

## HUMAN B CELL DIFFERENTIATION

### I. Analysis of Immunoglobulin Heavy Chain Switching Using Monoclonal Anti-Immunoglobulin M, G, and A Antibodies and Pokeweed Mitogen-induced Plasma Cell Differentiation\*

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Some of the members of a B cell clone switch from expression of IgM to the production of immunoglobulin molecules with other heavy chain isotypes (1-3) during development. The first model for this developmental process suggested an orderly sequence of switches from IgM to IgG to IgA (4), and this was envisioned to occur by sequential looping and excision of heavy chain constant (C)<sup>1</sup> region genes in tandem (5). These genes have since been identified on chromosome 12 in mice to the 3' side of the variable (V) region gene complex in the order:  $\mu$ ,  $\delta$ ,  $\gamma_3$ ,  $\gamma_1$ ,  $\gamma_{2b}$ ,  $\gamma_{2a}$ ,  $\epsilon$ , and  $\alpha$  (6-10). Each is separated by an intervening sequence of untranslated DNA. Except for the  $\delta$  gene, which is relatively close and transcribed together with the  $\mu$  gene (8), expression of non- $\mu$  genes involves rearrangement so that the C gene to be transcribed is brought next in line to the V genes (11-14). Two possible mechanisms have been proposed for this. One involves joining of a switch nucleotide sequence 5' to the C $\mu$  gene with a complementary switch sequence in front of C $\gamma_3$  or another C gene, and excision of the DNA between the two switch sites (11-15). In the other model, this approximation is postulated to occur by sister chromatid exchange (16). The latter was prompted by molecular and cellular evidence for "backward" switches, e.g., from  $\gamma_{2b}$  to  $\gamma_1$  (13, 16, 17). Studies of the differentiation potential of normal mouse B cells have rarely provided results consistent with "backward" switches; the outcome of most studies is consistent with the idea of switch pathways from IgM to other downstream heavy chain isotypes (18-25). Evidence for the occurrence of multiple isotype switches in the order of the C gene alignment in mice has been reported (24-26), and other results are more consistent with the idea of direct switches from  $\mu$  to each of the other isotypes (21, 27). Conflicting data have also been reported about the switch pathways followed by human B cells. Most of the precursors of IgM plasma cells appeared to express IgG on their surface in some studies (28, 29). Results of other studies suggest, instead, that IgG is expressed only by the B cell precursors of IgG

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<sup>1</sup> *Abbreviations used in this paper:* C, heavy chain constant region; c, cytoplasmic; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; MNC, mononuclear cells; PBS, Dulbecco's phosphate-buffered saline; PWM, pokeweed mitogen; s, surface; V, heavy chain variable region.

plasma cells, and that the common switch pathways are from IgM to IgG and from IgM to an IgA isotype (30).

In these studies we have used monoclonal antibodies specific for human  $\mu$ ,  $\gamma$ ,  $\alpha$ , and  $\alpha 1$  subclass to examine the immunoglobulin isotypes expressed by the B lymphocyte precursors of plasma cells producing IgM, IgG, IgA<sub>1</sub>, and IgA<sub>2</sub>. The stimulus used for inducing plasma cell differentiation was pokeweed mitogen. The results obtained in this model system suggest that single switches from  $\mu$  to expression of another heavy chain are the rule.

### Materials and Methods

**Cell Preparation.** Human mononuclear cells (MNC) were obtained from heparinized venous blood of healthy donors by Ficoll-Hypaque gradient centrifugation. The recovered MNC were washed three times with Hanks' balanced salt solution containing 5% fetal calf serum (FCS) and incubated at 37°C for 1 h in a plastic tissue culture dish to remove some of the adherent cells and to permit shedding of cytophilic IgG. Nonadherent cell suspensions were adjusted to a concentration of  $1 \times 10^6$  cells/ml in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS (Grand Island Biological Co., Grand Island, NY), 2 mM L-glutamine, 50  $\mu$ g/ml gentamicin, and  $5 \times 10^{-5}$  M 2-mercaptoethanol.

**Cell Culture.** Cultures were established in flat-bottomed microplates (Costar, Data Packaging, Cambridge, MA) with each well containing 0.2 ml of the cell suspension. Cells were stimulated with 5  $\mu$ l/ml of pokeweed mitogen (PWM; Grand Island Biological Co.), a dose determined in preliminary experiments to induce optimum differentiation, and incubated at 37°C for 7 d, unless otherwise mentioned, in a humid atmosphere of 5% CO<sub>2</sub> and 95% air.

