Differentiation Capacity of Cultured B Lymphocytes from Immunodeficient Patients

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Abstract Peripheral blood lymphocytes from 27 healthy individuals and from 18 patients with a diverse spectrum of defects in humoral immunity were examined for their capacity to undergo terminal differentiation in vitro. Pokeweed mitogen induced cells from normal persons to synthesize and secrete IgM, IgG, and IgA as detected by immunofluorescence and incorporation of [^{14}C]amino acids. Lymphocytes from three boys with X-linked agammaglobulinemia were stimulated to proliferate, but did not synthesize immunoglobulin. Lymphocyte cultures from three of four patients having agammaglobulinemia with B lymphocytes produced different immunoglobulin classes in ratios similar to the in vivo distribution of classes of B lymphocytes. Lymphocytes from a dysgammaglobulinemic boy deficient in serum IgG and IgA, but who had normal numbers of IgM-, IgG-, and IgA-bearing B lymphocytes, could not be stimulated by pokeweed mitogen to make IgG and IgA. Synthesis and secretion of IgA, as well as IgM and IgG, was detected in cell cultures from each of 10 patients with isolated IgA deficiency. The results suggest that deficiencies in immunoglobulin synthesis may reflect either (*a*) failure to develop B lymphocytes, (*b*) arrested development of B lymphocytes due to intrinsic metabolic abnormalities, or (*c*) disturbance of factors extrinsic to the B lymphocyte which are essential for normal induction of plasma cell maturation.

INTRODUCTION

The development of antibody-producing plasma cells is a discontinuous process that can be divided conveniently into two stages (1). The first stage involves the primary differentiation of stem cells to immunocompetent B lymphocytes, without requirement for exogenous antigenic stimulation. This thymus-independent population of lymphoid cells constitutes about 20–30% of the circulating lymphocytes in man (reviewed in reference 2). B lymphocytes can be identified by the presence of membrane-bound immunoglobulins in high density (3–8); these are the receptors through which antigens are recognized (9–11). The union of antigen and surface antibody triggers second-stage events which include B-lymphocyte proliferation and terminal differentiation into antibody-secreting plasma cells. Study of this cell population in patients with deficiencies in circulating immunoglobulins has suggested that several different defects in differentiation may contribute to failure of plasma cell maturation and antibody deficiency. B lymphocytes are very deficient or absent in most boys with X-linked agammaglobulinemia (2, 12–21). Other patients, both males and females, with an equivalent deficiency in all of the circulating immunoglobulins may have normal percentages of circulating B lymphocytes (2, 12, 15–21). Normal B-lymphocyte distribution has also been found in patients selectively deficient in IgG and IgA (2, 18, 22) and in most patients with isolated deficiency of IgA, regardless of whether this abnormality is familial or associated with environmental insults (13, 18–20, 23–26).

Pokeweed mitogen (PWM),[1] an extract of *Phytolacca americana*, reacts with nonimmunoglobulin surface determinants of T and B lymphocytes (27). Its reaction with B lymphocytes induces blast transformation, cell division, and terminal plasma cellular differentiation with immunoglobulin synthesis (28–38). In previous experiments we found that PWM stimulated peripheral blood lymphocytes from normal individuals to differentiate into cells containing easily detectable cytoplas-

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[1] *Abbreviations used in this paper:* PWM, pokeweed mitogen; X-LA, X-linked agammaglobulinemia.

mic immunoglobulins of the three major classes, IgM, IgG, and IgA. Lymphocytes from IgA-deficient patients were induced to differentiate into cells containing cytoplasmic IgA as well as IgM and IgG. Stimulated lymphocytes from agammaglobulinemic females having B lymphocytes and from patients with X-linked agammaglobulinemia were induced to proliferate, but we found very few or no cells producing detectable amounts of cytoplasmic immunoglobulins (38).

The present report expands the numbers of individuals investigated and the variety of defects in B-lymphocyte differentiation studied by PWM induction of terminal B-cell differentiation in short-term cultures. The development of cells containing cytoplasmic immunoglobulins has been correlated with incorporation of ^{14}C-labeled amino acids into immunoglobulins; newly synthesized immunoglobulins were actively secreted. We show that B lymphocytes from some agammaglobulinemic and IgA-deficient patients can be induced in vitro to overcome the in vivo developmental arrest with full-scale synthesis and secretion of IgM, IgG, and IgA. In contrast, the results suggest that B lymphocytes from other immunoglobulin-deficient patients have inherent defects of differentiation which cannot be bypassed under the same conditions of culture.

