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Research Article

Transformation of human peripheral blood lymphocytes with Epstein-Barr virus and rapid screening on rat insulinoma cells by an enzyme-linked immunosorbent assay were used to identify monoclonal autoantibodies that reacted with human pancreatic islets. Six such monoclonal autoantibodies were isolated and cloned. All six also were found to react with human thyroid. It is concluded that lymphocytes able to make autoantibodies that react with both the pancreas and thyroid are common in the human B cell repertoire.

Human Monoclonal Autoantibodies that React with Both Pancreatic Islets and Thyroid

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Abstract

Transformation of human peripheral blood lymphocytes with Epstein-Barr virus and rapid screening on rat insulinoma cells by an enzyme-linked immunosorbent assay were used to identify monoclonal autoantibodies that reacted with human pancreatic islets. Six such monoclonal autoantibodies were isolated and cloned. All six also were found to react with human thyroid. It is concluded that lymphocytes able to make autoantibodies that react with both the pancreas and thyroid are common in the human B cell repertoire.

Introduction

Antibodies that react with pancreatic islets are frequently found in the serum of patients with newly diagnosed insulin-dependent diabetes mellitus (IDDM)[1] (1–3). What is perhaps not as fully appreciated is that autoantibodies that react with a variety of other organs (e.g., gastric mucosa, adrenals, anterior pituitary, lymphocytes) are also found in the serum of IDDM patients (4–7). Autoantibodies that react with the thyroid are particularly common (8–11). The nature of this multiple organ autoreactivity of serum has never been fully explained. There are at least two possibilities. One is that the serum of IDDM patients contains a number of organ-specific antibodies. The second is that the serum contains antibodies that recognize common proteins or epitopes in different organs. Using hybridoma technology or Epstein-Barr virus (EBV) transformation, it is now becoming possible to address this question by studying the reactivity of human monoclonal autoantibodies (12–14). In this paper, we describe a simple and rapid screening procedure for detecting human monoclonal autoantibodies that react with pancreatic islets, and show that many of these antibodies also react with the thyroid and other human tissues.

Methods

Patients. Peripheral blood lymphocytes (PBL) were obtained from 10 patients: four with IDDM, five with both diabetes and thyroiditis, and one with hyperthyroidism but no diabetes. The six EBV-transformed cell lines reported herein were derived from five of these patients: three males and two females; mean age, 10.4 yr; mean duration of diabetes, 3.0 yr. Two cell lines were derived from one of the patients with diabetes; one cell line each was derived from three of the patients with diabetes and thyroiditis, and one cell line was derived from the patient with hyperthyroidism.

Preparation of EBV. EBV was obtained from the culture supernatant of the B95-8 marmoset cell line (15). B95-8 cells were cultured at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, VA), 2 mM L-glutamine, and antibiotics (culture medium). For virus production, the cells were cultured at a concentration of 10^6/ml and incubated for 10 d at 34°C. The virus-containing supernatant was removed, filtered through a 0.45-μm millipore filter, and stored at −70°C.

Establishment of lymphoblastoid cell lines by EBV transformation. Human PBL were isolated from 20 ml of heparinized blood by centrifugation on lymphocyte separation medium (LSM; Bionetics Laboratory Products, Charleston, SC), washed three times, and then separated into T and B lymphocyte-enriched populations over a nylon wool column (16). 5×10^6 B lymphocytes were suspended in 1 ml of culture medium, mixed with 0.5 ml of B95-8 cell line supernatant, incubated for 2 h at 37°C, and then washed once with culture medium. 5×10^4 B lymphocytes infected with EBV and 1×10^5 T-enriched autologous cells treated with mitomycin C (25 μg/ml, 20 min, 37°C) were then seeded in 0.2 ml of culture medium in wells of a 96-well plate (3596; Costar, Cambridge, MA). The plates were placed in a humidified atmosphere in the presence of 7.5% CO_2. The cells were fed twice weekly by replacing half of the culture medium with fresh medium. The occurrence of cell transformation was judged by cell aggregation, acid production, increase in cell number, and acquisition of the ability to be successfully subcultured (15). The production of immunoglobulin, its class and concentration were determined by an enzyme-linked immunosorbent assay (ELISA) (13).