**Monoclonal Hybridoma Antibodies.** The characteristics of the mouse monoclonal hybridoma antibodies against human immunoglobulin isotypes are listed in Table I. Anti- $\mu$ , - $\gamma$ , and - $\alpha$  antibodies were prepared by Doctors H. Kubagawa, M. E. Conley, and J. F. Kearney (University of Alabama in Birmingham, Birmingham, AL). The anti- $\alpha_1$  and - $\alpha_2$  antibodies have been previously described (31). The specificities of the monoclonal anti-Ig antibodies were established by enzyme-linked immunoabsorbent assays and by immunofluorescence staining of a panel of myeloma cells. The monoclonal antibodies were purified from mouse ascitic fluid by affinity column chromatography. The concentration of antibody was established from the optical absorbance at 280 nm ( $E_{280}^{1\% 1\text{cm}} = 14.0$ ).

**Depletion of Cells by the "Panning" Method.** IgM-, IgG-, or IgA-bearing cells were selectively depleted from blood MNC preparations by precoating the cells with one of the mouse monoclonal antibodies, followed by adherence to rabbit anti-mouse IgG antibody-coated dishes (32, 33). Affinity-purified rabbit anti-mouse IgG antibody at 50  $\mu$ g/ml in 10 ml of 0.05 M Tris-HCl buffer (pH 9.5) was used for coating the plastic dishes. In each experiment,  $1-1.5 \times 10^7$  blood MNC were treated with 0.2 ml of 200  $\mu$ g/ml monoclonal antibodies specific for one heavy chain isotype in Dulbecco's phosphate-buffered saline (PBS) at 4°C for 20 min. As controls, cells were incubated with the same concentration of purified mouse myeloma protein MOPC-300 ( $\gamma_1\kappa$ ) or with medium alone. After 20 min, cells were washed four times with PBS

TABLE I  
Mouse Monoclonal Hybridoma Antibodies Specific for Human  
Immunoglobulin Isotypes

Source clone	Isotype of monoclonal antibody	Human isotype specificity
$\mu$ -145-8	$\gamma_1\kappa$	anti- $\mu$
C3-124	$\gamma_2\lambda$	anti- $\gamma$
R1-29-2	$\gamma_1\kappa$	anti- $\alpha$
1-155-1	$\gamma_3\lambda$	anti- $\alpha_1$
14-3-26	$\gamma_2\kappa$	anti- $\alpha_2$

containing 5% FCS, resuspended in 3 ml of 5% FCS in PBS, and poured onto plastic dishes previously coated with anti-mouse IgG antibody. The dishes were held at 4°C for 70 min. Nonadherent cells were harvested with two gentle washes using 1% FCS in PBS. Three or more depletion cycles were used to deplete cells efficiently. The remaining nonadherent cells were resuspended in RPMI 1640 culture medium.

*Depletion of Cells by the Fluorescence-activated Cell Sorter (FACS).* A FACS IV instrument (BD-FACS system, Mountain View, CA) was used to remove surface (s) IgM<sup>+</sup> cells from MNC samples in some experiments (34). Blood MNC were incubated with monoclonal anti- $\mu$  antibody (200  $\mu$ g/ml), washed three times, and then stained with fluorescein-conjugated rabbit F(ab)<sub>2</sub> anti-mouse IgG antibody (0.4 mg/ml, fluorescein/protein = 2.4) for 20 min at 4°C. All antibody preparations were spun in a Eppendorf Microfuge at 15,000 *g* for 2 min to remove aggregates. Cells were suspended at  $3 \times 10^6$ /ml in PBS containing 5% FCS. For sorting, the light-scatter profile of the cell suspension was determined and the scatter gates for fluorescence were set to remove the Ig<sup>+</sup> cells. Cells were sorted at a rate of 2,800–3,000 cells/s. sIgM<sup>-</sup> cells were collected and suspended in RPMI 1640 culture medium. Approximately 80% of the cells were recovered in the Ig<sup>-</sup> fraction.

*Immunofluorescence Studies.* Affinity-purified and fluochrome-conjugated goat antibodies specific for human  $\mu$ ,  $\delta$ ,  $\gamma$ , and  $\alpha$  heavy chains were used in most experiments to enumerate B lymphocytes and mature plasma cells. These rhodamine- or fluorescein-conjugated antibodies gave values identical with those obtained with the monoclonal antibodies in indirect immunofluorescence assays and had the advantage of allowing two-color immunofluorescence analysis of each cell preparation. The staining combinations routinely used were (a) rhodamine-labeled anti- $\mu$  plus fluorescein-labeled anti- $\delta$ , and (b) fluorescein-labeled anti- $\gamma$  plus rhodamine-labeled anti- $\alpha$ . Surface staining of B lymphocytes was performed as described (35). At least 500 cells in each sample were counted using a Leitz fluorescent microscope (E. Leitz, Inc., Rockleigh, NJ).