METHODS

27 healthy individuals, ranging in age from 4 mo to 62 yr, served as controls. The patient group included 18 individuals. Three boys with probable X-linked agammaglobulinemia[2] had marked deficiency of circulating B lymphocytes bearing membrane-bound immunoglobulins. Four other agammaglobulinemic patients had detectable populations of B lymphocytes; two females (K. L. and N. G.) and one male (N. M.) had normal percentages of lymphocytes bearing membrane-bound IgM, IgG, and IgA, and one female (G. F.) had normal numbers of lymphocytes bearing surface IgM and IgA but decreased members of B lymphocytes bearing surface IgG. A dysgammaglobulinemic 2-yr-old boy had a normal serum IgM but was deficient in IgG and IgA; normal numbers of B lymphocytes bearing IgM, IgG, and IgA were present. 10 IgA-deficient patients had normal percentages of each class of B lymphocytes, including IgA-positive cells. The individuals with panhypogammaglobulinemia and the boy with selective IgG and IgA deficiencies were diagnosed during the course of evaluation for recurrent pyogenic infections since early childhood. Five of the patients with isolated deficiency of IgA had family members with a similar defect or other types of immunoglobulin deficiency, one had ataxia-telangiectasia, one had congenital toxoplasmosis, one had congenital rubella, and three had no demonstrable genetic or environmental correlates. Some of these patients have been described (12, 24).

Cell cultures, DNA synthesis, and immunofluorescence. Lymphocyte cultures in round-bottom tubes for DNA synthesis and immunofluorescence studies were performed as previously described (38). In some experiments cells were grown in flat-bottom microplates (Microtest II, Falcon 3040; Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) (36). Briefly, 1×10^5 lymphocytes isolated by Ficoll-Hypaque gradient centrifugation were plated in a volume of 0.2 ml in each well. Cells were cultured in Waymouth's medium (Medium MB 752/1, Grand Island Biological Co., Grand Island, N. Y.) or RPMI 1640 (Grand Island Biological Co.) supplemented with 20% of homologous plasma pooled from either one of two agammaglobulinemic individuals (one with X-LA, and the other, K. L., having B lymphocytes). The microculture plates were wrapped in clear plastic wrap (Glad Wrap, Union Carbide Corp., New York) purchased from a local supermarket. Kinetic- and dose-response studies indicated that a dose of 1λ or 5λ PWM (Grand Island Biological Co., lot no. R4212) per well and 7 days incubation at 37°C in 5% CO_2 atmosphere gave optimal results with regard to differentiation of immunoglobulin-producing cells. DNA synthesis was measured by adding [^{14}C]thymidine (sp act 62 mCi/mmol, Amersham/Searle Corp., Arlington Heights, Ill., 0.0125 μCi/culture) to triplicate cultures 18 h before harvesting. Subsequently the cell cultures were processed in a microplate precipitator for scintillation counting (39). Triplicate or quadruplicate cultures were pooled for cytoplasmic immunofluorescence study and processed as previously described (38).

In vitro biosynthesis of immunoglobulin. $10\text{–}20 \times 10^6$ cells were cultured in a concentration of $0.5\text{–}1 \times 10^6$/ml in 2-ounce prescription bottles. Half of the medium was replaced after 3 days of incubation. 18 h before termination of the culture the cells were washed and resuspended in McCoy's 5a media deficient in lysine and isoleucine (Microbiological Associates Inc., Bethesda, Md.). [^{14}C]-Lysine and [^{14}C]isoleucine (sp act 342 and 338 mCi/mmol, respectively, Amersham/Searle Corp.) were added in a dose of 1.5 μCi each per culture. Detection of newly synthesized immunoglobulins by immunoelectrophoresis and autoradiography was done by the method of Hochwald, Thorbecke, and Asofsky (40) with slight modification. The cells were lysed by freezing and thawing three times. After centrifugation to remove the debris, supernatant fluids were dialyzed against 0.085% saline to remove free [^{14}C]amino acids. The fluid was lyophilized and then redissolved in $\frac{1}{10}$ the original volume. Culture fluid was electrophoresed in the presence of normal serum as a carrier. Immunoglobulin lines were developed with monospecific antisera to human IgM, IgG, and IgA (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.). Autoradiographs were then prepared by exposing the film (Kodak Royal-X Pan Film 4166; Eastman Kodak Co., Rochester, N. Y.) to the washed stained plates for 2 wk. Film was developed in Kodak HC-110 developer.

In some experiments the proportions of newly synthesized immunoglobulins within the cells and in the culture supernate were determined independently by removing the supernate before freezing and thawing the cells.

