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## INDUCTION OF IMMUNOGLOBULIN ISOTYPE SWITCHING IN CULTURED I.29 B LYMPHOMA CELLS

### Characterization of the Accompanying Rearrangements of Heavy Chain Genes

BY JANET STAVNEZER, SONIA SIRLIN, AND JOAN ABBOTT

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A clone of B cells bearing IgM can be induced to switch to the expression of other isotypes, i.e., to other Ig heavy (H)<sup>1</sup> chain constant region (C<sub>H</sub>) genes, while maintaining expression of the identical H chain variable region (V<sub>H</sub>) gene (1, 2). Studies of the structure of Ig genes in cells that have undergone isotype switching have led to the conclusion that the isotype switch, except to  $\delta$ , is effected by DNA recombinations between tandemly repeated switch (S) sequences located 5' to each C<sub>H</sub> gene, resulting in the deletion of the  $\mu$  gene and the other C<sub>H</sub> genes located 5' to the C<sub>H</sub> gene to be expressed (in myelomas, hybridomas, B cell lymphoma, and normal splenic B cells treated with lipopolysaccharide [LPS]) (3–11). The production of  $\delta$  is an exception. Cloned cell lines that simultaneously produce both  $\mu$  and  $\delta$  contain  $\delta$  genes in the same context as in  $\mu$ -producing cells, and therefore must produce  $\mu$  and  $\delta$  by alternative RNA processing and/or termination of transcription (12–14). In response to antigenic challenge, a large number of cells in the spleen express  $\mu$  and another isotype (other than  $\delta$ ) simultaneously (15, 16). It is not yet known whether this is due to a transient  $\delta$ -like mechanism in which alternative RNA processing/termination occurs (17), or whether these double-producing cells have recently undergone switch-recombination and have deleted their  $\mu$  genes but still contain  $\mu$  messenger RNA (mRNA) and protein.

Another question still unanswered is what induces switching, i.e., whether antigen and/or T cells are required (18–20). Also unknown is how the isotype to be expressed is determined. As one B cell clone can generate multiple isotypes, it is generally believed that splenic B cells are not committed to one isotype (1, 21–23), but whether subpopulations of B cells become restricted in their isotype potential during maturation is unknown.

One major reason that these and other questions about the mechanism of isotype switching have remained unanswered is the lack of clonal or purified populations of IgM<sup>+</sup> cells that could be induced to undergo isotype switching *in vitro*. In this report, we demonstrate that the murine B cell lymphoma I.29

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<sup>1</sup> *Abbreviations used in this paper:* C<sub>H</sub>, heavy chain constant-region; D, diversity segment of Ig; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; H, heavy chain; Id, idiotype; IFM, immunofluorescence microscopy; J, joining region of Ig; LPS, lipopolysaccharide; mRNA, messenger RNA; PBS, phosphate-buffered saline; V<sub>H</sub>, heavy chain variable region.

provides such a system. The I.29 lymphoma arose spontaneously in 1961 as an ascites in the I/St strain of mice (24). It consists of cells expressing either IgM or IgA, with identical idiotype (25), and with identical  $V_H$  genes (9, and D. Klein and J. Stavnezer, manuscript in preparation).

We have previously established that the  $IgM^+$  cells contain two H chain chromosomes bearing two rearranged  $\mu$  genes: an expressed  $\mu$  gene (which has undergone  $VDJ_4$  [variable-diversity-joining] recombination), and a nonexpressed  $\mu$  gene that has undergone a  $DJ_2$  recombination (Fig. 1) (9, and J. Stavnezer, unpublished data). In the  $IgM^+$  cells, the  $C_H$  genes 3' to the  $\mu$  genes are present in the germline configuration on both chromosomes. In the  $IgA^+$  cells present in the lymphoma, both  $\mu$  genes have been deleted, having undergone switch-recombination events between  $S_\mu$  and  $S_\alpha$  sequences on the expressed H chain chromosome, and between  $S_\mu$  and  $S_{\gamma_3}$  sequences on the nonexpressed chromosome. These recombinations resulted in the deletion of all the  $C_H$  genes, except  $\alpha$ , from the expressed chromosome, and deletion of the  $\mu$  and  $\delta$  genes from the nonexpressed chromosome. The  $C_H$  genes 3' to the  $\gamma_3$  genes are in the germline configuration on the nonexpressed chromosome. The configuration of the Ig light chain genes is identical in the  $IgM^+$  and  $IgA^+$  cells (9). The structure of the  $\mu$  and  $\alpha$  genes established that the  $IgM^+$  and  $IgA^+$  cells in the I.29 lymphoma are related by switch-recombination events, but not whether this is an active ongoing process.

In this manuscript, we describe the development of an in vitro system in which purified or cloned  $IgM^+$  cells, derived from the I.29 lymphoma, have been induced to switch most often to IgA and, less frequently, to IgE or IgG2. The isotype switching can occur in the absence of T cells or macrophages. DNA recombinations that accompany the isotype switch are characterized.

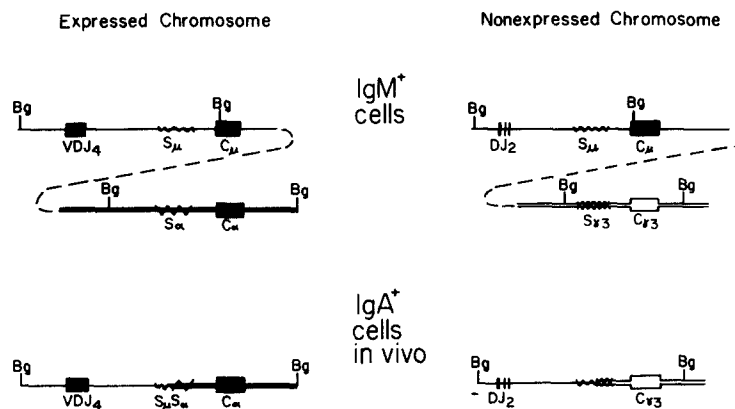


FIGURE 1. Diagram (not to scale) of H chain gene chromosomes in the  $IgM^+$  and  $IgA^+$  cells from the I.29 lymphoma. The upper figures illustrate that the  $IgM^+$  cells contain an expressed  $\mu$  gene produced by a  $VDJ_{H4}$  recombination, and a nonexpressed  $\mu$  gene that has undergone  $D_{Q52} \cdot J_{H2}$  recombination (26 and J. Stavnezer, unpublished data). The lower diagrams illustrate that in the  $IgA^+$  cells that have switched in vivo and are present in the lymphoma, both the expressed and nonexpressed H chain chromosomes have undergone DNA recombinations within or near the tandemly repeated switch (S) sequences; the expressed chromosome has switched to  $\alpha$  and the nonexpressed to  $\gamma_3$  (9). The approximate locations of the Bgl II (Bgl) sites are indicated.

