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Virus-Induced Autoimmunity: Monoclonal Antibodies That React with Endocrine Tissues

Abstract. *Mice infected with reovirus type 1 develop an autoimmune polyendocrine disease. Spleen cells from these mice were fused with myeloma cells and the culture fluids were screened by indirect immunofluorescence for autoantibodies reactive with normal mouse tissues. A large panel of cloned, stable antibody-producing hybridomas has been obtained. Fourteen of the hybridomas make autoantibodies that react with cells in the islets of Langerhans, 24 with cells in the anterior pituitary, 11 with cells in gastric mucosa, and 5 with nuclei. Except for the antibodies to nuclei, the monoclonal autoantibodies are organ-specific. Some, however, show broad cross-species reactivity, recognizing similar antigenic determinants in mouse, rat, pig, and human organs, whereas others recognize determinants only in rodent tissues. Several of the antigens recognized by these monoclonal autoantibodies have been identified as hormones (for example, glucagon, growth hormone, and insulin).*

There are a large number of important human diseases of undetermined etiology that have an autoimmune component (*1*). In some of these diseases, such as systemic lupus erythematosus, the autoimmune component is very broad, involving many different organs and tissues (*2*). In insulin-dependent diabetes mellitus and polyendocrinopathy, the autoimmune component involves primarily, but not exclusively, endocrine organs (for example, pancreas, anterior pituitary, thyroid, and gastric mucosa) (*3*). In still other diseases, such as myasthenia gravis, the autoimmune response is further restricted, being directed predominantly against the acetylcholine receptor (*4*).

Little is known about the factors that trigger the production of autoantibodies in these various diseases, but viruses have been suspected as one of the possible causes. Recently, we showed that SJL/J mice, infected with reovirus type 1, develop an autoimmune polyendocrine disease characterized by mild diabetes mellitus and retarded growth (*5*). Autoantibodies directed against normal pancreas, pituitary, and gastric mucosa were found in serum samples from the infected mice. The polyendocrinopathy has an immunological component, because immunosuppression prevents the development of autoantibodies, diabetes, and growth retardation (*6*). However, the role that these antibodies actually play in the pathogenesis of this disease has been difficult to evaluate because of the problem of obtaining large quantities of purified autoantibodies. To accomplish this end, we used hybridoma technology. Spleen cells from reovirus-infected mice were fused with myeloma cells, and the culture fluids were screened for autoantibodies. By this method we have obtained over 40 stable hybrids that produce monoclonal autoantibodies that react with a variety of normal mouse tissues.

Suckling SJL/J mice were inoculated with reovirus type 1 as previously described (*5*). Serum samples from animals with retarded growth were examined by indirect immunofluorescence (FA) for antibodies reactive with paraffin sections of normal mouse pancreas, pituitary, and stomach that had been fixed in Bouin's fluid. Mice with detectable autoantibodies were killed between 9 and 26 days after infection. Spleen cells were fused with P3-653Ag8 myeloma cells at a 10:1 ratio with the use of 50 percent polyethylene glycol 1000 (*7*). The fused cells were plated (2.5×10^5 cells per well) in 96-well microtiter plates containing unstimulated peritoneal exudate cells (5×10^3 cells per well) as a feeder layer. After selection of cells in HAT medium (hypoxanthine, aminopterin, thymidine), supernatant fluids from confluent cultures were examined by FA for antibodies to mouse adrenal glands, brain, muscle, pancreas, pituitary, salivary glands, skin, stomach, thymus, and thyroid. Hybrids producing autoantibodies were immediately cloned by limiting dilution (*8*) and were microscopically inspected for the presence of a single colony. Only cloned hybrids that remained productive upon passage are described in this report. Spleen cells obtained from mice less than 20 days after infection yielded

Table 1. Monoclonal autoantibodies against the anterior pituitary react with growth hormone. Abbreviation: N.D., not determined.

Hybridoma	Anterior pituitary (FA)*					Growth hormone (ELISA)†	
	Mouse		Rat	Pig	Human	Rat	Human
	Before	After‡					
8–4	400	8	20	2	<2	640	<10
6B–12	200	<2	20	<2	<2	80	<10
6B–7	80	<2	8	<2	<2	40	<10
2–9	200	16	20	<2	<2	320	20
6A–3	800	<2	50	16	<2	160	<10
5B–5	800	<2	800	400	800	2,560	20,480
5B–8	400	N.D.	400	20	20	<10	<10