RESULTS

Induction of immunoglobulin synthesis in lymphocyte cultures from normal individuals. By varying the technique of staining, direct immunofluorescence can be used to distinguish precursor B lymphocytes from their more mature immunoglobulin-secreting progeny

[2] Two were half-brothers having different fathers; an agammaglobulinemic brother of the other agammaglobulinemic boy died earlier with *Pseudomonas sepsis* (12).

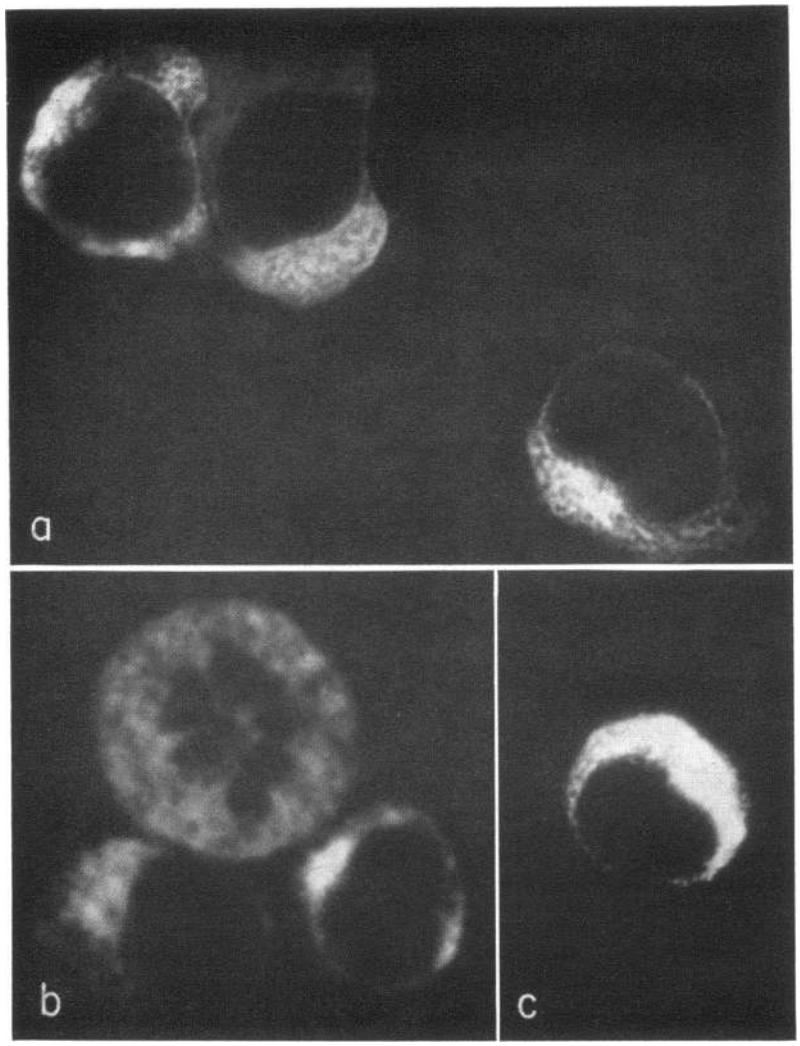

FIGURE 1 Fluorescent-positive cells in pokeweed-stimulated culture of lymphoid cells from a patient with ataxia-telangiectasia and IgA deficiency (R. H.). Cultured cells were fixed and stained with monospecific fluorescent antibody for (*a*) IgM, (*b*) IgG, and (*c*) IgA. Note that one of the IgG-containing cells is in metaphase (× 5,000).

(6). The membrane-associated immunoglobulin of B lymphocytes is readily visualized only when the cells are stained in the viable state. Under these conditions fluorescent anti-immunoglobulin is excluded from the cytoplasm by an intact cell membrane. When cells are stained after fixation, cytoplasmic immunoglobulin is detected. Most B lymphocytes do not contain sufficient immunoglobulin in the cytoplasm to be seen under these conditions.

Fresh peripheral blood lymphocytes from six normal subjects were fixed and stained for cytoplasmic immunoglobulins. The frequency of cells stained for each major immunoglobulin class was less than 1 per 1,000. Lymphocytes cultured in the absence of PWM had a similar low frequency of immunoglobulin-containing cells. PWM induced a large increase in the incidence of cells containing cytoplasmic immunoglobulin. In 32 cultures from 27 normal individuals, the median frequency of fluorescent cells was 2.3% for IgM, 1.6% for IgG, and 1.7% for IgA (Table I). Typical morphology of stained cells is illustrated in Fig. 1.

