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Epstein-Barr Virus-Transformed Lymphocytes Produce Monoclonal Autoantibodies that React with Antigens in Multiple Organs

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Peripheral blood lymphocytes from normal individuals and patients with autoimmune abnormalities such as insulin-dependent diabetes mellitus and thyroiditis were infected with Epstein-Barr virus, and the culture supernatants were tested for autoantibodies that reacted with normal tissues. Between 58 and 86% of Epstein-Barr virus-transformed cultures produced immunoglobulin M antibodies, and between 9 and 24% of the transformed cultures produced immunoglobulin G antibodies that reacted with normal tissues. Ten Epstein-Barr virus-transformed clones secreting human immunoglobulin M monoclonal autoantibodies were isolated. Four of these monoclonal autoantibodies were studied in depth and found to react with antigens in multiple organs, including thyroid, pancreas, stomach, smooth muscle, and nerves. It is concluded that Epstein-Barr virus can trigger the production of autoantibodies without infecting the target cells to which the autoantibodies are directed.

Epstein-Barr virus (EBV) is a common herpesvirus causing infectious mononucleosis. Multiple immunological phenomena have been observed in patients with infectious mononucleosis, including the transient occurrence of antibodies, mostly of the immunoglobulin M (IgM) class, to certain tissue antigens. Smooth muscle autoantibodies have been frequently found (1, 7, 20), and antibodies that react with nuclei, mitochondria, microsomes, lymphocytes, and specific organs (e.g., thyroid and stomach) have been occasionally observed (1, 12, 14, 20).

EBV is known to be a polyclonal B-cell activator (3, 4, 16). In vitro, the virus transforms human B lymphocytes into immortalized lymphoblastoid cells that proliferate and secrete immunoglobulins (2, 3). The present experiments were initiated to see whether EBV-transformed and cloned B lymphocytes would produce autoantibodies that react with antigens in normal tissues.

B95-8 marmoset cells (kindly provided by D. V. Ablashi) (13) producing EBV were grown at 37°C in culture medium (RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics), suspended at a concentration of 10^6 cells per ml, and incubated for 10 days at 34°C. The virus-containing supernatant was removed, passed through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.), and stored at −70°C. Lymphocytes from ca. 20 ml of heparinized peripheral blood from three patients with autoimmune abnormalities (i.e., insulin-dependent diabetes mellitus and thyroiditis) and two normal individuals were isolated by centrifugation on Ficoll lymphocyte separation medium (Litton Bionetics Laboratory, Kensington, Md.). Since T cells suppress the outgrowth of EBV-infected cells (4), EBV transformation was performed by the infection of nylon-adherent, B-lymphocyte-enriched cultures (21). Between 1×10^6 and 4×10^6 B lymphocytes were suspended in 1 ml of culture medium, mixed with 0.5 ml of EBV-containing supernatant from B95-8 cells, incubated for 2 h at 37°C, and then washed once with culture medium. Between 1×10^4 and 4×10^4 lymphocytes incubated with EBV were seeded in each well of a 96-well plate. These cells were usually cultured in the presence of 0.5×10^5 to 1×10^5 autologous, nylon-effluent, T-lymphocyte-enriched cells treated with mitomycin C (30 μg/ml, 20 min, 37°C), which served as feeder cells. Under these conditions, 100% of the seeded wells showed lymphoblastoid cell transformation.

After 4 weeks, the supernatants of EBV-transformed B-lymphocyte cultures were screened for immunoglobulin production by an enzyme-linked immunosorbent assay. Briefly, the wells of flat-bottomed plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) for the enzyme-linked immunosorbent assay were coated overnight at 4°C with 100 μl of a 1:2,000 dilution of goat $F(ab)_2$ anti-human IgM, IgG, and IgA antibodies (Cappel Laboratories, West Chester, Pa.) at pH 9.6 in carbonate buffer and then washed three times in phosphate-buffered saline containing 0.05% Tween 20. Each well then received 100 μl of supernatant from the EBV-transformed cultures diluted 1:3 in phosphate-buffered saline–0.05% Tween 20. After 1 h of incubation at room temperature, the wells were washed as described above and incubated for another hour with 100 μl of a 1:400 dilution of goat anti-human IgM, IgG, or IgA conjugated to peroxidase (Cappel). The plates were then washed three times and developed with 50 μl of 0.01% H_2O_2 substrate in the presence of 0.01% *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.), and the optical density at 492 nm was read with a Multiscan microplate reader (Flow Laboratories, Inc., McLean, Va.). Wells containing serial dilutions of IgM, IgG, and IgA ranging from 10 ng/ml to 100 μg/ml served as standards.