## Materials and Methods

*Mice.* I.29 tumor cells were passaged intraperitoneally in I/St (I × BALB/c F<sub>1</sub>) or in I × C57BL/6F<sub>1</sub> mice.

*Cell Culture.* I.29 $\mu$  cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 50% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT), 1 mM glutamine (Gibco Laboratories), 50  $\mu$ M  $\beta$ -mercaptoethanol, insulin (0.1  $\mu$ /ml) (Eli Lilly and Co., Indianapolis, IN), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) (Gibco Laboratories), and kanamycin (100 U/ml) (Gibco Laboratories). For induction of switching, I.29 $\mu$  cells were centrifuged from the culture medium, washed once in RPMI 1640, and resuspended in RPMI 1640 containing 10% FCS, 1 mM glutamine, 50  $\mu$ M  $\beta$ -mercaptoethanol, 0.1 mM nonessential amino acids (Gibco Laboratories), 1 mM sodium pyruvate (Gibco Laboratories), penicillin, streptomycin, and kanamycin. Various inducers were added for 3 or 4 d, after which time they were removed by aspirating the culture fluid from above the cells; subsequently, the cells were fed with 10% FCS/RPMI 1640, when necessary.

*Immunofluorescent Antibodies.* A rat monoclonal anti-mouse IgM (R33/24) from G. Hammerling (University of Cologne, Federal Republic of Germany), conjugated to fluorescein isothiocyanate (FITC) was used for all the immunofluorescence experiments (given by R. Sitia, University of Genoa, Italy, and U. Hammerling, Sloan-Kettering Institute, New York).

Two different rhodamine-conjugated anti-mouse IgA antisera were used: (a) a rabbit antiserum produced by immunization with the myeloma protein J558, which was absorbed to render it class-specific, and purified by affinity chromatography (from R. Sitia), and (b) goat antiserum (IgG fraction), purified by affinity chromatography to render it class-specific (Cappel Laboratories, Cochranville, PA).

An FITC-conjugated rabbit anti-mouse  $\gamma$ Fc antiserum (from U. Hammerling) was used to detect the binding of the mouse monoclonal anti-I.29 Id antibody (T10/219), or used directly to search for the presence of IgG in I.29 cells.

An FITC-conjugated goat anti-rabbit Ig antiserum (from U. Hammerling) was absorbed with I.29 cells and used to detect the binding of a rabbit anti-IgE antiserum (from Z. Ovary, New York University) that had been absorbed with I.29 $\mu$  cells.

*Nonimmunofluorescent Antibodies.* The mouse monoclonal anti-I.29 Id antibody ( $\gamma$ 2,  $\kappa$ ) (T10/219) (27) was produced by a hybridoma that was derived from a fusion of NS.1 myeloma cells and spleen cells from C57BL/6 mice previously injected with IgA from the ID150 hybridoma (derived by fusion of I.29 cells and NS.1) (28). This antibody precipitated IgM and IgA from the I.29 IgM (ID43) and IgA (ID150) hybridomas but did not react with normal mouse serum. Ammonium sulfate-precipitated material from hybridoma culture supernatants containing anti-I.29 Id antibody were given by N. Tada (Tokai University, Kanagawa, Japan). The precipitated proteins were dialyzed against phosphate-buffered saline (PBS) and filtered through a 0.45- $\mu$ m filter. This reagent consisted of ~96% FCS and 4% anti-Id antibody (determined by the yield of the purified anti-Id from this crude preparation, as assayed by absorbance at 230 nm). This reagent is termed ammonium sulfate-precipitated anti-Id antibody. The amounts used are given as actual amount of anti-Id antibody calculated to be present. The antibody was tested at 5–125  $\mu$ g/ml.

Anti-Id antibody was purified from the preparation of ammonium sulfate-precipitated anti-Id by binding to protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), and eluting the antibody with 0.1 M glycine-HCl, pH 3. Nonbound and bound fractions were dialyzed against RPMI 1640 and filtered to sterilize. Antibody was added to the cultures at 5–25  $\mu$ g/ml. Other antibodies used in attempts to induce isotype switching were: (a) a monoclonal anti-IgD<sup>5a</sup> (given by N. Tada) (29), which had been prepared identically with, and used at the same total protein concentration as the crude anti-Id antibody, (b) culture supernatants (not concentrated or purified) from the rat anti-mouse IgM hybridoma R33/24 (100  $\mu$ l/ml of culture), (c) goat anti-IgM (IgG fraction) (Cappel Laboratories), tested at 25–100  $\mu$ g/ml, (d) goat anti-IgM (affinity-purified; given by W.

Paul, National Institutes of Health, Bethesda, MD (30), tested at 50  $\mu\text{g}/\text{ml}$ , (e) goat anti-mouse IgA (IgG fraction) (Cappel Laboratories), tested at 50–500  $\mu\text{g}/\text{ml}$ .

**Immunofluorescence Microscopy.** Cells were pelleted from culture media and suspended in RPMI 1640 without serum, and 50  $\mu\text{l}$  was spotted onto poly-L-lysine-coated microspot slides. After 20 min, 2  $\mu\text{l}$  of 16% bovine serum albumin in PBS was added. 10 min later, the spots were washed extensively with 1% FCS in PBS containing 0.2% sodium azide. Slides were placed in 95% ethanol for 15 min to several days at  $-20^{\circ}\text{C}$  to fix the cells, in order to permit the anti-Ig reagents to stain the cytoplasm. In early experiments, cells were cytocentrifuged onto slides and fixed in 95% ethanol. Slides were soaked overnight or longer in 1% FCS in PBS with 0.2% sodium azide, which greatly reduced the nonspecific staining.

