

IN VITRO REGULATION OF IgA SUBCLASS SYNTHESIS

I. Discordance between Plasma Cell Production and Antibody Secretion

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In all normal individuals, the IgA in the serum and secretions can be divided into two subclasses, IgA₁ and IgA₂, on the basis of antigenic differences. IgA₁ constitutes 80–90% of the IgA in serum (1–3), whereas approximately equal amounts of IgA₁ and IgA₂ occur in the secretions (2).

Our previous studies have shown that although the IgA B cell distribution in the peripheral circulation reflects serum ratios of IgA₁ to IgA₂, that is, 80% of the cells express surface IgA₁ and 20% bear IgA₂ (4), the differentiation potential of peripheral blood lymphocytes in response to polyclonal B cell activators is quite different and more closely resembles the distribution of IgA₁ and IgA₂ in secretions. In cultures stimulated with pokeweed mitogen (PWM) for 7 d, 50% of the plasma cells that are positive for cytoplasmic IgA are positive for IgA₁ and 50% are positive for IgA₂ (4). This suggests that the cells in the circulation that differentiate into IgA plasma cells in response to PWM may be cells migrating to the mucosal surfaces. The observation that most of the IgA released into the supernatants of these cultures is dimeric, like IgA in secretions, rather than monomeric, like serum IgA (5), supports this hypothesis.

To further investigate regulation of IgA subclasses in PWM-stimulated cultures, IgA₁ and IgA₂ were measured in culture supernatants by a radioimmunoassay we have recently devised using monoclonal anti-IgA subclass antibodies (3). The results demonstrate that although there are equal numbers of IgA₁ and IgA₂ plasma cells in PWM-stimulated cultures, >90% of the IgA released into the culture supernatant is IgA₁. This discrepancy cannot be accounted for by failure of the assay to detect in vitro synthesized IgA₂, selective loss or destruction of IgA₂, delayed release of IgA₂, or failure of IgA₂ plasma cells to produce J chain.

Materials and Methods

PWM Cultures. Peripheral blood lymphocytes from healthy adult laboratory personnel were separated from heparinized venous blood by Ficoll-Hypaque centrifugation. The cells were suspended in RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) with 20% fetal calf serum (FCS) at $5\text{--}10 \times 10^6$ cells/ml and incubated in plastic petri dishes at 37° for 45 min to partially deplete monocytes. Nonadherent lymphocytes were resuspended in RPMI 1640 with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.05 mM 2 mercaptoethanol, and 20% FCS at a concentration of 10^6 cells/ml. Aliquots

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of 0.2 ml were distributed into the wells of flat-bottomed microtiter plates (Costar, Data Packaging, Cambridge, MA). 5 μ l of PWM (Gibco Laboratories) were added to each well. The cells were cultured at 37°C in humidified air with 5% CO₂ for various periods. The cells were harvested gently by pasteur pipette, counted, and then spun down. Supernatants were then removed and the cell pellet was either resuspended and spun onto glass slides by cytocentrifugation for cytoplasmic staining or lysed by sonication for quantitation of intracellular IgA.

Cells used for cytoplasmic staining were fixed for 20 min in 95% ethanol, 5% glacial acetic acid, then washed three times in phosphate-buffered saline (PBS). The hybridoma supernatant, anti-IgA₁ or anti-IgA₂, was layered over the cells, and the slides were incubated for 20 min at room temperature followed by two washes in PBS. The cells were then stained with fluorochrome-tagged goat anti-mouse immunoglobulin, washed twice, then stained with heterologous anti-human IgA or anti-J chain that had been labeled with a contrasting fluorochrome. The cells were washed again and then mounted under a coverslip and examined with a Zeiss fluorescence microscope equipped with epi-illumination and appropriate barrier filters (Carl Zeiss, Inc., New York). To determine the proportions of IgA plasma cells positive for each subclass, slides were first scanned under filters selective for fluorescein fluorescence. Cells positively stained for fluorescein-tagged anti-IgA were then examined under filters selective for rhodamine to detect positive staining for IgA₁ or IgA₂. At least 100 IgA-positive cells were counted on every slide.