*Estimates of Cytoplasmic (c) Ig<sup>+</sup> Cells.* The numbers of plasma cells were estimated in PWM-stimulated cell cultures maintained for 7 d, and occasionally longer. Cells were harvested from three culture wells, pooled, washed twice, and resuspended in PBS containing 5% FCS. The number of viable cells, estimated by counting cells that excluded trypan blue dye with a hemacytometer, ranged from 60 to 80%. The cells were spun onto glass slides by cytocentrifugation, fixed in 95% ethanol and 5% acetic acid at -20°C, washed in PBS, and stained with either fluorescein- or rhodamine-conjugated goat anti-human  $\mu$ ,  $\gamma$ , or  $\alpha$  antibodies in most experiments (35). In some experiments mouse monoclonal anti-human  $\alpha_1$  and  $\alpha_2$  antibodies were used in an indirect immunofluorescence assay. The percentage of cells brightly stained for cytoplasmic Ig determinants was determined using a Leitz microscope equipped for differential immunofluorescence and phase-contrast light microscopy. At least 1,000 cells were counted for each isotype, and the absolute number of cIg<sup>+</sup> cells per well was calculated by multiplying the percentage of cIg<sup>+</sup> cells by the number of viable cells per well. The effects of monoclonal antibodies on plasma cell differentiation were calculated by the following formula: the percentage of control = (number of cIg<sup>+</sup> cells/well containing antibody/number of cIg<sup>+</sup> cells/well in control culture)  $\times$  100.

## Results

*Effects of Monoclonal Anti- $\mu$ , Anti- $\gamma$ , and Anti- $\alpha$  Antibodies on PWM-induced Plasma Cell Differentiation.* In these studies, blood MNC were cultured with PWM in the presence of various concentrations (0.01–300  $\mu$ g/ml) of monoclonal anti- $\mu$ , anti- $\gamma$ , or anti- $\alpha$  antibody. The experimental values are expressed as percentages of control values for IgM, IgG, and IgA plasma cells at the end of a 7-d culture interval. The mean numbers of plasma cells in control cultures were  $10.3 (\pm 2.0) \times 10^3$  cells/culture for cIgM<sup>+</sup>,  $17.2 (\pm 3.6) \times 10^3$  cells/culture for cIgG<sup>+</sup>, and  $13.4 (\pm 1.8) \times 10^3$  cells/culture for cIgA<sup>+</sup> plasma cells. The mean number of viable cells in control cultures recovered after a 7-d culture was  $1.95 (\pm 0.02) \times 10^5$  cells/culture.

Anti- $\mu$  antibody suppressed the differentiation of cIgM<sup>+</sup> cells and also of cIgG<sup>+</sup> and cIgA<sup>+</sup> cells with plasma cell morphology in a dose-related fashion (Fig. 1).

However, the development of cIgG<sup>+</sup> or cIgA<sup>+</sup> cells was less effectively suppressed by anti- $\mu$  antibody than was the development of cIgM<sup>+</sup> cells. The results suggest that approximately one-half of the B cell precursors of IgG or IgA plasma cells bear sIgM, and the rest bear little or no sIgM. Monoclonal anti- $\gamma$  antibody selectively suppressed the development of cIgG<sup>+</sup> cells without affecting the differentiation of cIgM<sup>+</sup> or cIgA<sup>+</sup> cells (Fig. 2). Even if MNC were cultured with anti- $\gamma$  antibody for as long as 11 d, the differentiation of cIgM<sup>+</sup> or cIgA<sup>+</sup> cells was not affected (data not shown).