To stain the cells, slides were wiped dry around the cell spots, 10  $\mu\text{l}$  of an appropriate dilution of antibody was added to each spot, and the slides were placed in a humidified chamber at room temperature for 30 min. Nonbound antibodies were removed by soaking in PBS for 1.5 h to several hours.

**Restriction Enzyme Digestion, Gel Electrophoresis, and DNA Blotting.** These procedures were performed as described (9); the hybridization mixtures contained 50% deionized formamide, 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, 50  $\mu\text{g}/\text{ml}$  *Escherichia coli* DNA, 1 mg/ml yeast RNA, 900 mM NaCl, 90 mM sodium citrate, 0.6 mM disodium EDTA, and 5% dextran sulfate. The hybridization reactions were incubated overnight at  $42^{\circ}\text{C}$ . If the DNA fragment used as the probe contained highly reiterated sequences, annealing was performed using one-half the amount of NaCl (450 mM NaCl), and the blots were washed at  $60\text{--}65^{\circ}\text{C}$  instead of  $50\text{--}52^{\circ}\text{C}$ .

**RNA Blots.** RNA and  $^{32}\text{P}$ -labeled Hind III fragments of  $\lambda$  bacteriophage DNA, used as molecular weight markers, were denatured with glyoxal and dimethylsulfoxide, electrophoresed in 1% agarose gels with 10 mM phosphate buffer, pH 7 (31), and blotted onto diphenylthioether paper (32). To reuse blots, hybridized probes were removed by incubation of the blots for 2–3 h at  $68^{\circ}\text{C}$  in 75% formamide (Bethesda Research Laboratories, Gaithersburg, MD), 50 mM sodium phosphate, pH 7, and 5 mM disodium EDTA, pH 7.

**Hybridization Probes.** All hybridization probes were nick-translated fragments isolated from plasmids or  $\lambda$  phage, encoding Ig H-gene segments from the BALB/c genome, or Ig C<sub>H</sub> cDNA. M2-5B was a 2.1-kb Hind III fragment that encoded the 5' portion of the C $\mu$  gene and 5' flanking sequences (Fig. 5) (33). J<sub>H1-2</sub> was a 1.1-kb Bam HI fragment, and J<sub>H3-4</sub> was a 2-kb Bam HI–Eco RI fragment (Fig. 5) from p<sub>j6</sub> and p<sub>j11</sub>, respectively (33). The probe for C $\alpha$ , labeled  $\alpha$  in Fig. 5, was a 1.4-kb Msp I fragment from p $\alpha$ (J558)<sup>13</sup> (33). The probe for C $\gamma_3$  was a 2-kb Bam HI–Kpn I fragment encoding the genomic  $\gamma_3$  gene, from the recombinant  $\lambda$  phage SL51 (34), which we have subcloned into pKB111 (35). All the above probes were from K. Marcu (State University of New York, Stony Brook, NY). The probe for C $\epsilon$ , labeled  $\epsilon$  in Fig. 5, was a 2.9-kb Bam–Hind III fragment that encoded the 5' half of the genomic C $\epsilon$  gene and 5' flanking sequences (36) (from T. Honjo, Osaka University, Osaka, Japan). The probe for C $\gamma_{2b}$  was a 0.8-kb Pst I–Kpn I fragment from p $\gamma_{2b}$ (11)<sup>7</sup> (37) (from R. Perry, Institute for Cancer Research, Fox Chase, PA). This probe also hybridized with  $\gamma_{2a}$  sequences. The probe for C $\gamma_1$  was a 0.7-kb Hinc II–Hind III fragment from the cDNA plasmid pH21-1 (from W. Salser, University of California at Los Angeles)(38). The probe for C $\delta$  was a 0.6-kb Pvu II–Hind III fragment from the plasmid clone p $\delta$ 54J (39) (from P. Tucker, University of Texas, Dallas, TX).

## Results

**Source of Pure IgM<sup>+</sup> Cells.** Two preparations of pure IgM<sup>+</sup> cells isolated from the I.29 lymphoma by U. Hammerling (9, and U. Hammerling, unpublished data) were adapted to culture and used as starting material for the isotype switching experiments: (a) I.29 $\mu$  cells were obtained by sorting IgM<sup>+</sup> cells from the I.29 tumor on the fluorescence-activated-cell sorter, and were subsequently

passed intraperitoneally in mice. The expression of cytoplasmic IgM and IgA was assayed at several passages by immunofluorescence microscopy (IFM) of ethanol-fixed cells, staining simultaneously with an FITC-conjugated monoclonal rat anti-mouse IgM and rhodamine-conjugated, affinity-purified goat or rabbit anti-mouse IgA. The I.29 $\mu$  cells were found to express only IgM; for example, at passage 17, no IgA<sup>+</sup> cells were detected among 18,400 cells, i.e., <0.01% of the I.29 $\mu$  cells were IgA<sup>+</sup>. (b) Cells that we called IgM.S were obtained in a separate sorting experiment, and were subsequently further purified by placing 100, 1,000, or 10,000 of the IgM<sup>+</sup> cells into 36 Millipore filter chambers, which were implanted into mice. Only one of the chambers yielded I.29 cells, and these cells were 100% IgM<sup>+</sup>. After 25 intraperitoneal passages, the IgM.S cells remained >99% IgM<sup>+</sup>. These IgM<sup>+</sup> cell lines were adapted to culture.

*IgM<sup>+</sup> Cells Can Switch to Expression of IgA.* When I.29 $\mu$  or IgM.S cells were placed into culture, a low level of switching to IgA occurred; after 1 or 2 mo they were 1–2% IgA<sup>+</sup> and 98% IgM<sup>+</sup>, and after 3 mo they were >99% IgM<sup>+</sup>. We attempted to induce the cultured IgM<sup>+</sup> cells to undergo isotype switching by treatment with LPS, a B cell mitogen that induces isotype switching in cultured normal spleen cells (15). At various times after the addition of LPS, cells were cytocentrifuged onto slides, fixed with cold 95% ethanol, and stained with antibodies against IgM and IgA. 6 d after induction, an increased number of IgA<sup>+</sup> cells were observed, and many of the IgA<sup>+</sup> cells also stained within the cytoplasm with anti-IgM (Fig. 2). The double-staining cells must have been derived by isotype switching from the IgM<sup>+</sup> cells, and not by the outgrowth of any IgA<sup>+</sup> cells that could be contaminating the IgM<sup>+</sup> cell lines, since we have previously demonstrated that IgA<sup>+</sup> cells present in the I.29 tumor have deleted the C $\mu$  genes from both of their H chain chromosomes, and thus cannot synthesize IgM (9). The double-producing cells apparently had recently switched from IgM to IgA synthesis, because at later times after the addition of LPS, the IgA<sup>+</sup> cells no longer also contained IgM (see below).