Screening for antibodies. Confluent microcultures of rat insulinoma cells (RIN 5F) (17) were grown in 96-well plates, washed three times with phosphate-buffered saline (PBS), and then fixed with 0.5% glutaraldehyde in PBS for 5 min at room temperature. The fixed cells were washed once, incubated for 5 min at room temperature in 0.15 M glycine in PBS, and then washed two more times before storage in PBS at 4°C. The fixed RIN 5F cells were used within 3 d.

To detect antibodies in the culture supernatants of EBV-transformed cells, fixed RIN 5F cells were washed three times in PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) (PBS-Tween), and incubated with 100-μl aliquots of the culture supernatants diluted 1:3 in PBS-Tween. A human monoclonal IgM (MOR-h1) reacting with islets (12) served as a positive control and a nontissue reacting human monoclonal IgM, designated 69-1A-33, served as the negative control. After 1-h incubation at 37°C, the cells were washed three times with PBS-Tween and then incubated at room temperature for 1 h with 100 μl of a 1:400 dilution of goat anti-human IgM, or a goat anti-human IgG, conjugated to peroxidase (Cappel Laboratories, Malvern, PA). After three additional washes, the plates were developed with 0.01% H_2O_2 and 0.01% o-phenylenediamine (Sigma Chemical Co.). The optical density at 492 nm was read using a Multiscan Microplate reader (Flow Laboratories). Wells with optical readings greater than three standard deviations above the mean of the negative controls were scored as positive for antibody activity.

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1. *Abbreviations used in this paper:* ABC, avidin-biotin complexes; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; IDDM, insulin-dependent diabetes mellitus; LSM, lymphocyte separation medium.

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Table I. Reactivity of EBV-induced Human Monoclonal Antibodies with RIN 5F Rat Insulinoma Cells

Monoclonal antibody	Ig class*	Binding to fixed RIN 5F cells‡
		cpm
Culture medium	—	149±41
69-1A-33 (Negative control)§	IgM(κ)	194±38
Mor-h1 (Positive control)§	IgM(κ)	1,589±242
AWA12	IgM(λ)	1,063±102
WKG9	IgM(λ)	1,338±98
LAG9	IgM(λ)	1,248±181
GDC4	IgM(κ)	1,874±202
CNH11	IgM(κ)	1,694±91
CNH8	IgM(κ)	3,112±181

* Determined by ELISA.
‡ Antibodies were tested by RIA at a concentration of 1.0 μg/ml. Counts per minute (cpm) expressed as mean±SD. Tests were done in triplicate.
§ See Methods.

The binding of antibodies to RIN 5F cells was also determined by radioimmunoassay (RIA). Glutaraldehyde-fixed RIN 5F cells in 96-well plates were washed twice with PBS and then incubated with 100 μl of RPMI 1640 containing 10% bovine serum albumin (BSA) for 1 h at 37°C. After one wash with RPMI 1640 containing 1% BSA, 50 μl of the culture supernatant from EBV-transformed cells were added. After 1 h at 37°C, the wells were washed twice, and 50 μl of ^{125}I-labeled goat anti-human IgM (Cappel Laboratories), containing ~5 × 10^4 cpm, were added to each well. After 1 h at 37°C, the supernatant was aspirated and the wells were washed three times. 100 μl of 2 N NaOH were added to each well, and the fluid and dissolved cells were absorbed by cotton swabs and counted in a Beckman 4000 gamma counter (Beckman Instruments, Inc., Fullerton, CA).

Cloning. Lymphoblastoid cell cultures making antibodies to RIN 5F cells were cloned by limiting dilution (1 cell/0.2 ml per well) on feeder layers consisting of 10^5 human allogeneic peripheral blood mononuclear cells treated with mitomycin C (25 μg/ml, 20 min, 37°C) in 96-well tissue culture plates (18). Evidence that this procedure results in the production of monoclonal antibodies with multiple organ reactivity comes from earlier studies (12–14, 19–23) and from recent experiments in our laboratory showing that 10 different EBV-transformed cell lines cloned three times by the limiting dilution method retained the same multiple organ reactivity pattern as cells cloned only once (Casali et al., unpublished data).