The differentiation process induced by PWM appeared not to involve interaction with membrane-bound immunoglobulin. A similar increase in percent of stained cells occurred in cultures treated with antibodies to immunoglobulins. Similarly, cells cultured in

TABLE I

PWM Responses of Circulating Lymphocytes from Normals and Patients with Various Immunoglobulin Deficiencies

Donors	B-cell development in vivo: Circulating Ig levels* IgM	IgG	IgA	Lymphocytes bearing surface Ig‡ IgM	IgG	IgA	Lymphocyte response to PWM in vitro: Cells containing cytoplasmic Ig§ IgM	IgG	IgA	DNA synthesis (stimulation index‖)
	mg/100 ml			*%*						
Normals (27)	126±57	1280±115	228±54	8.9±4.5	17.3±9.6	5.5±4.0	23(4–230)	16(3–94)	17(6–101)	30±34
X-Linked agammaglobulinemia (3)										
	0	270	0	<0.2	0.3	<0.2	0	0	0	24–70
Agammaglobulinemia with B lymphocytes										
K. L.	0	275	0	8.1	7.0	2.2	64	34	19	38
N. M.	6	99	7	9.4	10.7	3.9	56	12	23	55
N. G.	0	130	0	6.1	3.1	3.4	<1	<1	<1	9
G. F.	0	96	0	7.0	0.5	1.3	15	1	7	18
Dysgammaglobulinemia with B lymphocytes										
P. B.	47	20	0	10.8	8.3	2.7	17	<1	<1	54
							12¶	1	<1	56

* Patients other than N. M. and P. B. were receiving monthly γ-globulin injections. Values for normals are mean ±SD of normal adults in our laboratory.

‡ Values for normals are mean ±SD of normal children and adults in our laboratory (24).

§ Values for normals are expressed as median (range) per 1,000 cells counted. The frequency in unstimulated cultures was <1. The values obtained in patients K. L. and P. B. were the average of two or three separate experiments. Includes some data for comparison previously published (38).

‖ [^{14}C]Thymidine CPM stimulated culture/ CPM unstimulated culture. Mean ±SD of responses from normals are given.

¶ Values obtained in cultures of lymph node cells.

round-bottom tubes for 48 h, a procedure which results in loss of detectable surface immunoglobulin, were still capable of responding to PWM.

The two culture techniques resulted in a similar frequency of immunoglobulin-containing cells when normal lymphocytes were studied. However, the microplate technique offered considerable savings in time and may have provided better conditions for culture of lymphocytes from some patients (discussed below).

To confirm the usefulness of the direct fluorescent assay in measuring induction of immunoglobulin synthesis by PWM, we studied immunoglobulin biosynthesis by the classical method of [^{14}C]amino acid incorporation and compared the results. As shown in Table II and Fig. 2, fluid from PWM-stimulated cultures consistently produced more intensely labeled specific precipitin lines for each immunoglobulin class than did control fluids. With some exceptions (e.g., M. W., Table II) the intensity of labeling on the autoradiographs correlated roughly with the numbers of fluorescent cells in companion cultures. In four cultures from normal subjects the relative distribution of newly synthesized immunoglobulins in culture supernate and cell sap was compared (Table IV). The results indicated that the bulk of immunoglobulin synthesized during an 18 h period was secreted into the surrounding media. In summary, the data on cultured lymphocytes from normal individuals indicates that PWM induces a marked increase in the relative numbers of cells containing cytoplasmic IgM, IgG, and IgA, which is associated with an increased rate of synthesis and secretion of immunoglobulins into the extracellular fluid.

Induction of immunoglobulin synthesis in cultures from immunodeficient patients

X-linked agammaglobulinemia. We previously reported failure to find immunoglobulin-producing cells in PWM-stimulated cultures from three boys with X-linked agammaglobulinemia (38). This observation was confirmed in one of the patients by the failure of his cultured cells to incorporate [^{14}C]amino acids into immunoglobulin. DNA synthesis induced by PWM stimulation was normal (Table I).

Agammaglobulinemia with B lymphocytes. Data on these patients are presented in Tables I and IV. Three patients, K. L., N. M., and N. G. had normal numbers of B lymphocytes bearing each immunoglobulin class, while C. F. had a selective deficiency in γ-bearing B lymphocytes. Cultured cells from N. M., a 26-yr-old man, were normally stimulated by PWM to mature into cells containing abundant cytoplasmic IgM, IgG, and IgA. In previous lymphocyte cultures from K. L., a 16-yr-old female, we were unable to detect a significant increase in the proportion of immunoglobulin-containing cells (maximum, 4/1,000 IgM-positive cells in four separate attempts) although DNA synthesis was normal, and simultaneous cultures of lymphocytes from normal individuals developed IgM-, IgG-, and IgA-containing cells in the usual frequencies (38). The earlier results were obtained using cultures established