To obtain further proof of active switching, eight clones of IgM<sup>+</sup> cells were produced by plating one or three I.29 $\mu$  cells in 20  $\mu$ l of medium, in microtiter wells. Two clones were obtained from the 50 wells that had received one cell per well, and six clones from 50 wells that had received three cells per well. The clones were >99% IgM<sup>+</sup>, as no IgA<sup>+</sup> cells were detected among >1,000 cells examined. The clones were tested for their ability to switch to IgA. Six of the eight clones yielded IgA<sup>+</sup> cells. This provided unequivocal proof that the IgM<sup>+</sup> cells could switch to IgA, as the level of contamination of the starting population of cultured I.29 $\mu$  cells with IgA<sup>+</sup> cells was <1.0%. The experiments described below were performed on the noncloned I.29 $\mu$  cell line, except as indicated.

*LPS Induces Switching.* The standard induction protocol was to place  $0.5 \times 10^6$  cells in 1 ml of culture fluid and add LPS. On day 4, the LPS was removed and the cells were resuspended in 1–2 ml of RPMI 1640 containing 10% FCS. At various intervals, a sample of cells from each well was counted, spotted onto a poly-L-lysine-coated slide, and assayed by IFM to determine the percent of cells that were IgA<sup>+</sup> and/or IgM<sup>+</sup>.

One of several similar experiments is presented in Table I. When I.29 $\mu$  cells were simply changed from medium containing 50% FCS and insulin (0.1 U/ml),

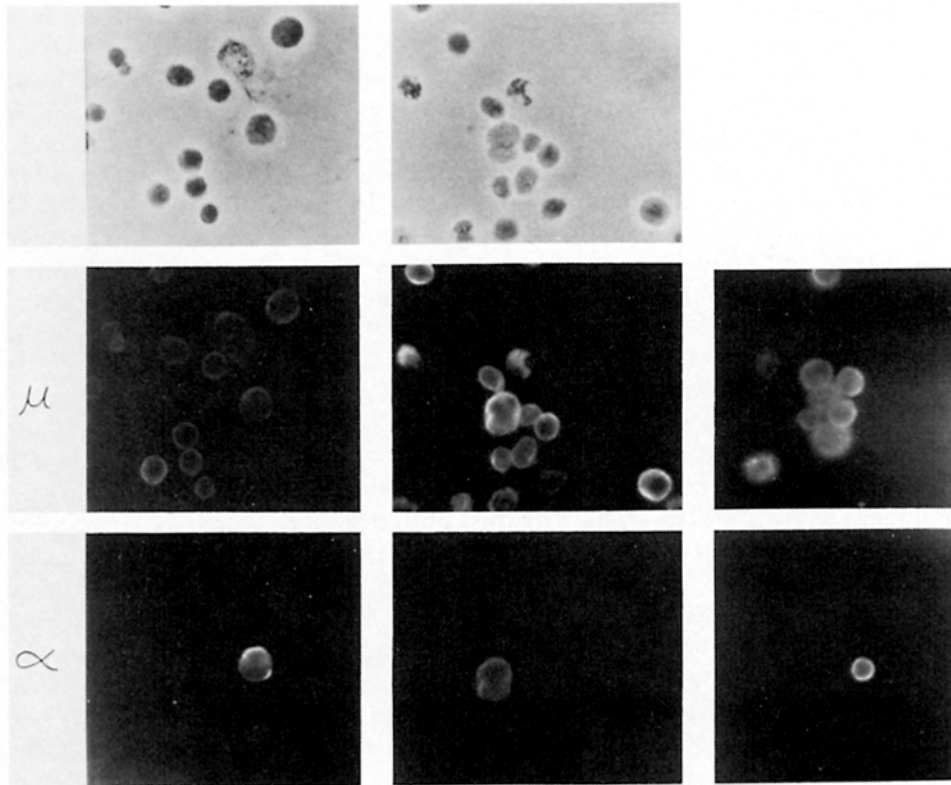


FIGURE 2. IFM of I.29 $\mu$  cells treated with LPS for 6 d. Top row: phase-contrast pictures of the same fields photographed in the lower two rows with ultraviolet epi-illumination after the reaction of cytocentrifuged, fixed cells with anti-IgM and anti-IgA. Middle row: IgM was detected with an FITC-conjugated monoclonal rat anti-mouse IgM (R33/24, from G. Hammerling). Bottom row: IgA was detected with a rhodamine-labeled rabbit anti-mouse IgA (from R. Sitia).

in which they were cultured, and placed into medium with 10% FCS and without insulin (well 1), a small transient increase in the percent of IgA<sup>+</sup> cells occurred. When cells were treated with 10  $\mu$ g/ml LPS (well 2), 8% became IgA<sup>+</sup> by 8 d after LPS addition, and 5 of the 16 IgA<sup>+</sup> cells counted also contained cytoplasmic IgM. The proportion of IgA<sup>+</sup> cells increased until day 15, at which time 43% of the cells were IgA<sup>+</sup>. The fraction of the IgA<sup>+</sup> cells that also stained with anti-IgM was maximal at day 4, and thereafter decreased so that on day 15 only a small proportion of the IgA<sup>+</sup> cells contained IgM. By 18 d after initiation of the LPS pulse, the proportion of IgA<sup>+</sup> cells in the culture began to decrease, and by 35 d after LPS addition, this culture was 100% IgM<sup>+</sup>. The return to IgM expression occurred in all cultures that were followed for this duration, except for a few cultures that switched to such a great extent that they became  $\sim$ 100% IgA<sup>+</sup> (see below).