Tissue preparation for immunohistochemistry. Tissues were obtained from human autopsy specimens. Saline perfusion was sometimes used to decrease extracellular immunoglobulins. Fresh tissues were snap-frozen in embedding medium (OCT; Miles Laboratories, Naperville, IL) by immersion in isopentane cooled with a dry ice-ethanol slurry. Frozen sections were cut at 6 μm, allowed to air dry for 5 min, and then fixed for 5 min in acetone. In some cases sections were stored at −40°C.

Staining techniques. The reactivity of autoantibodies with tissues was determined by immunoperoxidase employing amplification with avidin-biotin complexes (ABC) (24) or by indirect immunofluorescence.

Results

EBV-transformed B lymphocytes make immunoglobulins that react with rat insulinoma (RIN 5F) cells. PBL were infected with EBV and cultured for three weeks in 96-well plates. All the microcultures showed lymphoblastoid cell transformation and immunoglobulin secretion, as in previous experiments (13). The supernatants of EBV-transformed B lymphocyte cultures were screened for both IgM and IgG antibodies reacting with fixed RIN 5F cells by ELISA. A total of 960 EBV-transformed B lymphocyte cultures were derived from PBL of 10 patients. Of these 960 cultures, 38 (4%) were found to produce IgM antibodies that reacted with RIN 5F cells. These cells were cloned by limiting dilution (18). In spite of the low cloning efficiency typical of EBV-transformed cells (13), six EBV-transformed B lymphocyte clones, derived from the PBL of five of the 10 patients and producing monoclonal antibody reactive with RIN 5F cells, were isolated (Table I). The clones generally produced from 0.5 to 5.0 μg of IgM per milliliter of culture medium.

Reactivity of monoclonal antibodies with RIN 5F cells and human islets. Tables I and II show the reactivity of the six EBV-transformed cultures that were successfully cloned. All six human monoclonal antibodies were of the IgM class and bound by RIA to fixed RIN 5F cells (Table I). The binding was 5 to 15 times greater than with a nontissue reacting human monoclonal IgM. By indirect immunofluorescence (Fig. 1 *A*), all six antibodies also strongly stained RIN 5F cells. Further studies showed that these monoclonals did not react with the surface of viable RIN 5F cells as measured by RIA, immunofluorescence, or complement-dependent ^{51}Cr-release assay (data not shown).

To see if the antibodies, positive on rat insulinoma cells,

Table II. Reactivity of EBV-induced Monoclonal Antibodies with Normal Human Tissues

	Reactivity*						
				Stratified squamous epithelium (esophagus)			
Monoclonal antibody	Pancreas islets	Thyroid acinar	Peripheral nerve axons	Basal	Superficial cells‡	Cell borders	T lymphocytes
AWA12	+	+	+	−	+	−	−
WKG9	+	+	+	+	−	−	+
LAG9	+	+	+	+	+	+	−
GDC4	+	+	±	−	−	−	−
CNH11	+	+	−	−	−	−	−
CNH8	+	+	−	−	+	−	−

* Tested on frozen sections of normal human tissue by ABC immunoperoxidase and on smears of nylon wool-enriched human T lymphocytes (~95% OKT3$^+$ cells) by ELISA and indirect immunofluorescence. +, positive reactivity; ±, variable reactivity; −, negative reactivity. ‡ Includes both superficial and intermediate cells.