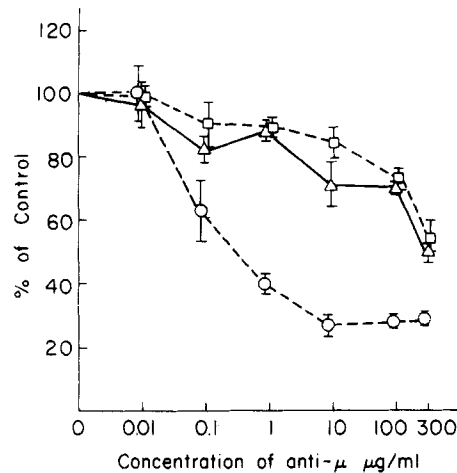


FIG. 1. Effect of monoclonal anti- $\mu$  antibody on PWM-induced plasma cell differentiation. Values are expressed as percentage of control for cIgM<sup>+</sup> (○), cIgG<sup>+</sup> (Δ), and cIgA<sup>+</sup> (□) cells at the end of a 7-d culture. Mean values  $\pm$  SE from three different experiments are shown. The absolute value for the untreated controls in this set of experiments were  $8.0 (\pm 0.5) \times 10^3$  cells/culture for cIgM<sup>+</sup>,  $14.9 (\pm 4.1) \times 10^3$  cells/culture for cIgG<sup>+</sup>, and  $13.6 (\pm 2.4) \times 10^3$  cells/culture for cIgA<sup>+</sup> plasma cells. The mean number of viable cells in control cultures recovered after 7-d culture was  $1.94 (\pm 0.02) \times 10^5$  cells/culture.

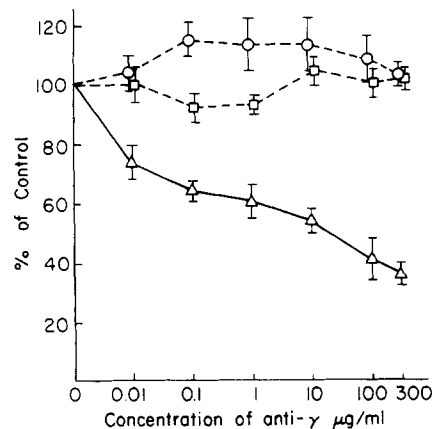


FIG. 2. Effect of monoclonal anti- $\gamma$  antibody on PWM-induced plasma cell differentiation. Symbols are as in Fig. 1. Mean values  $\pm$  SE from three different experiments are shown. The absolute values for the untreated controls in this set of experiments were  $11.3 (\pm 2.3) \times 10^3$  cells/culture for cIgM<sup>+</sup>,  $17.9 (\pm 4.8) \times 10^3$  cells/culture for cIgG<sup>+</sup>, and  $11.5 (\pm 1.0) \times 10^3$  cells/culture for cIgA<sup>+</sup> plasma cells. The mean number of viable cells in control cultures recovered after 7-d culture was  $1.95 (\pm 0.02) \times 10^5$  cells/culture.

Similarly, anti- $\alpha$  antibody selectively suppressed the development of cIgA<sup>+</sup> cells in a dose-related fashion (Fig. 3). In both instances, anti- $\gamma$  and anti- $\alpha$  antibodies were more effective in suppressing the homologous classes of plasma cells than was anti- $\mu$  antibody. As little as 0.01  $\mu$ g/ml of anti- $\gamma$  and anti- $\alpha$  antibodies caused significant suppression of IgG and IgA plasma cells, respectively, and the degree of suppression was greater with the highest antibody concentrations when compared with the same concentration of anti- $\mu$  antibody. The selective suppressive effects of anti- $\gamma$  and anti- $\alpha$  antibodies were very similar on a weight-for-weight basis, although the former was a  $\gamma_{2b}\lambda$  antibody and the latter a  $\gamma_{1k}$  antibody, as was the anti- $\mu$  antibody.

Taken together, the results of the three sets of experiments suggest that many of the PWM-induced IgG and IgA plasma cells are derived directly from sIgM-bearing B cells, but in this system the differentiation pathway to IgG or IgA plasma cells does not involve intermediate expression of sIgA or sIgG, respectively.

*Effect of Depletion of sIgM<sup>+</sup> Cells.* To confirm that IgG and IgA plasma cells may be derived from sIgM-bearing B cells, blood MNC were depleted of sIgM<sup>+</sup> cells by using the panning method or a FACS. Blood MNC were treated with monoclonal anti- $\mu$  antibody and depleted of sIgM<sup>+</sup> cells by three sequential incubations on rabbit anti-mouse IgG-coated dishes, or stained with fluorescein isothiocyanate (FITC)-conjugated rabbit F(ab')<sub>2</sub> anti-mouse IgG antibody and sorted with a FACS IV. The MNC preparations depleted of sIgM<sup>+</sup> cells, which contained only 0.1–0.2% sIgM<sup>+</sup> cells (Table II), were then cultured for 7 d with PWM. When sIgM<sup>+</sup> cells were depleted with the panning method, the number of cIgM<sup>+</sup> cells with plasma cell morphology at the end of the culture period was reduced to 6% of control values, and cIgG<sup>+</sup> and cIgA<sup>+</sup> cells were reduced 37 and 57%, respectively (Fig. 4). Similar results were obtained in the experiment using the FACS for depletion of sIgM<sup>+</sup> cells (Fig. 5). The number of cIgG<sup>+</sup> cells was decreased to 45% of control values obtained when untreated MNC were cultured with PWM and to 46% in the case of the unsorted MNC control; cIgA<sup>+</sup> cells were decreased to 58 and 67%, respectively. These results confirm that some IgG and IgA plasma cells are derived directly from sIgM-bearing lymphoid precursors.