*Anti-I.29 Id (Anti-idiotypic) and LPS Together Induce Maximal Switching.* Because we expected that the antigen receptor would be involved in triggering isotype switching, we attempted to induce switching with anti-Ig

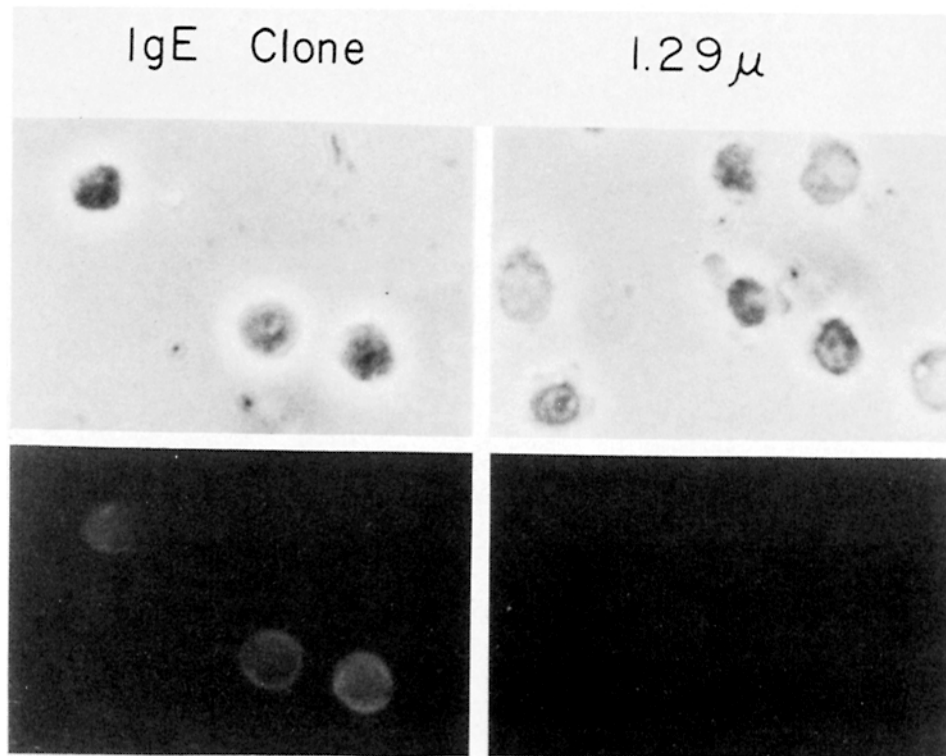


FIGURE 3. Detection of cytoplasmic IgE in cultured I.29 cells. Cloned IgE<sup>+</sup> cells or I.29 $\mu$  cells were spotted onto poly-L-lysine-coated slides, fixed in 95% ethanol, and stained with rabbit anti-IgE (from Z. Ovary), followed by an FITC-labeled anti-rabbit IgG (U. Hammerling). The phase-contrast pictures in the upper two panels correspond to the fields below, which were photographed with ultraviolet epi-illumination.

antibodies. Two preparations of anti-Id were derived from the culture supernatant of a mouse hybridoma producing an anti-I.29 Id (T10/219) (27): (a) ammonium sulfate-precipitated proteins, and (b) purified anti-Id. Both of these preparations were highly effective in inducing switching in I.29 $\mu$  cells when added together with LPS (10  $\mu$ g/ml) for 4 d (Table I). 8 d after induction, 30% of the cells were IgA<sup>+</sup> in well 3, which had been treated with LPS plus the ammonium sulfate-precipitated anti-Id, and 14% of the cells were IgA<sup>+</sup> in well 4, which had been treated with LPS plus purified anti-Id. The percent of IgA<sup>+</sup> cells may have been greater in well 3 than in well 4 because the amount of anti-Id present in the ammonium sulfate-precipitated preparation added to well 3 (~25  $\mu$ g) was greater than the amount of purified anti-Id added to well 4 (5  $\mu$ g). Anti-Id and LPS appeared to synergize, because treating the cells with a greater amount of LPS (50  $\mu$ g/ml) did not produce a greater proportion of IgA<sup>+</sup> cells (not shown) and purified anti-Id alone did not induce switching (well 5). The ammonium sulfate-precipitated anti-Id did induce switching by itself, but it is likely that this preparation was contaminated with LPS from the large amount of FCS it contained.

The total number of viable cells present in each culture, originating from 0.5



TABLE I  
Induction of Switching in  $I.29 \mu$  Cells

Well number	Treatment	Percent of IgA <sup>+</sup> or "null" (IgE <sup>+</sup> ) cells at:									
		4 d	8 d	11 d	15 d	18 d	22 d	35 d			
1	Control	IgA	4.8 (2/10)*	1 (1/2)	1 (0/2)	0	0.4 (0/1)	0	0		
		Null†	0	0.5	2	2	0	0	0		
		Total cells‡	3.9	9	7	9	9.3	36	—		
2	LPS (10 $\mu$ g/ml)	IgA	1.5 (3/3)	8 (5/16)	37 (9/42)	43 (9/49)	27 (0/51)	10 (0/44)	0		
		Null	0	8	2	0	0	0	0		
		Total cells	2	0.3	0.9	13	13.5	26	—		
3	Ammonium sulfate-precipitated anti-Id (25 $\mu$ g/ml) + LPS	IgA	1.9 (6/6)	30 (12/25)	29 (22/48)	91 (58/113)	96 (9/162)	99 (0/234)	100		
		Null	0	7	4	0	0	0	0		
		Total cells	2.8	0.6	0.12	0.5	0.2	1.8	—		
4	Purified anti-Id (5 $\mu$ g/ml) + LPS	IgA	1.4 (3/3)	14 (5/25)	54 (1/102)	66 (2/134)	74 (0/143)	NT	NT		
		Null	0	6	0	0	0	0	0		
		Total cells	1.95	1.5	1.5	5.3	15	—	—		
5	Purified anti-Id (5 $\mu$ g/ml)	IgA	1.2 (0/3)	1.9 (0/4)	0.9 (0/2)	1 (0/2)	0	NT	NT		
		Null	0	0	0	0	0	0	0		
		Total cells	3.2	17	10	6	6.5	—	—		

\* The number in parenthesis is the number of double-staining (IgM<sup>+</sup> + IgA<sup>+</sup>) cells/IgA<sup>+</sup> cells.

† When tested (at days 4 and 11), the percent of cells that stained with neither anti-IgM nor anti-IgA was approximately equal to the percent of cells that were IgE<sup>+</sup>.

‡ Total number of viable cells ( $\times 10^{-6}$ ) in the culture. At day 0, cultures contained  $0.5 \times 10^6$  cells that were >99% IgM<sup>+</sup>.