For quantitation of intracellular IgA subclasses, the cells were washed three times, then resuspended at 10⁷ cells/ml in PBS containing 1% FCS, and disrupted by sonication at 0°C for 1 min at 20,000 cycles/s (Sonifer Cell Disruptor, Health Systems Ultrasonics, Inc., Plainview, NY). The sonicate was clarified by centrifugation for 5 min at 12,800 *g*. Initial experiments were conducted in which known quantities of IgA were added to cells before sonication. Subsequent radioimmunoassay indicated that the procedure did not damage either subclass of IgA.

Antibodies. The production and characterization of the monoclonal anti-IgA₁ and anti-IgA₂ antibodies (1-155-1 and 14-3-26, respectively) have been previously described (4). The heterologous anti-human IgA was produced by immunizing a goat with a highly purified IgA₁K myeloma protein. Antibodies to the kappa light chain and contaminating immunoglobulins were removed by sequential absorption over a series of Sepharose 4B columns to which Cohn Fraction II, IgGK, or IgMK myeloma proteins had been covalently bound. The anti-IgA antibody was eluted from a column to which a purified IgA₂ protein had been fixed to insure that the anti-IgA had no subclass specificity.

The fluorochrome-tagged goat anti-mouse antibodies were a gift from Dr. M. D. Cooper and Dr. H. Kubagawa, University of Alabama, Birmingham, AL. These reagents did not cross react with human immunoglobulins. The rhodamine-labeled anti-J chain was a gift from Dr. J. Mestecky, University of Alabama.

Radioimmunoassay. The radioiodination of the heterologous goat anti-human IgA and radioimmunoassay were done as previously described (3). The wells of microtiter plates were coated with a 1:500 dilution of hybridoma anti IgA₁ or anti IgA₂ ascites. After remaining protein binding sites were blocked with bovine serum albumin (Sigma Chemical Co., St. Louis, MO), the culture supernatants or cell lysates were added in duplicate to wells at dilutions of 1:2, 1:8, 1:32, and 1:128. After overnight incubation, the wells were washed and 10 ng of ¹²⁵I-labeled heterologous goat anti-IgA was added. After 4 h the wells were washed, then counted in a Beckman Gamma 4000 (Beckman Instruments, Fullerton, CA). A serum standard with known concentrations of IgA₁ and IgA₂ was run in all assays to generate a standard curve. The concentrations of IgA₁ and IgA₂ in the supernatants or cell pellet lysates were determined from this standard curve.

Results

Peripheral blood lymphocytes from 27 normal adults were stimulated with PWM for 7 d. The cultures were then harvested and the plasma cells were stained by immunofluorescent techniques for cytoplasmic IgA and the IgA subclasses IgA₁ and IgA₂. Only mature, brightly stained plasma cells with eccentric nuclei were counted.