Our experiments showed that all the microculture wells that contained transformed cells (i.e., 480 wells) produced some immunoglobulin. As seen in Fig. 1, 36, 23, and 32% of the cultures produced between 1.0 and 4.9 μg of IgM, IgG, and IgA per ml, respectively. IgM concentrations ranging from 5.0 to 19.9 μg/ml were found in 20% of the cell culture supernatants, and occasionally even higher concentrations were observed. IgG and IgA concentrations greater than 10.0 μg/ml were very rare.

The supernatants of EBV-transformed B-lymphocyte cul-

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TABLE 1. Tissue reactivity of supernatants from EBV-transformed lymphocyte cultures

Subject	EBV-Transformed cultures producing tissue-reactive immunoglobulin[a]			
	IgM		IgG	
	No. tested	% Positive	No. tested	% Positive
Patient				
1	17	82	34	24
2	65	58	11	9
3	ND[b]	ND	40	23
Control				
1	64	86	15	20
2	70	61	40	23

[a] Supernatants of EBV-transformed microculture wells containing more than 1 μg of IgM or IgG per ml were tested for reactivity with frozen, acetone-fixed sections of monkey pancreas and thyroid by the avidin-biotin-immunoperoxidase method. The results are expressed as the percentage of microculture wells containing tissue-reacting IgM or IgG antibodies.
[b] ND, Not determined.

tures containing more than 1 μg of IgM or IgG per ml were initially screened for reactivity with frozen sections of normal monkey pancreas and thyroid fixed in acetone. Monkey tissues were used because of the availability of fresh, well-preserved specimens. However, the same results were subsequently obtained when the supernatants were tested on acetone-fixed sections of frozen normal human tissue. Reactivity of the supernatants with tissue was determined by the immunoperoxidase method employing amplification with avidin-biotin complexes (8). As seen in Table 1, between 58 and 86% of EBV-transformed cell cultures from both patients and normal individuals produced IgM antibodies, and between 9 and 24% produced IgG antibodies that reacted with normal tissues.

Selected EBV-transformed cell cultures making autoantibodies were cloned by limiting dilution (one cell in 0.2 ml per well) in 96-well plates in the presence of 10^5 mitomycin C-treated allogenic peripheral blood leukocytes as feeder cells. Under these conditions, between 2 and 6% of the seeded wells showed cell growth after 4 weeks. By this procedure, 10 clones producing tissue-reacting IgM monoclonal antibodies were isolated. Most of the clones were found to secrete IgM at concentrations ranging from 0.5 to 2.0 μg/ml,

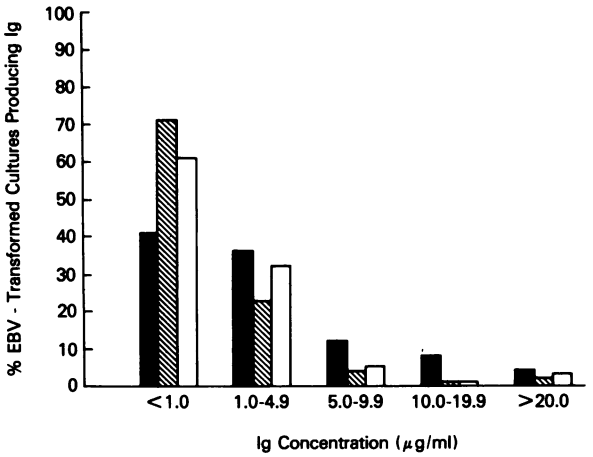

FIG. 1. Immunoglobulin production by EBV-transformed cell cultures. The supernatants of 480 EBV-transformed cell cultures from five individuals were tested for IgM, IgG, and IgA concentrations by enzyme-linked immunosorbent assay. The results are expressed as percentages of EBV-transformed cell cultures producing IgM (■), IgG (▨), and IgA (□).

and occasionally IgM concentrations as high as 5 to 10 μg/ml were found. These 10 clones have been passaged continuously for at least 6 months without loss of specific antibody activity.

All 10 monoclonal autoantibodies were tested against a panel of normal tissues and found to react with antigens in more than one organ. Each antibody showed a peculiar and individual pattern of tissue reactivity. The partial reactivity pattern of four of these monoclonal antibodies is shown in Table 2 and Fig. 2. Monoclonal D5-2-19, for example, reacted with thyroid acinar cells (Fig. 2-2) and pancreatic islet cells and ductules (Fig. 2-13). In addition, it reacted with stomach smooth muscle, esophageal stratified epithelium, and axons of peripheral nerves. In contrast, monoclonal E10-1-19 displayed a specific reaction with the colloid, but not with the acinar, tissue of the thyroid (Fig. 2-3); the pancreatic reactivity was restricted to the basement membrane of ducts and ductules. Monoclonal H10-3-5 reacted with a large number of tissues (Fig. 2-6, 2-9, and 2-11), including some not listed in Table 2: the parathyroid gland (Fig. 2-15), ductal cells in the salivary gland (Fig. 2-17), and