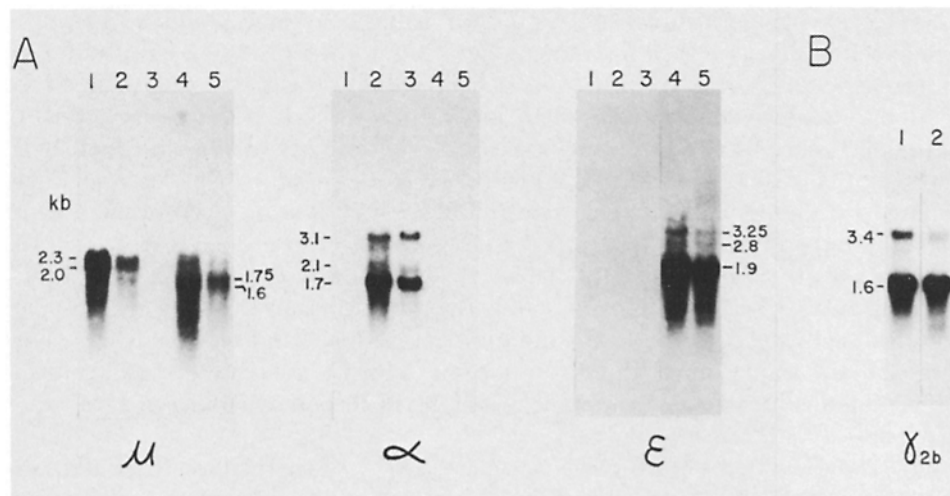
NT = not tested.

$\times 10^6$  cells, is given in brackets ( $\times 10^{-6}$ ) in Table I for time points up to 22 d. The cell number increased by four- to eightfold in the first 4 d of culture in the presence or absence of LPS. At 4 d the cell viability was slightly lower in LPS-treated cultures (average, 69%) than in cultures without LPS (average, 83%), but by 8 d there was a significant decrease in cell viability in all cultures treated with LPS. For example, cells treated with LPS alone (well 2), or with ammonium sulfate-precipitated anti-Id plus LPS (well 3) were 16% viable. Cells treated with purified anti-Id plus LPS (well 4) were 31% viable, whereas cells treated with purified anti-Id alone were 84% viable. The proportion of cells that were IgA<sup>+</sup> or IgM<sup>+</sup> at days 4–11 was approximately the same whether live only or both live and dead cells were counted, which indicates that IgM<sup>+</sup> and IgA<sup>+</sup> cells were not differentially viable under these conditions. Thus, the increase in IgA<sup>+</sup> cells in LPS-treated cultures was not simply due to the differential viability of IgM<sup>+</sup> and IgA<sup>+</sup> cells.

*Do I.29 $\mu$  Cells Switch to Isotypes Other Than IgA?* At early times (8–11 d) after the addition of LPS, cells appeared that did not stain with either anti-IgM or anti-IgA ("null" in Table I). These cells were shown to express IgE with the I.29 Id by indirect IFM on fixed cells using anti-IgE and anti-I.29 Id antibodies. Duplicate slides from the experiment presented in Table I (for days 4 and 11) were stained with rhodamine-conjugated goat anti-IgA and a rabbit anti-IgE antiserum, followed by an FITC-conjugated anti-rabbit Ig antiserum. The percent of cells that were IgE<sup>+</sup> corresponded with the percent of cells that were null in cell populations stained with anti-IgM and anti-IgA. On day 4, the percent of cells that were IgE<sup>+</sup> was slightly greater than the percent that were null, as expected if some of the IgE<sup>+</sup> cells also contained IgM (data not shown). Although IgA<sup>+</sup> cells preferentially accumulated in the treated cultures, a transient increase in IgE<sup>+</sup> cells, which was maximal at 8 d, was observed in a number of experiments, and IgE<sup>+</sup> cells did predominate in a few cultures. One culture of I.29 $\mu$  cells that had been maintained in 10% FCS/RPMI 1640 in the absence of insulin and inducers for 10 wk, became 90% IgE<sup>+</sup>, 10% IgM<sup>+</sup>. From this culture, seven IgE<sup>+</sup> clones were obtained by the limiting dilution method (as described for the IgM<sup>+</sup> clones). Fig. 3 shows that the cloned IgE<sup>+</sup> cells, but not the I.29 $\mu$  cells, stained with rabbit anti-IgE.

*Ig H Chain RNA Expressed in I.29 Cells.* Ig H chain RNA present in I.29 cells were examined in an attempt to corroborate the results of the IFM assays. Fig. 4 A illustrates a blot of total cell RNA from I.29 cells that had been hybridized with the  $\mu$ ,  $\alpha$ , or  $\epsilon$  probes (indicated below each panel). The *in vivo* line of I.29 $\mu$  cells contained Ig  $\mu$  mRNA but not  $\alpha$  or  $\epsilon$  mRNA (lane 1 in all three panels). I.29 $\mu$  cells that had been induced to switch, and were 85% IgA<sup>+</sup>, 15% IgM<sup>+</sup> (lane 2), contained  $\alpha$  mRNA and reduced amounts of  $\mu$  mRNA. Cloned IgA<sup>+</sup> cells (lane 3) contained  $\alpha$  mRNA but no  $\mu$  or  $\epsilon$  mRNA. Two IgE<sup>+</sup> clones (lanes 4 and 5) contained  $\epsilon$  mRNA (40, 41), but also  $\mu$  RNA that were probably sterile transcripts from the nonexpressed DJ-C $\mu$  allele (42, 43). As the  $\mu$  gene on the nonexpressed chromosome had been deleted from the cloned IgA<sup>+</sup> cells (9), no sterile  $\mu$  RNA were present in these cells (lane 3).