*Effect of Depletion of sIgG<sup>+</sup> or sIgA<sup>+</sup> Cells.* As shown in Fig. 6, removal of sIgG<sup>+</sup> or

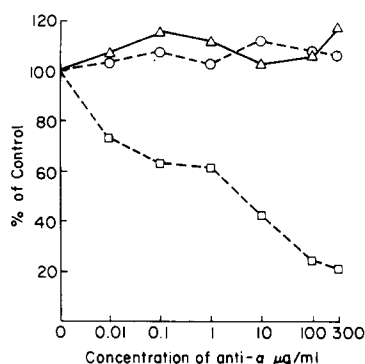


FIG. 3. Effect of monoclonal anti- $\alpha$  antibody on PWM-induced plasma cell differentiation. Symbols are as in Fig. 1. Mean values from two different experiments are shown. The absolute values for the untreated controls in this set of experiments were  $13.1 \times 10^3$  cells/culture for cIgM<sup>+</sup>,  $15.1 \times 10^3$  cells/culture for cIgG<sup>+</sup>, and  $10.8 \times 10^3$  cells/culture for cIgA<sup>+</sup> plasma cells. The mean number of viable cells in control cultures recovered after a 7-d culture was  $1.98 \times 10^5$  cells/culture.

TABLE II  
Distribution of sIg isotypes expressed by B cells remaining in blood MNC preparations selectively depleted of IgM, IgG, or IgA B cells

Experiment	Separation procedure	MNC	Percentage of positive cells			
			sIgM	sIgD	sIgD	sIgA
I*	Panning	Control	9.9	9.2	2.5	1.7
		sIgM <sup>-</sup>	0.2	0.5	1.9	1.4
II‡	FACS	Untreated control	9.4	8.9	1.5	1.1
		Unsorted control§	9.4	9.1	1.4	1.1
		sIgM	0.1	1.5	1.2	1.0
III	Panning	Control	7.8	6.8	2.5	2.1
		sIgG <sup>-</sup>	6.9	6.0	0.4	2.3
		sIgA <sup>-</sup>	6.4	6.2	2.5	0.1

\* MNC were treated with monoclonal anti- $\mu$  antibody or MOPC-300 myeloma protein and depleted with rabbit anti-mouse IgG antibody-coated dishes.

‡ MNC were treated with monoclonal anti- $\mu$  antibody, stained with FITC-rabbit F(ab)<sub>2</sub> anti-mouse IgG antibody, and sorted with a FACS.

§ MNC were incubated with antibodies but not sorted.

|| MNC were treated with monoclonal anti- $\gamma$  or anti- $\alpha$  antibody or with medium alone and depleted with rabbit anti-mouse IgG antibody-coated dishes.

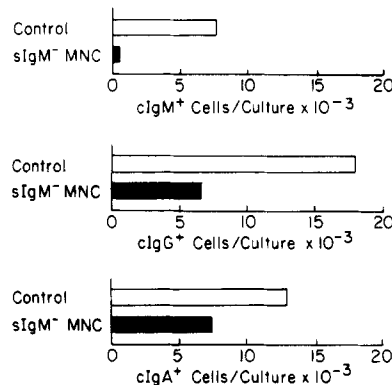


FIG. 4. Effect of depletion of sIgM<sup>+</sup> lymphocytes on PWM-induced plasma cell differentiation. MNC were depleted of sIgM<sup>+</sup> cells by the panning method as described in Materials and Methods. Control and sIgM<sup>+</sup>-depleted (sIgM<sup>-</sup>) MNC were cultured with PWM for 7 d. Represented is one of three reproducible experiments.

sIgA<sup>+</sup> cells from blood MNC selectively depleted the precursors of cIgG<sup>+</sup> or cIgA<sup>+</sup> cells, respectively, and did not affect differentiation of cells producing other isotypes. These results also suggest that in this PWM system, the differentiation pathway to IgG or IgA plasma cells does not involve intermediate expression of sIgA or sIgG, respectively.