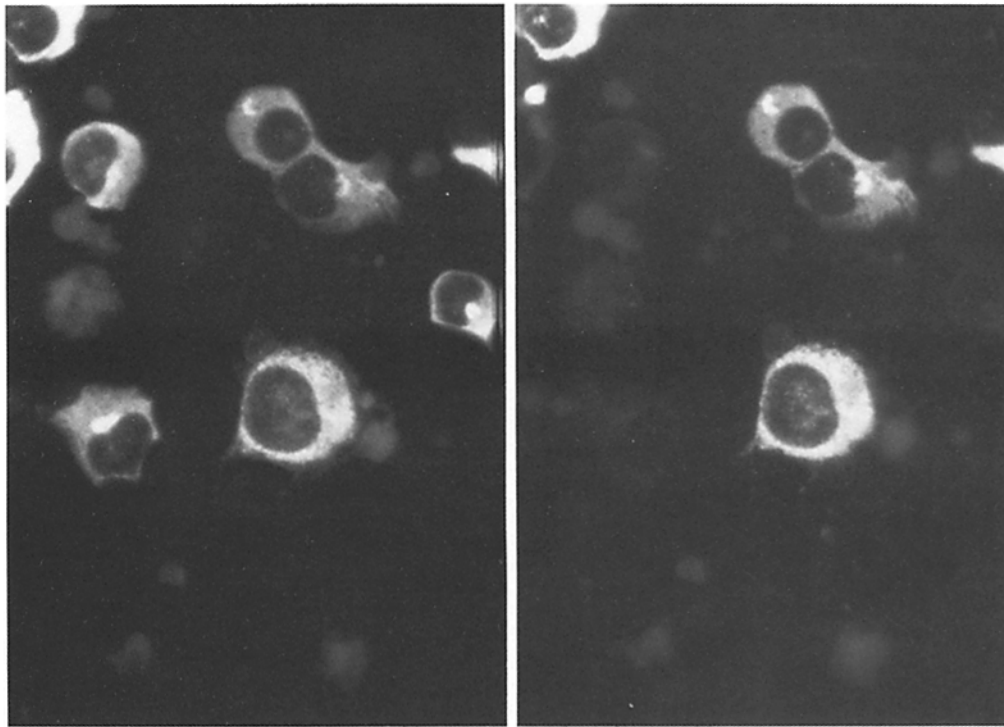


Fig. 1. Immunofluorescent staining of plasma cells from PWM-stimulated peripheral blood lymphocytes. (Left) Cells stained with heterologous fluorescein-tagged goat anti-human IgA; (right) the same field examined with filters selective for rhodamine fluorescence demonstrates doubly stained cells positive for IgA₂.

Cells stained for IgA₂ were always as intensely stained with the heterologous anti IgA as those stained for IgA₁ (Fig. 1). Of the plasma cells stained for IgA, $50.0 \pm 2.44\%$ were also stained for IgA₁, whereas $50.9 \pm 2.59\%$ stained for IgA₂.

To investigate the relationship between IgA plasma cells and IgA released into supernatants, IgA₁ and IgA₂ were measured in the supernatants of cultures with varying proportions of IgA₁ and IgA₂ plasma cells. As noted in Table I and Fig. 2, in all cultures $<10\%$ of the secreted IgA was IgA₂.

Previous experiments demonstrated a predominance of IgA₁ plasma cells early in the PWM response, at days 3 and 4; however, by day 7 of culture, the proportion of IgA₂ plasma cells was equal to that of IgA₁ (5). This suggested that the maturation of IgA₂ plasma cells might be delayed compared with that of IgA₁, and that if the culture period were extended, higher concentrations of IgA₂ might be found in culture supernatants. Therefore, culture supernatants were harvested from cells cultured for 5, 7, 9, or 12 d. To verify that the radioimmunoassay used to measure IgA₂ would detect IgA₂ synthesized *in vitro*, lysates of the cell pellets from these cultures were also assayed for IgA₁ and IgA₂. A representative experiment is shown in Table II. In each of four experiments, the amounts of IgA₁ and IgA₂ in the cell pellets were approximately equal, as would have been predicted from the results of cytoplasmic staining. However, the amount of IgA₂ in the culture supernatant was always $<10\%$ of the total IgA, and this proportion did not increase with time. In all experiments there was

TABLE I

Experiment	Cytoplasmic staining		IgA in supernatants*	
	c IgA/total cells	c IgA ₂ /c IgA	IgA ₁	IgA ₂
	%			
1	7	55	1,836	59
2	3	29	846	74
3	0.4	43	188	18

* Expressed as ng/10⁶ cells cultured.

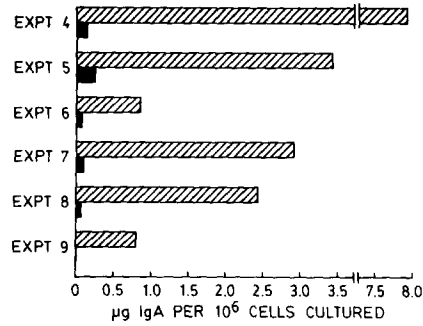


FIG. 2. Culture supernatants from PWM-stimulated lymphocytes from six different individuals were assayed for IgA₁ (▨) and IgA₂ (■).