TABLE II

*Correlation of In Vitro Immunoglobulin Biosynthesis with Development of Immunoglobulin-Containing Cells in PWM-Stimulated Lymphocytes from Normal Individuals**

Donors	PWM	Cells containing cytoplasmic immunoglobulins‡ IgM	IgG	IgA	Immunoglobulin biosynthesis§ IgM	IgG	IgA	DNA synthesis (stimulation index‖)
M. Mc.	−	1	3	1	−	+	−	
	+	4	10	12	+ + +	+ + +	+ + +	60
S. L.	−	3	1	1	−	+	+	
	+	21	46	69	+ + +	+ + +	+ + +	61
M. W.	−	2	1	1	+ +	+ +	+ +	
	+	5	7	11	+ + + +	+ + + +	+ + + +	27
S. W.	−	<1	<1	<1	+ + +	+ +	+ +	
	+	144	88	101	+ + + +	+ + + +	+ + + +	15
M. C.	−	1	<1	<1	+	+	+ +	
	+	42	22	38	+ + + +	+ + + +	+ + + +	33
R. K.	−	<1	<1	1	+ +	+	+	
	+	16	10	20	+ + + +	+ + + +	+ + + +	21
P. J.	−	1	1	1	+	+ +	+ +	
	+	22	16	20	+ + + +	+ + + +	+ + + +	9
D. Ma.	−				+	+	−	
	+		Not done		+ + +	+ +	+ +	42

* All of the studies were done on cells cultured for 7 days; but the culture conditions for the studies of cytoplasmic immunoglobulins were slightly different from those cultures examined for immunoglobulin biosynthesis (see Methods). In a few instances the studies were performed on different samples of blood.

‡ Number of cells showing positive fluorescence per 1,000 cells counted. Includes some data for comparison previously published (38).

§ Intensity of radiolabeling of specific precipitin arcs on autoradiographs of immunoelectrophoretic patterns was graded −, +, + +, + + +, and + + + +. Total immunoglobulin in cells + supernate was measured.

‖ [^{14}C]Thymidine CPM stimulated culture/CPM unstimulated culture.

in round-bottom tubes. However, an entirely normal response to PWM was found on two occasions several months later when the cells were cultured by the microplate technique (Table I). Biosynthesis and secretion of immunoglobulins were also demonstrated in stimulated cultures from this agammaglobulinemic girl (Table IV). This change in the response of her B lymphocytes could be due either to an improvement in culture technique, or to a change in her clinical status. In regard to the latter there has been no evidence of increased numbers of B lymphocytes or an increase in immunoglobulin synthesis in vivo. The previously observed defect in triggering immunoglobulin synthesis was clearly not due to a serum inhibitor. In fact, a pool of K. L. serum obtained at a time when her cells did not respond has been used to supplement all other cultures. Patient N. G. has also been described previously (12, 38). She is the only one of this group in whom abnormalities in T-lymphocyte function have been demonstrated. Induction of cells containing cytoplasmic immunoglobulins by PWM did not occur in round-bottom tube cultures on three occasions. It has not been possible to restudy her responses using the microplate technique.

Patient G. F. differed from the other three panhypogammaglobulinemic individuals in that she had normal numbers of IgM-bearing (7.0%) and IgA-bear-

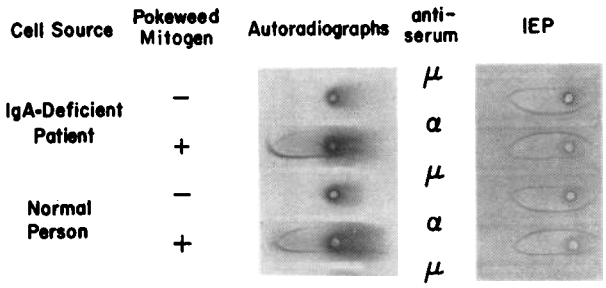

FIGURE 2 Autoradiographs of immunoelectrophoretic patterns demonstrating immunoglobulin synthesis in 7-day cultures of circulating lymphocytes from a patient with IgA deficiency (M. T.) and a normal person (M. M.). Faint labeling of IgM and IgA lines is present in the unstimulated culture fluids from the normal individual, but not the IgA-deficient subject. PWM treatment induced a major increase in IgM and IgA synthesis in both individuals. Similar patterns were seen for IgG (not shown).