*The antibodies, precipitated from supernatant fluids by 50 percent saturated ammonium sulfate, were tested for reactivity with anterior pituitary and purified growth hormone. All of these antibodies were immunoglobulin M. Paraffin sections from normal mouse, rat, pig, and human pituitaries (fixed in Bouin's fluid) were incubated with serial dilutions of autoantibody and then with antibody to mouse immunoglobulin conjugated with fluorescein isothiocyanate. The fluorescent antibody (FA) titer represents the reciprocal of the highest dilution giving positive fluorescence. †For enzyme-linked immunosorbent assay (ELISA), Immunlon-2 plates (Dynatech Laboratories) were coated with 5 μg of purified human or rat growth hormone (courtesy of National Pituitary Agency, Baltimore), incubated with serial dilutions of monoclonal antibodies, and then reacted with peroxidase-labeled antibody to mouse immunoglobulin as described (*5*). Absorbance was read with a Titertek microplate reader and titers were expressed as the reciprocal of the highest dilution giving a value twice the control readings. ‡The FA titer after incubation for 48 hours with 0.5 mg of purified rat growth hormone per milliliter.

only immunoglobulin M–synthesizing hybridomas.

The frequency of stable clones producing autoantibodies was remarkably high; in some fusions, up to 20 percent of the tested culture wells gave rise to productive hybridomas. Monoclonal antibodies against pancreas, pituitary, stomach, and cell nuclei were readily obtained. Most of the monoclonal antibodies to pancreas (13 hybrids) react with cells in the periphery, but not in the central area, of the islets. The fluorescent labeling of peripheral islet cells by one of the autoantibodies is shown in Fig. 1A. This antibody, with an FA titer of 1:3200 against mouse pancreas, cross-reacts with rat (1:3200), porcine (1:400), and monkey (1:100) pancreas. This antibody also reacts with porcine glucagon in an enzyme-linked immunosorbent assay (ELISA) with a titer of 1:128. Furthermore, the FA titer, as tested on mouse pancreas, was reduced (from 1:256 to 1:2) following adsorption with porcine glucagon (1 mg/ml). Only one hybridoma (not shown) has been isolated that synthesizes antibody reactive with the central portion of pancreatic islets (that is, β cells). This antibody has a titer of 1:2028 against rat insulin in ELISA, and its reactivity against mouse pancreas was removed (FA titer reduced from 1:50 to 1:2) by incubation with rat insulin (0.1 mg/ml).

Five hybridomas that synthesize antibodies to nuclei have been isolated. One such hybridoma was isolated from an animal that had no detectable nuclear antibodies in its serum. A typical pattern of nuclear staining is shown in Fig. 1B. These antibodies react with nuclei of many organs; the nuclear antigen (or antigens) with which they react has not been determined.

Eleven hybridomas have been isolated that synthesize antibodies against cells in the gastric mucosa. Figure 1C shows the type of fluorescent staining seen with a number of these antibodies. Histochemical staining of the cells suggests that some of these antibodies react with "chief" (pepsinogen-producing) cells.

Twenty-four hybridomas have been isolated that secrete autoantibodies against the anterior pituitary gland. Most of these monoclonal antibodies react with a pattern similar to that seen with antibody 5B-5 (Fig. 1D). It is interesting that another hybridoma (5B-8), isolated from the same animal, secretes antibody that appears to react with a different subpopulation of cells (not shown).

The capacity of seven representative antibodies to cross-react with pituitaries from other species is shown in Table 1. All of these antibodies react, albeit often at lower titers, with rat pituitary. Three of the antibodies cross-react with pig pituitary. Two of the antibodies (5B-5 and 5B-8) cross-react with human pituitary, but antibody 5B-5 is unique in its capacity to react strongly with all species tested.

The antibodies to pituitary gland were tested by ELISA against rat and human growth hormones (GH) (Table 1). All of these antibodies, except 5B-8, react with rat GH, whereas only two monoclonal antibodies react with human GH. Antibody 2-9 reacts very weakly but reproducibly with human GH, while antibody 5B-5 reacts very strongly with human GH. Adsorption of these antibodies with rat GH significantly reduces their FA titers against mouse pituitary (Table 1). Adsorption of antibody 5B-5 with rat GH also reduces its FA titer (from 1:800 to 1:2) against human pituitary (not shown).

Our studies show that it is possible to prepare a large panel of monoclonal autoantibody-synthesizing hybridomas by using spleen cells from mice with reovirus-induced polyendocrine disease. With the exception of the antibodies to nuclei, these monoclonal antibodies are organ-specific. Moreover, except for one nuclear antibody, none of the autoantibodies thus far tested reacts with reovirus-infected cell cultures.