TABLE 2. Tissue reactivity of EBV-induced monoclonal autoantibodies

Monoclonal autoantibody[a]	Immunoglobulin class[b]	Immunoglobulin production (μg/ml)	Tissue showing reactivity[c]				
			Thyroid	Pancreas	Stomach	Stratified squamous epithelium	Nerve
D5-2-19	IgM(λ)	1.2	Acinar	Islets, ductules	Smooth muscle	Perinuclear region	Axons
E10-1-19	IgM(κ)	1.5	Colloid	Basement membrane of ducts	None	None	None
H10-3-5	IgM(κ)	1.1	Acinar	Acinar, ducts	Smooth muscle	Basement membrane	Axons
D1-2	IgM(κ)	5.0	Acinar	None	None	Reserve cells	None

[a] Obtained from a patient with insulin-dependent diabetes mellitus and Hashimoto's thyroiditis.
[b] The immunoglobulin class and light-chain type were determined by enzyme-linked immunosorbent assay.
[c] Monoclonal autoantibodies were routinely tested at a concentration of 1 μg/ml by the avidin-biotin-immunoperoxidase method on frozen, acetone-fixed sections of normal monkey tissue.

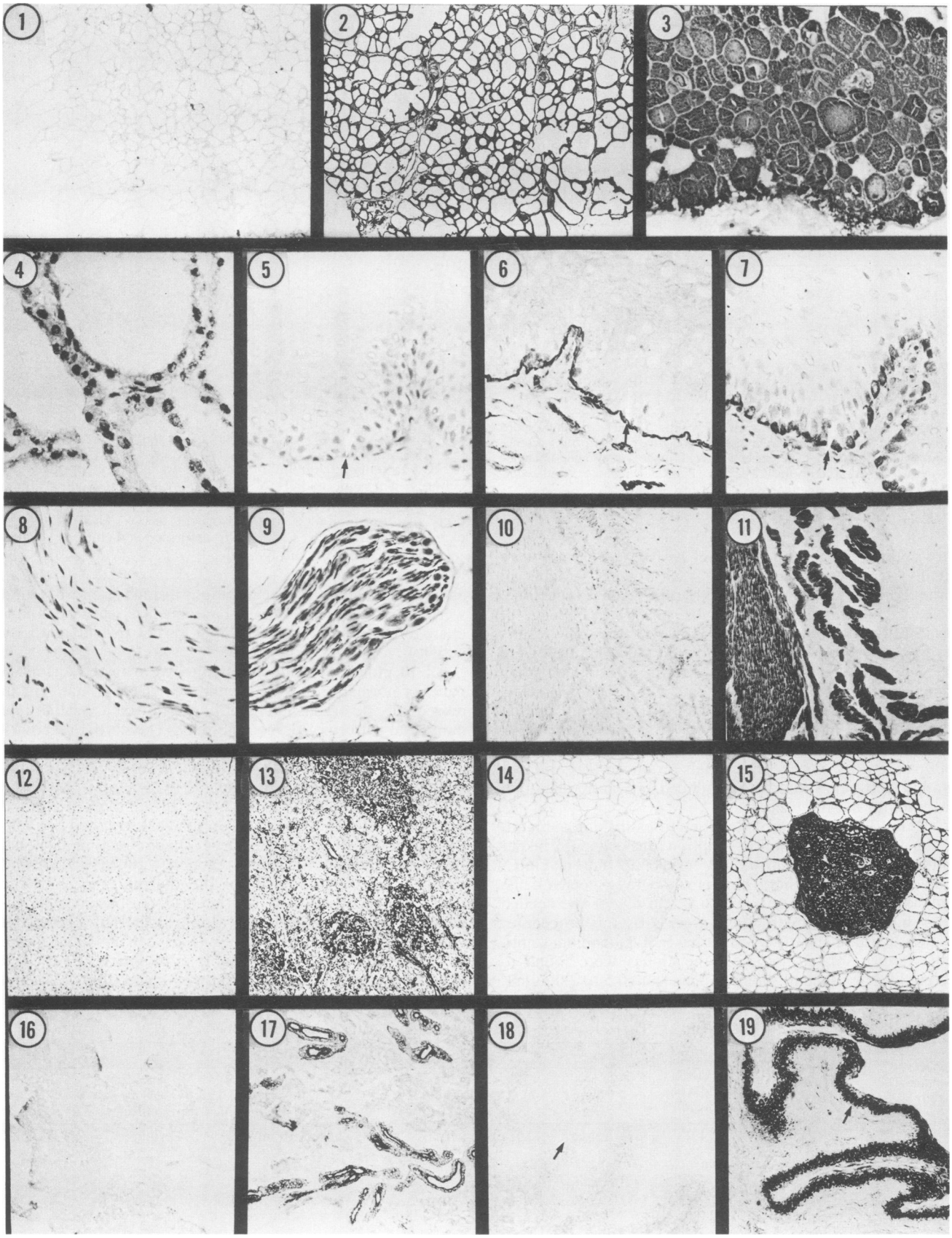

FIG. 2. Reactivity of EBV-induced autoantibodies with tissue sections. Panels 1 through 4, Thyroid: (1) non-tissue-reacting monoclonal IgM (control antibody); (2) D5-2-19 monoclonal antibody reacting with acinar cells; (3) E10-1-19 monoclonal antibody reacting with colloid; (4) antinuclear antibody from uncloned EBV-transformed culture. Panels 5 through 7, Esophagus: (5) control antibody; (6) H10-3-5 monoclonal antibody reacting with basement membrane; (7) D1-2 monoclonal antibody reacting with reserve cells in esophagus. Panels 8 and 9, Peripheral nerve: (8) control antibody (the counterstain reacts with Schwann cell nuclei); (9) H10-3-5 monoclonal antibody reacting with axons (myelin sheath can be seen around the reactive fibers). Panels 10 and 11, Stomach: (10) control antibody; (11) H10-3-5 monoclonal antibody reacting with smooth muscle. Panels 12 and 13, Pancreas: (12) control antibody; (13) D5-2-19 monoclonal antibody reacting with islets and ductules. Panels 14 and 15, Parathyroid and surrounding thyroid: (14) control antibody; (15) H10-3-5 monoclonal antibody reacting with parathyroid and, to a lesser degree, thyroid. Panels 16 and 17, Salivary gland: (16) control antibody; (17) H10-3-5 monoclonal antibody reacting with ductal cells, particularly at luminal border. Panels 18 and 19, Bladder: (18) control antibody; (19) H10-3-5 monoclonal antibody reacting with transitional epithelium. Arrows indicate basement membrane. Magnification: panels 1 through 3, 14, and 15, ×18; panels 10 through 13 and 16 through 19, ×45; panels 4 through 9, ×180.

transitional epithelium of the bladder (Fig. 2-19). The reactivity of monoclonal D1-2 was more limited, being restricted primarily to thyroid acinar cells and reserve cells of the esophageal stratified epithelium (Fig. 2-7).

