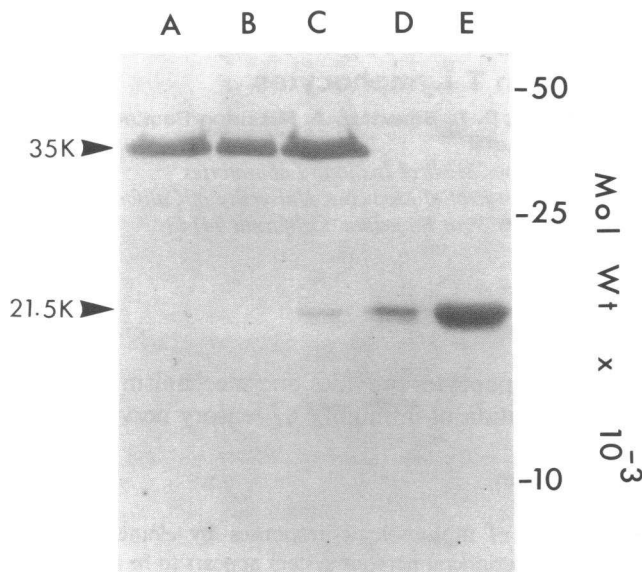


Figure 4. Isolation of MOR-h1 reactive antigens. Thyroid (lane A), stomach (lane B), and pituitary (lane C) extracts were passed through an MOR-h1 affinity column, the bound antigens were eluted, concentrated and electrophoresed on 15% SDS-PAGE. A 35,000-mol wt polypeptide was isolated from the three organs. The pituitary extract also contained an additional polypeptide of ~21,500 mol wt (lane C), which has an electrophoretic mobility identical to that of natural GH (lane D) and biosynthetic GH (lane E). K, $\times 10^{-3}$.

antibodies turn out to be polyreactive, then B lymphocytes that recognize multiple epitopes could upon exposure to any one of these epitopes and in conjunction with a high rate of somatic cell mutation (19, 20) differentiate into high affinity IgG-producing cells (21, 22). Polyreactive antibodies would endow the immune system with an economy for antigen recognition.

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