TABLE III

Responses of Circulating Lymphocytes from IgA-Deficient Patients to PWM

Patients	In vivo assessment		In vitro lymphocyte responses			
	Circulating IgA	Lymphocytes bearing surface IgA*	PWM	Cells containing cytoplasmic IgA‡	Biosynthesis of IgA in culture§	DNA synthesis (stimulation index‖)
	mg/100 ml	%				
J. H.	0	1.5	−	2	−	
			+	79	+ +	3¶
M. T.	0	6.7	−	<1	−	
			+	47	+ + + +	16
D. M.	0	5.2	−	1	−	
			+	24	+ +	21
R. H.	0	3.2	−	1		
			+	18	Not done	Not done
Da. W.	11	5.1	−	1	−	
			+	15	+ + + +	21
Do. W.	0	7.3	−	<1	−	
			+	7	+ + +	4¶
D. Mc.	0	5.7	−	1	+	
			+	6	−	55
C. N.	0	13.1	−	1	−	
			+	4	+ +	10
R. T.	0	3.0	−	<1		
			+	17	Not done	52
S. M.	7	5.5	−	<1		
			+	33	Not done	66

* Our mean value for normals is 5.5%±4.0 SD.

‡ Fluorescent-positive cells per 1,000 counted. Includes some data for comparison previously published (38).

§ Intensity of radiolabeling of specific precipitin arcs on autoradiographs of immunoelectrophoretic patterns was graded −, +, + +, + + +, and + + + +. Total immunoglobulin in cell + supernate was measured.

‖ [^{14}C]Thymidine CPM stimulated culture/CPM unstimulated culture.

¶ Unusually high background resulted in low stimulation indicies, the counts in the stimulated cultures were comparable with those of other experiments.

ing (1.3%) B lymphocytes, but had only 0.5% of IgG-positive cells. Like the others, serum IgM and IgA were undetectable, and her IgG level was consistent with bimonthly gammaglobulin injections (96 mg/100 ml). Stimulated cultures of her lymphocytes contained IgM- and IgA-positive cells in numbers similar to normals, but IgG-positive cells were rare (Table I).

Dysgammaglobulinemia with B lymphocytes. In a boy with normal circulating IgM, trace amounts of IgG (he had received a gammaglobulin injection several mo earlier), and no detectable IgA, normal percentages of B lymphocytes bearing each immunoglobulin class were found (IgM 10.8%, IgG 8.3%, and IgA 2.7%). In three separate experiments PWM stimulated a normal increase in IgM-containing cells, but did not induce development of IgG- and IgA-containing cells. A virtually identical result was obtained when lymphoid cells from antigen-stimulated lymph nodes were cultured in the presence of PWM and assayed by immunofluorescence and biosynthesis (Tables I and IV). Thus, his lymphocyte response to PWM paralleled the pattern of circulating immunoglobulins rather than the distribution of circulating B lymphocytes.

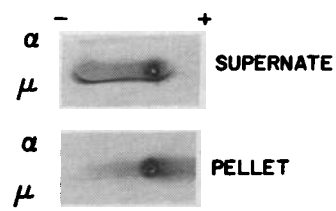

FIGURE 3 Autoradiographs demonstrating newly synthesized IgA and IgM in supernatant fraction and cellular (pellet) fraction of pokeweed-stimulated culture of peripheral blood lymphocytes from a patient with isolated IgA deficiency (D. M.). Note that the bulk of IgA and IgM is in the supernatant fraction indicating active secretion of these immunoglobulins. A similar pattern was seen for IgG (not shown).

TABLE IV

Immunoglobulin Synthesis and Secretion of Pokeweed-Stimulated Cultures from Normals and Patients with Various Immunoglobulin Deficiencies

Donors	Fraction*	IgM	IgG	IgA
Normals				
S. W.	Cell pellet	+‡	+ +	+
	Supernate	+ + + +	+ + + +	+ + + +
D. C.	Cell pellet	+ +	+ +	+ +
	Supernate	+ + + +	+ + + +	+ + + +
M. W.	Cell pellet	+ +	−	+ +
	Supernate	+ + +	+ +	+ + +
P. J.	Cell pellet	+ +	+ +	+
	Supernate	+ + + +	+ + + +	+ + + +
Agammaglobulinemia with B lymphocytes				
K. L.	Cell pellet	+ +	+	−
	Supernate	+ + + +	+ + +	+ +
Dysgammaglobulinemia with B lymphocytes				
P. B.§	Cell pellet	−	−	−
	Supernate	+ +	−	−
IgA-deficient patients				
M. T.	Cell pellet	+ +	+ +	+ +
	Supernate	+ + + +	+ + + +	+ + + +
Do. W.	Cell pellet	+ + +	+ +	+ +
	Supernate	+ + + +	+ + +	+ + +
Da. W.‖	Cell pellet	+ + +	+	+ +
	Supernate	+ + + +	+ + +	+ + + +
D. M.	Cell pellet	+ +	+ + +	−
	Supernate	+ + + +	+ + + +	+ +

* On termination of the experiments, the cell cultures were centrifuged and ^{14}C-labeled immunoglobulins were separately determined in the cell pellet and supernatant fraction (see Methods).