*Effect of Monoclonal Anti- $\alpha_1$  Antibody on IgA Plasma Cell Differentiation.* To examine the effect of subclass-specific antibodies on plasma cell precursors, monoclonal anti- $\alpha_1$  antibody (31) was used. After culturing MNC for 7 d with PWM and anti- $\alpha_1$  antibody, the numbers of IgA<sub>1</sub> and IgA<sub>2</sub> plasma cells were established by staining cIg with monoclonal anti- $\alpha_1$  and anti- $\alpha_2$  antibodies in an indirect immunofluorescence

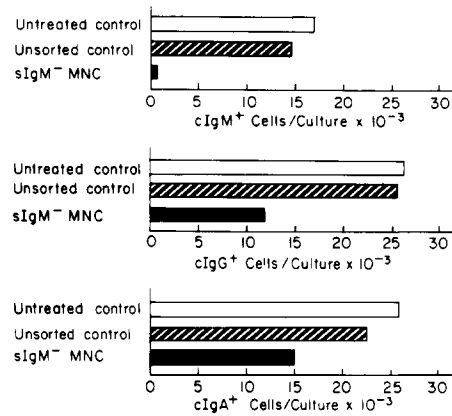


FIG. 5. Effect of depletion of  $sIgM^+$  lymphocytes on PWM-induced plasma cell differentiation. MNC were depleted of  $sIgM^+$  cells by the FACS as described in Materials and Methods. Untreated control, labeled but unsorted control, and  $sIgM^+$ -depleted ( $sIgM^-$ ) MNC were cultured with PWM for 7 d.

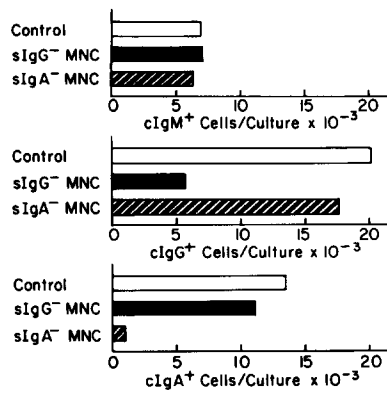


FIG. 6. Effect of depletion of  $sIgG^+$  or  $sIgA^+$  lymphocytes on PWM-induced plasma cell differentiation. MNC were depleted of  $sIgG^+$  or  $sIgA^+$  cells by the panning method as described in Materials and Methods. Control,  $sIgG^+$ -depleted ( $sIgG^-$ ), and  $sIgA^+$ -depleted ( $sIgA^-$ ) MNC were cultured with PWM for 7 d.

assay. Monoclonal anti- $\alpha_1$  antibody inhibited only the development of  $IgA_1$  plasma cells, and  $IgA_2$  plasma cell differentiation was not impaired (Fig. 7). These results confirm the earlier demonstration that  $IgA_1$  B cells and  $IgA_2$  B cells are discrete subpopulations (31).

### Discussion

These experiments have shown that mouse monoclonal antibodies specific for human  $\mu$ ,  $\gamma$ ,  $\alpha$ , and  $\alpha_1$  chains all inhibited pokeweed mitogen (PWM)-induced differentiation of human B cells, but each in a different way. Anti- $\mu$  antibody suppressed differentiation not only of  $IgM$  plasma cells but also of  $cIgG^+$  and  $cIgA^+$  cells in a dose-related fashion. However, the suppression of precursors for the  $cIgG^+$  and  $cIgA^+$  cells required higher concentrations of anti- $\mu$  antibody than the concentration required for suppression of precursors of  $cIgM^+$  cells. As estimated by the

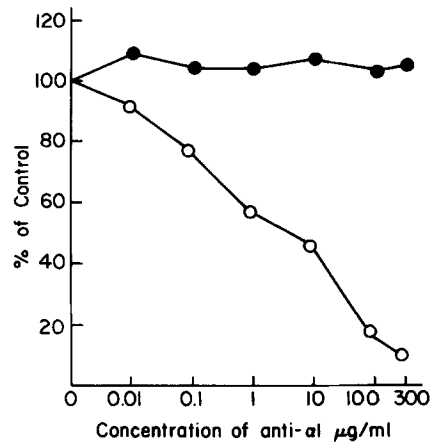


FIG. 7. The effect of monoclonal anti- $\alpha_1$  antibody on PWM-induced IgA plasma cell differentiation. Values are expressed as percent of control for cIgA $_1^+$  (○) and cIgA $_2^+$  (●) cells at the end of a 7-d culture.

concentration of anti- $\mu$  antibody required for 50% suppression, 100 times more anti- $\mu$  antibody was required to suppress the development of cIgG $^+$  and cIgA $^+$  cells. Selective depletion of sIgM $^+$  cells from blood MNC diminished the number of cIgG $^+$  and cIgA $^+$  cells to 40–60% of control values. These findings indicate that one-half or more of the precursor cells of IgG and IgA plasma cells express sIgM. The results also suggest that the IgM on IgG- and IgA-precursors imparts a weaker signal, perhaps because the density of sIgM on these cells is less than that for precursors of IgM plasma cells.