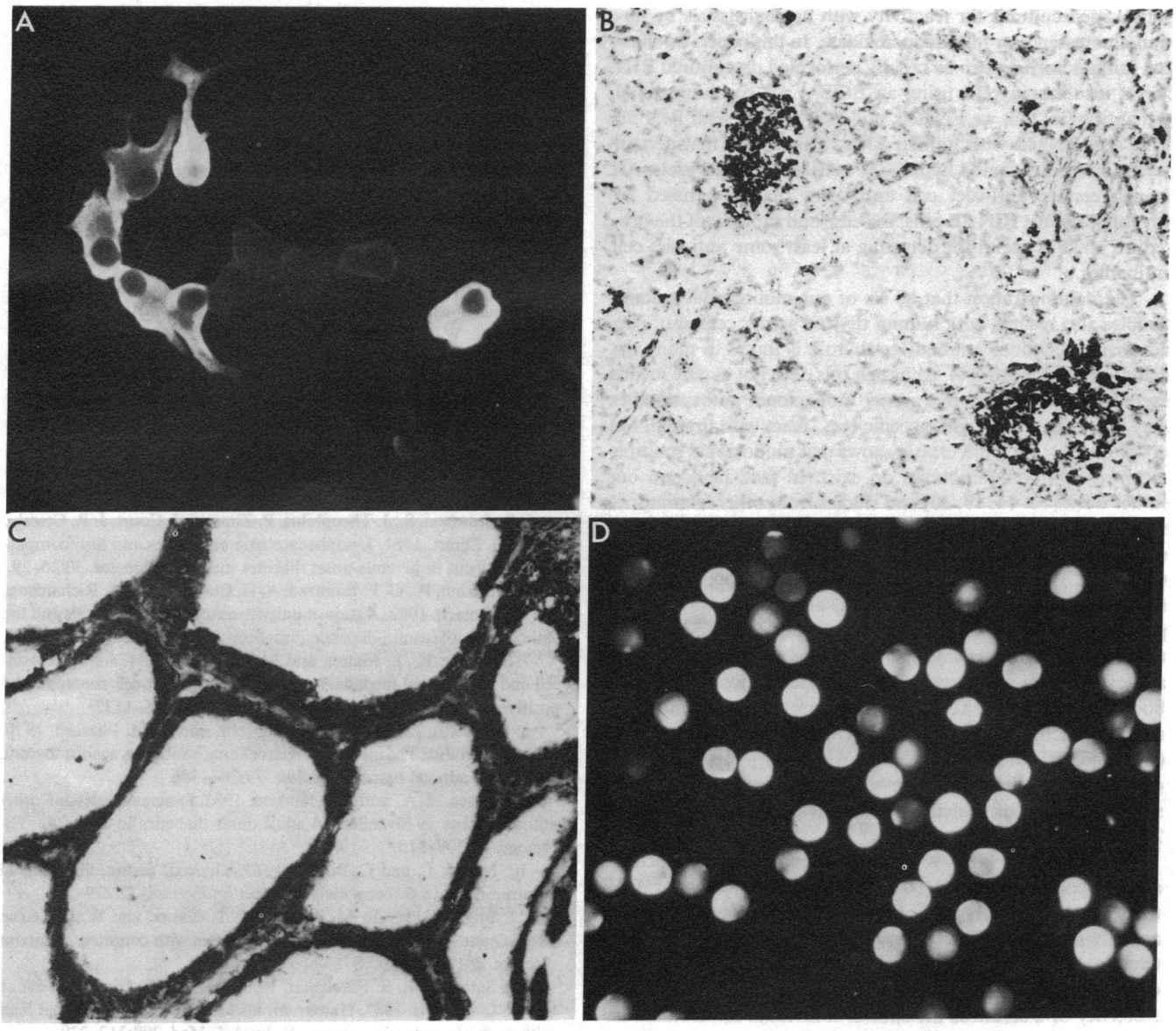

Figure 1. Reactivity of human monoclonal autoantibodies with rat and human tissues. (*A*) Reactivity of monoclonal antibody WKG9 with glutaraldehyde-fixed rat insulinoma cells (RIN 5F) by indirect immunofluorescence (× 100). (*B*) Reactivity of monoclonal antibody CNH8 with islets of human pancreas (frozen section) by immunoperoxidase technique (× 25). (*C*) Reactivity of monoclonal antibody LAG9 with acinar cells of human thyroid (frozen section) by immunoperoxidase technique (× 50). (*D*) Reactivity of monoclonal antibody WKG9 with glutaraldehyde-fixed human T cells by indirect immunofluorescence (× 100).

would also react with human islet cells, they were tested on normal human pancreas by immunoperoxidase or immunofluorescence (Fig. 1 *B*). All six human monoclonal antibodies reacted with human islet cells (Table II).