‡ Intensity of radiolabeling of specific precipitin arcs on autoradiographs of immunoelectrophoretic patterns was graded −, +, + +, + + +, and + + + +.

§ Lymphoid cells were obtained from an antigen-stimulated lymph node.

‖ At the time of this determination, this boy, brother of Do. W., was no longer deficient in circulating IgA (55 mg/100 ml).

IgA deficiency. In PWM-stimulated lymphocytes from each of 10 patients studied, the numbers of cells containing cytoplasmic IgA, as well as IgM and IgG were within the range found for normal individuals (Table III). Biosynthesis of IgA was demonstrated by immunoelectrophoresis and autoradiography in six of seven patients thus studied (Fig. 2, Table III). In cultured lymphocytes from four individuals, immunoglobulins within the cells and in the culture supernate were assayed separately. Most of the newly synthesized immunoglobulin was found in the supernate (Fig. 3, Table IV). In one experiment, lymphocytes from patient J. H. were cultured simultaneously in media supplemented with either autologous or pooled agammaglobulinemic plasma. PWM-stimulated cultures had an equivalent increase in DNA synthesis and a similar frequency of cells containing each class of immunoglobulin.

DISCUSSION

In these studies we have compared the differentiation capabilities of peripheral blood lymphocytes from normal individuals with those from patients exhibiting a variety of defects in immunoglobulin synthesis. PWM was used to induce differentiation of B lymphocytes over a 7-day culture interval. Our interpretation of these results is based upon integration of information regarding: (*a*) in vivo development and distribution of B lymphocytes and plasma cells in normals and immunoglobulin-deficient patients; (*b*) differences between B lymphocytes and plasma cells in their rates of synthesis, intracellular compartmentalization, and rates of release of immunoglobulins; and (*c*) characteristics of the B-lymphocyte differentiation induced by PWM under defined conditions of culture.

B lymphocytes outnumber their mature plasma cell progeny in normal peripheral blood by more than 100 to 1. These lymphocytes lack the fine structural characteristics of secretory cells and, by comparison with plasma cells, the amount of immunoglobulin synthesized by B lymphocytes is small. A portion of the immunoglobulin produced is incorporated into the plasma membrane, from which it is released with a half-life of 6 h or less (41–43). The distribution of immunoglobulin in B lymphocytes is such that it can be detected by immunofluorescent techniques only on the cell membrane (6).

Under normal circumstances, antigen-induced differentiation of B lymphocytes is accompanied by well-defined morphologic alterations, including formation of a rich network of polyribosomes along membrane-lined channels which connect to the Golgi apparatus (44). Plasma cells contain relatively large amounts of intracellular immunoglobulin easily detectable by immunofluorescent staining of fixed cells (45, 46); when stained in the viable state, so that fluorochrome-labeled anti-immunoglobulin is excluded from cell entry, plasma cells usually lack detectable surface fluorescence (6). The immunoglobulin synthesized by plasma cells is secreted very rapidy into the surrounding media (47–49).

The morphologic and functional alterations induced in B lymphocytes by PWM stimulation observed in these and other studies (29–34, 37, 38) are similar to those of antigen-induced plasma cell differentiation. Although PWM apparently does not "trigger" B lymphocytes via an interaction with surface antibodies, its use offers two significant advantages over antigen stimulation. The nonspecific stimulation of differentiation by PWM involves multiple clones rather than the few which might be triggered by antigen (37). A second

major advantage is the impressive induction of IgA-secreting cells (38, Table IV), which has been difficult to achieve in vitro by other means.

Since B lymphocytes are usually absent or quite rare in patients with X-linked agammaglobulinemia (2, 12–21), our failure to detect immunoglobulin-producing cells in unstimulated or PWM-treated cultures from this group is expected. These experiments thus serve as a negative control for the culture system. The stimulation of normal DNA synthesis in cultured lymphocytes from these agammaglobulinemic boys confirms results in mice indicating that PWM is mitogenic for T as well as B cells (36).