The addition of monoclonal anti- $\gamma$  or anti- $\alpha$  antibody to PWM cultures of MNC selectively diminished the number of cIgG $^+$  or cIgA $^+$  cells, respectively, and did not affect differentiation of plasma cells producing other immunoglobulin isotypes. Concentrations as low as 0.01  $\mu\text{g/ml}$  of the anti- $\gamma$  and anti- $\alpha$  antibodies caused significant suppression of IgG and IgA plasma cell development, and the degree of suppression achieved with the highest concentrations of anti- $\gamma$  and anti- $\alpha$  antibodies was greater than with the same concentrations of anti- $\mu$  antibody. These results indicate that in this mitogen-driven system the differentiation pathway from sIgM $^+$  cells to IgA plasma cells rarely, if ever, involves intermediate expression of sIgG.

It was of interest to determine whether the sIgM $^+$  precursors of IgG and IgA plasma cells expressed IgG or IgA before or after stimulation by PWM in culture. Approximately 20% of the circulating IgG B cells in normal adults normally express sIgM, and ~10% of the IgA B cells bear IgM that can be visualized by two-color immunofluorescence (35, 36). We found that removal of sIgG $^+$  or sIgA $^+$  cells before culture of blood MNC was as effective in reducing IgG and IgA plasma cell differentiation, as was incubation with anti- $\gamma$  or anti- $\alpha$  antibodies throughout the 7-d-culture interval. Removal of sIgG $^+$  cells selectively impaired IgG plasma cell differentiation, and depletion of IgA B cells likewise affected only the development of IgA plasma cells.

Similarly, anti- $\alpha_1$  antibodies inhibited only the differentiation of IgA $_1$  plasma cell precursors. IgA $_2$  plasma cell differentiation was not impaired. This same result has been obtained in another laboratory, in which selective inhibition of IgA $_2$  plasma cell differentiation by monoclonal anti- $\alpha_2$  antibodies was also demonstrated (M. E.



Conley, personal communication). These results are in keeping with an earlier demonstration that IgA<sub>1</sub> B cells and IgA<sub>2</sub> B cells are discrete subpopulations in individuals of all ages (31).

These results provide interesting parallels and contrasts with those of related experiments in which heterologous antisera and purified antibodies to different heavy chain isotypes have been used in the study of the differentiation potentials of normal human B cells (28–30, 37–41). Inhibition of IgM and IgG plasma cell precursors, and of IgA precursors in some experiments, has been observed consistently after the addition of anti- $\mu$ -specific preparations to PWM-stimulated human spleen, tonsillar, and blood MNC (30, 37, 38). The higher concentrations of purified monoclonal or goat anti- $\mu$  (30) antibodies required for inhibition of IgG and IgA precursors may reflect a relatively low density of IgM receptors on these subsets of B cells. This could explain a failure to isolate IgG and IgA precursors with anti- $\mu$  coated erythrocytes to form sufficiently stable rosettes for density-gradient separation (41). The inability of anti- $\mu$  to inhibit almost one-half of the IgG and IgA plasma cell precursors nevertheless suggests that PWM may stimulate a relatively mature subpopulation of B cells. By comparison, lipopolysaccharide (LPS) may stimulate plasma cell differentiation of a less-mature subset of B cells, almost all of which in mice can be inhibited by anti- $\mu$  (22). This seems to be the case for the LPS-responsive subset of B cells in humans as well (37; T. Kuritani and M. D. Cooper, unpublished results).