TABLE II

	IgA ₁ *	IgA ₂ *
Day 5		
Pellet	53	480
Supernatant	469	8
Day 7		
Pellet	335	387
Supernatant	2,680	31
Day 9		
Pellet	603	1,240
Supernatant	3,108	93
Day 12		
Pellet	603	387
Supernatant	3,108	62

PWM-stimulated cultures of peripheral blood lymphocytes were harvested after various periods. IgA₁ and IgA₂ were measured in the culture supernatant and in lysates of the washed cell pellets.

* Expressed as ng/10⁶ cells cultured.

more IgA₂ in the cell pellet than in the culture supernatants, in some cases as much as 90 times more (mean ratio of IgA₂ in pellet to IgA₂ in supernatant, 30.2:1). In contrast, there was always more IgA₁ in the culture supernatants than in the cell pellet, sometimes as much as 30 times more (mean ratio of IgA₁ in supernatant to IgA₁ in pellet 12.5:1).

To determine if IgA₂ in culture supernatants was selectively lost, purified IgA₂ myeloma proteins were added at the beginning of the culture period. When 4,000 ng/ml of an IgA₂λ (allotype Am2⁻) or an IgA₂K (allotype Am2⁺) were added at day 0, 3,320 ng and 2,680 ng, respectively, were retrieved in the supernatant at day 7.

Because production of J chain may be required for secretion of polymeric immunoglobulins, plasma cells from 7-d cultures were stained by double contrast immunofluorescent techniques with rhodamine-tagged anti-J chain and fluorescein-tagged

anti IgA₁, anti IgA₂, or anti-total IgA. J chain staining was detected in 93% of the plasma cells stained for IgA₁, 91% of the cells positive for IgA₂, and 91% of the cells stained with heterologous anti IgA.

Discussion

The results demonstrate a surprising discordance between two measures of *in vitro* B cell maturation. Although 50% of the IgA plasma cells in a 7-d PWM-stimulated culture express IgA₂, as demonstrated by cytoplasmic immunofluorescent staining, <10% of the IgA released into the culture supernatants is IgA₂. This discrepancy cannot be explained by selective loss or destruction of IgA₂ in the culture supernatants; if known amounts of purified IgA₂ myeloma proteins of either AM2⁺ or Am2⁻ allotype (6) were added at the beginning of the culture period, then 67–83% of the added protein could be retrieved from the supernatant at the end of culture.

The observation that there were approximately equal amounts of IgA₁ and IgA₂ in lysates of the cell pellets demonstrates that the radioimmunoassay used in these experiments is able to detect *in vitro* synthesized IgA₂. It also verifies the results of the immunofluorescent staining, which indicated that there were equal numbers of IgA₁ and IgA₂ plasma cells. The IgA₂ plasma cells were not morphologically different from the IgA₁ plasma cells. Cell size, nucleus/cytoplasm ratio, and intensity of staining were equal in the two subpopulations.

In past experiments it was noted that early in the PWM-stimulated culture there was a predominance of IgA₁ plasma cells, but that by 7 d of culture there were equal numbers of plasma cells expressing IgA₁ and IgA₂ (5). This suggested a relative delay in the maturation of IgA₂ plasma cells. However, even when culture periods were extended to 12 d, <10% of the IgA in the culture supernatants was IgA₂, and this proportion did not increase over time. Furthermore, there was always more IgA₂ in the cell pellet than in the culture supernatant, whereas there was always more IgA₁ in the supernatant than in the cell pellet. In several experiments <1% of the IgA in the culture supernatant was IgA₂. It is possible that in the cultures in which 5–10% of the IgA in the supernatant was IgA₂ that the IgA₂ was not released in the course of normal metabolic processes, but instead was the result of spontaneous cell death and lysis.