To determine if I.29 cells that had been treated with switch inducers switched to other isotypes besides IgA and IgE, we searched for the expression of  $\delta$ ,  $\gamma_3$ ,



**FIGURE 4.** Blots of I.29 cell RNA hybridized with probes for H chain genes. Total cell RNA were denatured with dimethylsulfoxide and glyoxal (30), electrophoresed on an agarose gel in 10 mM phosphate, pH 7, and transferred to diazophenylthioether paper (32). (A) Blot hybridized sequentially with the  $\mu$ ,  $\alpha$ , and  $\epsilon$  probes. The lanes contained 20  $\mu$ g of total cell RNA (except as noted) from: lane 1, in vivo I.29 $\mu$  cells; 2, I.29 $\mu$  cells induced to switch (85% IgA<sup>+</sup>, 15% IgM<sup>+</sup> cells); 3, BFO.3 cells (cloned IgA<sup>+</sup> cell line); 4 and 5, cloned IgE<sup>+</sup> cells, 40 and 20  $\mu$ g, respectively. The sizes of the  $\epsilon$  mRNA were as expected (40, 41). The  $\mu$  RNA detected in the cloned IgE<sup>+</sup> cells (lanes 4 and 5) are smaller than  $\mu$  mRNA and must be transcribed from the nonexpressed DJ<sub>2</sub>-C $\mu$  gene present in these cells. The very faint RNA band detected by the  $\epsilon$  probe in I.29 $\mu$  cells (lane 1 in  $\epsilon$  blot) is smaller than  $\epsilon$  mRNA and is transcribed from nonrearranged  $\epsilon$  genes (44). (b) Blot hybridized with  $\gamma_{2b}$  probe. Lanes contained 20  $\mu$ g of total cell RNA from: lane 1, IgM.S cells treated with LPS 18 d earlier (92% null, 8% IgM<sup>+</sup>); lane 2, I.29 $\mu$  cells treated with LPS 17 d earlier (2% IgA<sup>+</sup>, 75% null, 23% IgM<sup>+</sup>). Null cells did not stain with either anti-IgM or anti-IgA. In these experiments, cells were incubated at a higher cell density and with a higher concentration of LPS (50  $\mu$ g/ml) than normally used. The size of the  $\gamma_2$  mRNA (3.4 and 1.6 kb) agrees with the sizes reported by Rogers et al. (45). The  $\mu$ ,  $\alpha$ ,  $\epsilon$ , and  $\gamma_2$  mRNA were all hybridized with a probe for the I.29 V<sub>H</sub> gene, and all were poly(A)<sup>+</sup> (44 and J. Stavnezer, unpublished data).

$\gamma_1$ , and  $\gamma_2$  mRNA. Small amounts of poly(A)<sup>+</sup> RNA the size of  $\delta$  mRNA were detected in untreated IgM.S cells (44), although no IgD has been detected in I.29 cells (25). No  $\gamma_3$  or  $\gamma_1$  mRNA were detected. However, significant amounts of  $\gamma_2$  mRNAs were detected by a  $\gamma_{2b}$  cDNA probe (37) in some cultures of I.29 $\mu$  or IgM.S cells that had been treated with LPS 2–3 wk previously (Fig. 4 B). Since the  $\gamma_{2b}$  probe hybridizes with both  $\gamma_{2b}$  and  $\gamma_{2a}$  sequences, we do not yet know whether these mRNA encode  $\gamma_{2b}$  and/or  $\gamma_{2a}$  chains. In conclusion, I.29 cells cultured in vitro under our conditions switch mostly to IgA and, to a lesser extent, to IgE and IgG2, but not to IgG3 or IgG1.

**DNA Rearrangements Accompany Switching.** To determine if rearrangements of H chain genes accompany the isotype switch and to search for intermediate DNA rearrangements in the process of switching, we performed Southern blots on DNA from I.29 cells at various times after induction with LPS and/or anti-Ig reagents. DNA from the total population of cells in the cultures was analyzed, using the enzymes indicated in Figs. 5 and 6.