The most remarkable differences in results of experiments on the immunoglobulin isotypes expressed by PWM-responsive cells concern the expression of sIgG. Our studies, using purified mouse monoclonal and goat antibodies (30), suggest that IgG is expressed only on precursors of IgG plasma cells, whereas those of others (28, 38), using rabbit anti- $\gamma$  antisera, suggest that most IgM plasma cell precursors also express IgG. It is clear from the analysis of dose-response curves that even minor contamination of anti- $\gamma$  preparations with anti- $\mu$  antibodies could significantly distort the outcome of the experiment. In experiments suggesting that IgG-bearing cells were responsible for the majority of the response to PWM and gave rise to both IgG- and IgM-secreting cells, the sIgG<sup>+</sup> fraction separated by FACS sorting contained a higher percentage of sIgM<sup>+</sup> cells than did the sIgG<sup>-</sup> fraction (28). More difficult to explain are experiments in which inhibition of IgM plasma cell differentiation was inhibited by an anti- $\gamma$  antiserum, the biological activity of which could be inhibited by the Fc of IgG but not by the Fab fragment (38). However, we noted that a nonspecific suppression of all classes of PWM-induced plasma cells was exerted by purified goat anti- $\gamma$  antibodies (30). An analysis of the remaining plasma cells in the anti- $\gamma$ -treated cultures revealed a dose-related selective suppression of IgG plasma cells. Although the basis for a nonspecific suppression of plasma cell differentiation by anti- $\gamma$  antibodies is still unclear, our results indicate that mouse monoclonal anti- $\gamma$  antibodies may not display this characteristic.

Previous results indicate that PWM is a polyclonal mitogen of human B cells in the presence of helper T cells and monocytes (42–44). The results presented here indicate that PWM induces plasma cell differentiation of subpopulations of human B cells that are isotypically diverse. We have shown that all of these have an absence of surface IgD expression in common (T. Kuritani and M. D. Cooper, unpublished results). The major B cell subpopulations defined in the present experiments are: (a) sIgM<sup>+</sup> precursors of IgM plasma cells that do not express IgG or IgA isotypes, (b)

sIgG<sup>+</sup> precursors of IgG plasma cells that may or may not express residual sIgM but do not express IgA, and (c) sIgA<sup>+</sup> precursors of IgA plasma cells that may or may not express functional IgM receptors but do not express IgG. The IgA B cell subpopulation is further divisible into two separate sublines of IgA<sub>1</sub> and IgA<sub>2</sub> B cells. These results agree with previous results indicative of direct switch pathways from IgM to IgG and from IgM to an IgA isotype (30), and a limiting-dilution analysis indicates that the PWM-responsive cells in blood are isotypically committed (45). It is possible that clones of human B cells can make multiple switches in expression of heavy chain isotypes, even in a "backward" direction, but these were not detected in our studies because such switch pathways are infrequently used. Another possibility is that cells switching from  $\gamma$  to  $\alpha$ ,  $\gamma$  to  $\mu$ ,  $\alpha$  to  $\mu$ , or  $\alpha_1$  to  $\alpha_2$  may be sequestered in lymphoid tissues and are not present in the blood. However, our analysis of spleen cells, although not as extensive, has yielded the same results as those reported here for blood B cells. Although other tissues, such as gut-associated lymphoid tissues, would be interesting to examine in this way, we favor the idea that members of B cell clones that undergo isotype switch usually switch from the expression of C $\mu$  (and C $\delta$ ) directly to any one of the other C genes.

### Summary

Monoclonal antibodies were used to examine the immunoglobulin isotypes expressed by B lymphocyte precursors of IgM, IgG, IgA, and IgA<sub>2</sub> plasma cells. Plasma-cell differentiation was induced by the addition of pokeweed mitogen to cultures of blood mononuclear cells. Anti- $\mu$ , - $\gamma$ , - $\alpha$ , and - $\alpha_1$  antibodies were used in some experiments to inhibit differentiation of B lymphocytes bearing these heavy chain isotypes, and for selective removal of B lymphocyte precursors before culture with pokeweed mitogen in other experiments. Three major subpopulations of B lymphocyte precursors were identified: (a) a subpopulation of surface (s) IgM<sup>+</sup> precursors of IgM plasma cells that did not express IgG or IgA isotypes, (b) a subpopulation of sIgG<sup>+</sup> precursors of IgG plasma cells of which approximately one-half bore some IgM and none had detectable IgA receptors, and (c) a subpopulation of sIgA<sup>+</sup> precursors of IgA plasma cells; one half of these precursors could be shown to express functional IgM receptors but none were found to express IgG receptors. The sIgA subpopulation could be further subdivided into sIgA<sub>1</sub><sup>+</sup> precursors of IgA<sub>1</sub> plasma cells and IgA<sub>1</sub>-negative precursors of IgA<sub>2</sub> plasma cells. These results suggest that normal human B cells can switch from  $\mu$  directly to each of the other heavy chain isotypes, and that these represent the main switch pathways.

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