Recently, it has been shown that EBV-transformed B lymphocytes can produce monoclonal antibodies (9, 10, 18, 19, 22). The present study shows that EBV-transformed B lymphocytes also can produce monoclonal autoantibodies that react with antigens in tissues from normal individuals. Many of these antibodies are of the multiple organ-reactive (MOR) type; i.e., they react with antigens in several different organs. Similar types of autoantibodies have been obtained recently by fusing lymphocytes from mice or humans with appropriate myeloma cells (5, 6, 17).

The ease with which MOR antibodies have been obtained from patients and normal individuals and the capacity of both hybridomas and, now, transformed human B lymphocytes to secrete MOR antibodies argue that lymphocytes capable of making MOR antibodies are a common feature of the normal B-cell repertoire of the host. Studies of how these antibodies react with multiple organs suggest that MOR antibodies may react with the same protein present in different organs or the same epitope on different proteins, or perhaps they accommodate structurally similar but nonidentical epitopes on different proteins (6, 11). Regardless of the mechanism, MOR antibodies may be a partial explanation for the multiple-organ reactivity of serum obtained from patients with certain autoimmune diseases (6, 17).

The high frequency with which autoantibodies were detected in the present study is due to at least two factors: the method of screening and the assay used. The screening procedure consisted of testing each supernatant fluid from transformed cultures against a panel of normal tissue sections. Since each cell type or organ contains thousands of antigenic determinants, reactivities were detected that would have been missed by more limited and specific screening procedures. The avidin-biotin-immunoperoxidase assay used in the present study to detect autoantibodies also is considerably more sensitive than the standard immunofluorescence assay that we used in the past (5, 6, 17) and allows both preservation of specimens and easy identification of cell morphology. On a practical level, many of the monoclonal autoantibodies obtained by EBV transformation of lymphocytes should prove useful in preparing immunoaffinity columns for the isolation of specific tissue antigens.

A transient appearance of autoantibodies has been observed in animals and patients after a variety of viral infections, including EBV (15). It has often been speculated that viruses trigger an autoimmune response by making infected cells antigenic (15). The present study makes it clear that a virus can trigger the production of autoantibodies through its lymphoproliferative effect without infecting the target cells to which the autoantibodies are directed. Moreover, the demonstration here that EBV-transformed cells from normal individuals can make autoantibodies suggests that under some circumstances the stimulation of lymphocytes by autoantigens may not even be required to trigger the production of autoantibodies.

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