Other investigators have postulated that J chain may have a regulatory role in antibody secretion (7). Therefore, IgA plasma cells were counter-stained with anti-human-J chain to determine if the failure to secrete IgA₂ might be associated with a failure to produce J chain. As demonstrated by Mestecky et al. (8), almost all IgA plasma cells could be stained for J chain, including 93% of the IgA₁ plasma cells and 91% of the IgA₂ plasma cells.

It is not clear why IgA₂ is not secreted into culture media. It is possible that some terminal biochemical modification such as glycosylation (9) is incomplete. Ciccimarra et al. (10) demonstrated that some patients with common variable agammaglobulinemia have B cells that differentiate into IgG-containing plasma cells but not IgG-secreting cells. The failure to secrete immunoglobulin was associated with a defect in the incorporation of [³H]mannose and [³H]glucosamine into newly synthesized IgG. But, if terminal modifications are unfinished, the critical question becomes, why is the differentiation of IgA₁ plasma cells complete and that of IgA₂ plasma cells incomplete? It is possible that the two subpopulations of plasma cells are derived

from B cells that are at different stages of maturation. Mature IgA B cells in the peripheral circulation express predominantly IgA₁, whereas the less mature cells that express surface IgM as well as IgA are equally divided between those bearing IgA₁ and IgA₂ (4). It is possible that both the mature and immature B cells can differentiate into plasma cells in response to PWM, but only the more mature cells differentiate into immunoglobulin-secreting cells. This is unlikely to be the complete explanation because between 6 and 37% of the mature IgA B cells in the peripheral circulation express IgA₂, whereas only 0.2–9% of the IgA in culture supernatant is IgA₂. In addition, other investigators (11) have demonstrated that relatively immature B cells that express surface IgM can give rise to IgG-secreting cells in a PWM-driven system.

Other investigators (12, 13) have suggested that the different assays used to measure B cell differentiation might not be measuring equivalent phenomena. The reverse hemolytic plaque assay (12, 13), quantitation of immunoglobulin released into culture supernatant (13–15), and enumeration of cells positive for cytoplasmic immunoglobulin (12, 15, 16) are all frequently cited measurements of B cell function, but the relationship between them is not well established. The number of plaque-forming cells derived from 10^6 PWM-stimulated cells is usually reported as between 6 and 60×10^3 (12, 13), whereas the number of plasma cells positive for cytoplasmic immunoglobulin derived from the same number of cells is usually between 5 and 50×10^4 (12, 15, 16). This suggests that not all cells that have the morphologic and histochemical appearance of plasma cells are actually secreting immunoglobulin. Further, Munoz et al. (13) demonstrated a lack of correlation between the quantity of immunoglobulin secreted into culture supernatants and the number of plaque-forming cells from the same experiment. This suggests that not all immunoglobulin-secreting cells secrete equal amounts of immunoglobulin.

The results presented in this paper indicate that the precursors of IgA₂-producing cells can be successfully stimulated by PWM to differentiate into IgA₂ plasma cells but only inefficiently into IgA₂-secreting cells. This suggests that additional signals may be required for terminal differentiation of these cells; signals that may be provided at mucosal surfaces. The results also indicate that a cell with the phenotypic appearance of a plasma cell may not be at the final stage of differentiation.

Summary

To investigate the in vitro regulation of IgA subclass synthesis, peripheral blood lymphocytes from healthy adults were cultured with the polyclonal B cell activator, pokeweed mitogen. Although 50% of the IgA plasma cells from a 7-d culture were positive for cytoplasmic IgA₁ and 50% were positive for IgA₂, <10% of the IgA released into the culture supernatant was IgA₂. This discrepancy could not be explained by failure of the assay to detect in vitro synthesized IgA₂, selective loss or destruction of IgA₂ in culture media, delayed release of IgA₂, or failure of IgA₂ plasma cells to produce J chain. The results suggest that additional signals may be required for the differentiation of plasma cells into immunoglobulin-secreting cells.

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