The hybridomas most frequently iso-

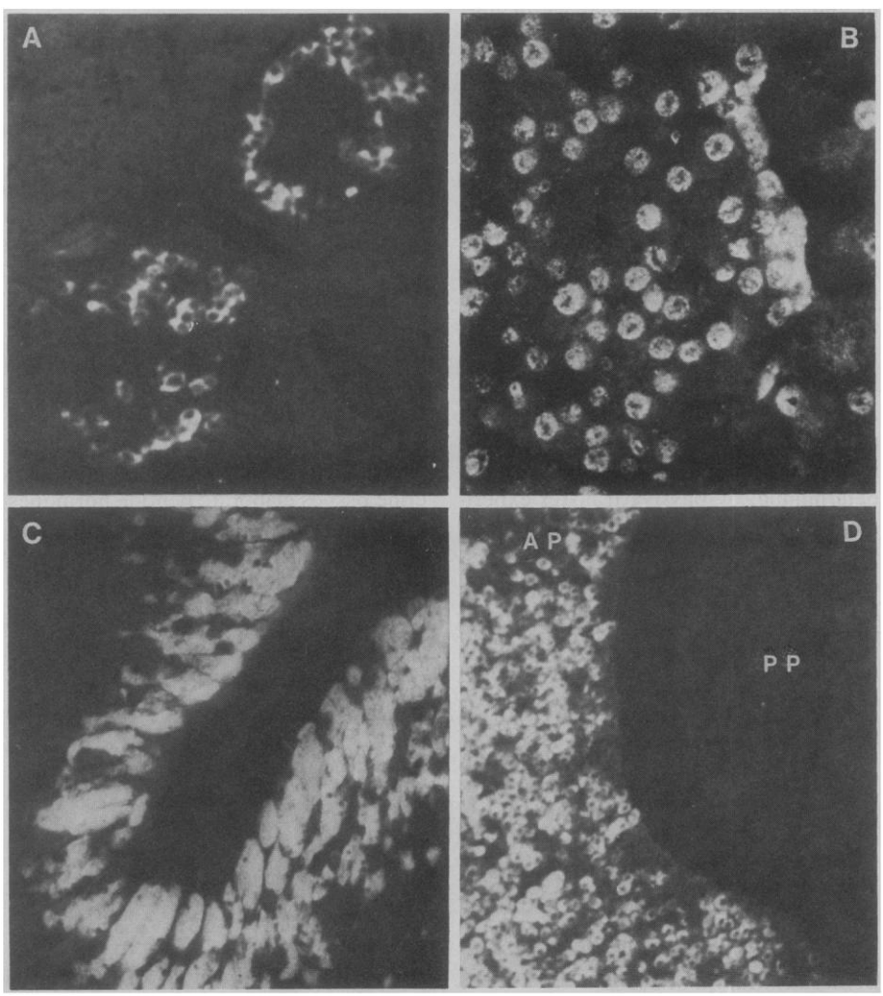

Fig. 1. Monoclonal autoantibodies reactive with normal mouse tissues. Normal mouse pancreas, pituitary, and stomach were fixed with freshly prepared Bouin's solution for 6 hours. The tissues were washed with six changes of 70 percent ethanol over 72 hours, embedded in paraffin, and sectioned. The deparaffinized sections were first incubated with the autoantibodies and then with antiserum to mouse immunoglobulin labeled with fluorescein isothiocyanate, as described (*6*). (A) Section of mouse pancreas incubated with a monoclonal autoantibody against glucagon. The photomicrograph shows cytoplasmic staining of cells in the periphery of the islets of Langerhans. No staining of acinar or β cells is seen (×340). (B) Section of mouse pancreas stained with a monoclonal autoantibody against nuclei. This antibody also reacts with nuclei of other tissues (×1360). (C) A monoclonal autoantibody reacting with a section of mouse stomach wall. Note the strong fluorescence in the deeper parts of the gastric mucosa (×170). (D) Section of mouse pituitary stained with a monoclonal autoantibody against growth hormone. Brilliant fluorescence is seen in the anterior lobe of the pituitary (*AP*); no staining of the posterior lobe (*PP*) is observed (×340).

lated synthesized autoantibodies that react with cells in the anterior pituitary. Some react with GH-producing cells, and others react with different but still unidentified subpopulations of cells. The differences in the species specificities of the monoclonal autoantibodies directed against GH suggests that these antibodies recognize different antigenic determinants on the GH molecule. Although human and rat GH are serologically different (*9*), the fact that at least one of the monoclonal antibodies (that is, 5B-5) reacts with GH from both species suggests that this antibody recognizes a shared antigenic determinant. The different reactivity patterns of these autoantibodies also suggest that they may serve as useful probes in the fine analysis of antigenic and evolutionary relatedness of hormones.