Reactivity of the monoclonal antibodies with other human tissues. The six monoclonal autoantibodies also were tested for reactivity with other normal human tissues. As seen in Table II, each of the monoclonal autoantibodies also reacted with human thyroid acinar cells (Fig. 1 *C*). In addition, each of the monoclonals showed a unique pattern of reactivity with other tissues. For example, monoclonal AWA12 reacted with peripheral nerves and superficial cells of the stratified squamous epithelium of the esophagus, but not with the cell borders or basal cells of the esophagus or with T lymphocytes. In contrast, WKG9 reacted with esophageal basal cells and T lymphocytes (Fig. 1 *D*), but not with the cell borders or superficial cells of the esophagus. LAG9 reacted with the basal cells, the superficial cells, and the cell borders of the stratified squamous epithelium of the esophagus, whereas GDC4 and CNH11 failed to react with these cells. Neither CNH11 nor CNH8 reacted with peripheral nerves.

Discussion

By transforming human peripheral blood lymphocytes with EBV, it is possible to prepare human monoclonal autoantibodies that react with human islets of Langerhans (13). One of the major hurdles in isolating these autoantibodies is the time and skill required to screen hundreds of supernatants from EBV-

transformed cultures for reactivity with normal tissues by immunofluorescence or immunoperoxidase. In this paper, we show that initial screening can be rapidly performed on cultured RIN 5F rat insulinoma cells using an ELISA. All six monoclonal autoantibodies that we isolated, and that reacted with RIN 5F cells, also reacted with human islets. This shows that certain determinants are shared by RIN 5F cells and human islets. Although certain anti-islet cell antibodies may be missed by screening against RIN 5F cells, the ease and rapidity of the procedure make it useful for detecting at least some anti-islet cell antibodies.

The demonstration that all six of our monoclonal autoantibodies also reacted with human thyroid acinar cells is of particular interest since antibody to thyroid is found in many patients with IDDM (8–11). Although this does not by any means exclude the possibility that many monoclonal autoantibodies may be more or less organ-specific (e.g., react with thyroid, but not pancreas), our work clearly shows that monoclonal multiple organ-reactive autoantibodies do exist. In fact, based on our earlier work (12–14, 19, 20) and studies from other laboratories (25–27), it appears that lymphocytes able to make multiple-organ reactive autoantibodies are very common in the normal B cell repertoire. For example, monoclonal autoantibodies, selected for their ability to react with DNA, also react with different polynucleotides and cardiolipin (25, 28); IgM monoclonal autoantibodies that react with IgG molecules also react with DNA and cytoskeletal elements (26) and a human monoclonal antibody that reacts with a 35,000-mol wt protein common to thyroid, stomach, and pituitary also reacts with growth hormone (14). The basis of the multiple organ reactivity of at least some of these autoantibodies appears to be their capacity to recognize similar epitopes or proteins in different organs (14, 19, 21–23, 28–30).

We still do not know whether the reactivities of monoclonal autoantibodies, prepared by hybridoma technology or EBV transformation, are typical of the reactivities of autoantibodies actually found in human serum. Nonetheless, monoclonal autoantibodies are providing an approach for studying the reactivity of human serum by constructing its individual components. The reactivity of a serum is the sum of individual monoclonal antibodies, each of which, in isolation, would show a unique pattern of reactivity differing from that of the sum of the components. The observation that the serum of IDDM patients shows strong reactivity with islet cells, with descending reactivity to other organs (4–11), could be explained by the multiple organ-reactive character of the individual antibodies. For example, if all the antibodies shown in Table II were mixed together, they would show strong reactivity with the pancreas and thyroid, but, depending on concentration, little, if any, reactivity with T lymphocytes (unpublished data) or with the cell border of the esophagus. Thus, the apparent specificity of a mixture of monoclonal antibodies (e.g., a serum) would be determined by the most commonly expressed reactivities shared by the individual components. A similar proposal, concerning the common reactivities of different antibodies in a serum, was made by Talmage (31) in 1959 and amplified by Richards et al. (32, 33) in the mid 1970s, before the development of monoclonal antibodies.

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