Previous observations on agammaglobulinemic patients having normal or nearly normal numbers of B lymphocytes have indicated that their B lymphocytes can react with antigens in vivo and proliferate in germinal center distribution (12, 50). It is in this group of patients that nodular lymphoid hyperplasia of the lymph nodes, spleen, and intestine often occurs. From these observations it would appear that the initial recognition of antigens by B lymphocytes, and the proliferation which follows, is not impaired. In the present study lymphocytes from such patients were induced by PWM not only to proliferate, but also to differentiate into mature immunoglobulin-secreting cells. These results indicate that the mechanisms which control the transformation of B lymphocytes into cells capable of large-scale assembly and secretion of immunoglobulins are potentially operative. Thus, the defect in vivo would appear to involve failure to generate the signal(s) required for terminal differentiation which normally can accompany the proliferative phase of B lymphocyte activation.

Another agammaglobulinemic woman had normal numbers of lymphocytes bearing surface IgM and IgA, but few bearing IgG. PWM-stimulated cultures from this patient contained numbers of IgM- and IgA-producing cells similar to controls, but IgG-positive cells were rare. The correlation between the distribution of B lymphocytes which stained for each immunoglobulin class in the starting preparation and the proportions of IgM-, IgG-, and IgA-containing cells found on termination of the culture suggests that the class of immunoglobulin on the surface of a B lymphocyte for the most part reflects the genetic commitment of that cell as regards the class to be synthesized by its progeny.

A different correlation was observed in cultures from the boy with a normal level of circulating IgM but deficiency of IgG and IgA. Although normal proportions of B lymphocytes staining for each class were present, only the IgM precursors were induced to undergo terminal differentiation in culture. It is interesting to compare the arrest of B-cell development in this boy with aspects of normal ontogeny of the B-cell line. By about 14 wk from conception, normal adult proportions of lymphocytes bearing IgM, IgG, or IgA are present in human fetal spleen and blood (51). If the presence of these precursor cells were the only requirement for induction of synthesis of the respective immunoglobulin classes, it would be expected that circulating levels of each class would rise nearly simultaneously following birth. This clearly does not occur, as IgM reaches adult levels in serum by about 1 yr of age, while IgA levels do not peak until near puberty. A similar lack of correspondence between serum levels and precursor B lymphocytes has recently been found for IgD (52). We have begun to examine the control of the ontogenetic sequence of immunoglobulin synthesis by determining the capacity of fetal and neonatal B lymphocytes to differentiate in response to PWM. Preliminary results indicate that only the IgM response is quantitatively normal. Thus the differentiative capacity of B lymphocytes from our patient, both in vivo and in vitro, seems roughly comparable with that which occurs earlier in the normal fetus.

Experimental observations in rodents concerning the role of T cells in helping to promote terminal B-cell differentiation may be pertinent to consideration of the human immunoglobulin deficiencies under discussion. In thymectomized mice, thymus-dependent antigens may stimulate an increase in numbers of B lymphocytes capable of binding the antigen, but fail to induce production of circulating antibody (53, 54). The similarity between this experimental immunologic lesion and that observed in some patients having agammaglobulinemia with B lymphocytes is apparent; both serve to emphasize the dissociability of antigen-induced B-lymphocyte proliferation and the terminal differentiation processes. The parallel between selective IgA deficiency in man and in the model of IgA deficiency observed in thymus-deprived rodents is also worthy of note. In congenitally athymic "nude" mice deficient in both serum and secretory IgA, the numbers of B lymphocytes carrying membrane-bound IgA are at least normal in number (55, 56). Furthermore, the immunoglobulin deficiencies in such mice are correctable by thymus grafting (57). Such speculations on the role of T cells in promoting plasma cell differentiation must be tempered with the knowledge that humans who are apparently athymic may be capable of synthesizing all three major classes of immunoglobulin (58). Nevertheless, the defects of immunoglobulin synthesis in T-cell-deficient mice and men (reviewed in reference 22) plus our experimental observations on the differentiation capabilities of B lymphocytes from immunoglobulin-deficient patients indicate the need to search for defects extrinsic to the B lymphocyte in such patients.

Stites, Levin, Austin, and Fudenberg have established long-term cultures of lymphoblastoid cell lines from immunodeficient patients and demonstrated their capacity for immunoglobulin synthesis (59). Differences in approach, particularly the possibility of selection of minor subpopulations in long-term culture, preclude direct comparison with our results. Nevertheless, data obtained from a variety of in vitro methods should eventually provide complementary information on the nature of the cellular defects in immunoglobulin deficiency diseases.

In conclusion, PWM has proven to be one effective method of analyzing the differentiative capability of B lymphocytes. The information gained from its use in the study of B-cell defects provides a glimpse into another order of complexity of the immunoglobulin deficiency diseases. Continued study of the defective B lymphocytes in such patients may offer greater understanding of the mechanisms of normal differentiation of antibody-producing cells. The observation that some defects of B-cell differentiation may be surmounted in vitro offers the hope of identifying specific metabolic abnormalities which are amendable to in vivo repair.

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