The availability of large quantities of purified autoantibodies should make it possible to address a number of fundamental questions concerning autoimmunity. First, it should be possible to determine whether different individuals with the same disease develop autoantibodies against the same molecules, and if so, whether the autoantibodies are directed against the same antigenic determinants. Second, these monoclonal autoantibodies can be used in affinity columns to isolate and identify some of the still unknown autoantigens. Third, these monoclonal autoantibodies should aid in determining, by passive transfer experiments, what role autoantibodies actually play in pathogenesis. Although the present study involves murine autoantibodies triggered by a viral infection, it should be possible to obtain and study monoclonal autoantibodies from human polyendocrine disease.

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Biologically Active Chorionic Gonadotropin: Synthesis by the Human Fetus

Abstract. *The kidney, and to a slight extent the liver, of human fetuses were found to synthesize and secrete the α subunit common to glycoprotein hormones. Fetal lung and muscle did not synthesize this protein. Since fetal kidney and liver were previously found to synthesize β chorionic gonadotropin, their ability to synthesize bioactive chorionic gonadotropin was also determined. The newly synthesized hormone bound to mouse Leydig cells and elicited a biological response: namely, the synthesis of testosterone. These results suggest that the human fetus may participate in metabolic homeostasis during its development.*

The concept that hormonal peptides are a unique product of a single cell type contained within a specialized organ known as an endocrine gland has proved to be too restrictive. Cells producing certain hormones occur outside these glands, and malignancies of nonendocrine tissues elaborate peptide hormones (*1*). Other cell types (nonendocrine and nonmalignant) also produce hormonal peptides (*1–4*). Recent studies on the human fetal-placental unit show that the placenta is a source of endorphin, adrenocorticotropin (ACTH), and hypothalamic releasing hormones, and that the fetal lung may produce ACTH and bombesin (*5*). Investigators in our laboratory have shown that large amounts of immunoreactive human chorionic gonadotropin (hCG) occur in a variety of human fetal tissues (*6*) and that synthesis of the β subunit of this hormone can occur in

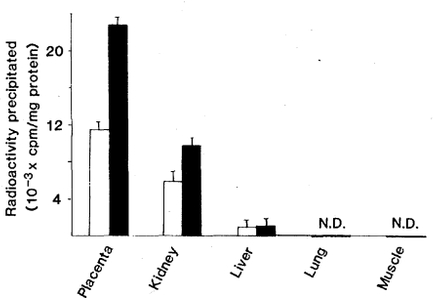

Fig. 1. Synthesis of α subunit by human fetal tissue and its characterization. Immunoprecipitation of newly synthesized α subunit. Placenta and fetal kidney, liver, lung, and muscle were obtained in the fresh state. The gestations were uncomplicated and ranged from 16 to 19 weeks. Approximately 1 g of each tissue was cut into 2-mm slices and placed in 10 ml of Krebs-Ringer bicarbonate buffer, *p*H 7.4, containing 0.2 percent glucose. The slices were incubated for 30 minutes at 37°C in an atmosphere of 95 percent O_2 and 5 percent CO_2, after which time the medium was changed and 20 μCi of [^{35}S]methionine was added. After incubation for another 12 hours the medium and tissues were separated. The tissues were homogenized in 0.15*M* NaCl, 5m*M* EDTA, and 0.1*M* tris containing 0.1 percent Triton X-100 (NET-TX). Samples were centrifuged at 40,000*g* for 1 hour at 40°C. The supernatants were decanted and 5 μl of a 10 percent suspension of *Staphylococcus aureus* capsule (SAC) was added per milliliter of supernatant collected. The centrifugation was then repeated. The α subunit was precipitated by mixing 100 μl of the tissue homogenates or incubation media with 50 μl of antiserum diluted 1:250 and allowing the mixture to react overnight at 4°C. Ten microliters of SAC were added and allowed to react for 15 minutes at room temperature and sedimented by centrifugation at 12,000*g* for 1 minute. The complexes were washed three times with 100 μl of NET-TX buffer to remove nonspecifically bound radioactivity. After the final wash, the SAC complex was disrupted by exposing it to 0.1*N* KOH for 30 minutes. The supernatant was decanted and counted in a liquid scintillation counter (PRIAS, Packard Instruments). The radioactivity is expressed per milligram of protein as determined by a micro-Bradford assay. The results represent triplicate determinations on six separate fetuses ± standard error of the mean for media (solid bars) and tissue homogenates (open bars).