The DNA fragments used as probes in these experiments are indicated as

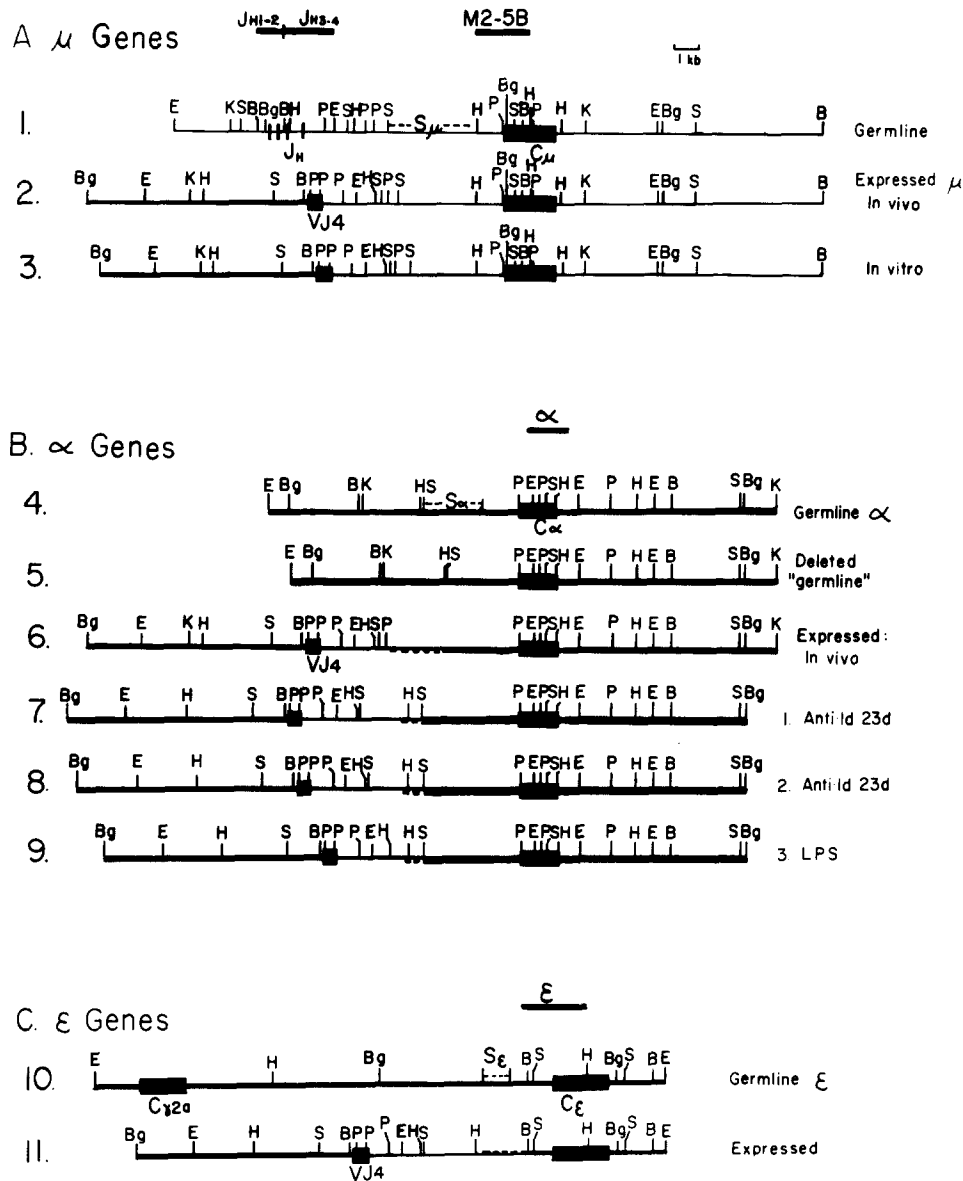


FIGURE 5. Restriction enzyme maps of expressed and germline  $\mu$ ,  $\alpha$ , and  $\epsilon$  genes within I.29 cells. The DNA regions within the probes are indicated by black bars above the maps. The  $J_H$  and  $C_H$  gene segments are indicated by black rectangles on the maps.  $S_\mu$ ,  $S_\alpha$ , and  $S_\epsilon$  indicate the approximate location of the tandemly repeated S sequences (7, 46). The approximate location of  $S_\mu$ - $S_\alpha$  and  $S_\mu$ - $S_\epsilon$  recombinations derived from the mapping data are indicated by dots or dashes along the maps and the change in thickness of the lines. The maps were derived from numerous genomic DNA blotting experiments and from published data (9, 36). As the maps of the  $\alpha$  and  $\epsilon$  genes differ in I/St mice (*Igh-C<sup>+</sup>*) and BALB/c mice (*Igh-C<sup>-</sup>*) (47), the sites in the germline  $C_\alpha$  gene were established by mapping a cloned germline  $\alpha$  gene (J. Stavnezer, unpublished data). The position of the expressed  $V_H$  gene was confirmed by DNA sequencing (D. Klein and J. Stavnezer, manuscript in preparation). (A) Maps of (1) the germline  $\mu$  gene present within I/St liver cells, and the expressed  $\mu$  gene within (2) in vivo  $IgM^+$  cells and (3) in vitro  $IgM^+$  cells. (B) Maps of (4) the germline  $\alpha$  gene in the in vivo  $IgM^+$  liver cells, and (5) shortened nonexpressed  $\alpha$  gene present 3 d after the addition of anti-Id to the I.29 cells. Maps of the expressed  $\alpha$  genes from: (6)  $IgA^+$  cells cloned from the in vivo I.29 tumor, (7) I.29  $\mu$  cells treated with anti-Id (labeled I.29 anti-Id 23d [1] in Fig. 7), (8) cells labeled I.29  $\mu$  anti-Id 23d (2) in Fig. 7, (9) cells treated with LPS (labeled I.29  $\mu$ -LPS) or treated with LPS and passaged twice in vivo (labeled I.29  $\mu$ -LPS, 2P in Fig. 7). The *Kpn* I sites were not mapped in fragments 7-9. (C) Maps of (10) the germline  $\epsilon$  gene and the nearby  $C\gamma_{2a}$  gene (positioned using data from Shimizu et al. [48]) and (11) the rearranged expressed  $\epsilon$  gene present in cloned  $IgE^+$  cells (labeled clones 4, 5, and 6 in Fig. 7). Restriction enzymes: B, Bam HI; Bg, Bgl II; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; S, Sst I (Sac I).

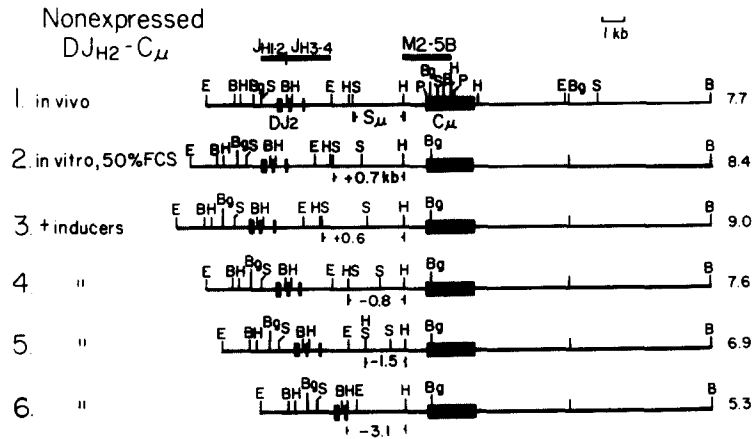


FIGURE 6. Maps of the predominant nonexpressed  $DJ_2-C\mu$  segments present in I.29 cells. The numbers on the right refer to the lengths in kilobases of the *Bgl* II fragments encoding the  $DJ_2$  segment and the 5' portion of the  $C\mu$  gene corresponding to each map. The lines without restriction sites indicated within and 3' to the  $C\mu$  gene indicate identity with (1) the *in vivo* segment. (1) Map of the nonexpressed  $DJ_2-C\mu$  gene segment present in the *in vivo* lines of I.29 $\mu$  and IgM.S. The recombined  $DJ_2$  segment is indicated by the most 5' rectangle, labeled DJ;  $J_3$  and  $J_4$  are indicated by the thinner bars to the right of  $DJ_2$ . The presence of the recombined  $D_{Q52-J_2}$  segment has been determined by partial nucleotide sequencing (J. Stavnezer, unpublished data). This map is of the DNA segment that contains the 7.7-kb *Bgl* II fragment in Fig. 7, lanes 3, 4, and 19. (2) Map of the predominant form of the nonexpressed  $DJ_2$  segment in I.29 $\mu$  and IgM.S cells in culture. The  $S\mu$  region, which has increased in size relative to that in the *in vivo* cells, is indicated by the length of the added sequences, plus 0.7 kb. (3-6) Maps of the  $DJ_2-C\mu$  segments detected in cells treated for 3 d with ammonium sulfate-precipitated anti-Id (Fig. 7, lanes 9 and 24). (3) The largest  $DJ_2-C\mu$  segment appears to have resulted from a further addition of 0.6 kb to the  $S\mu$  region. This fragment corresponds to the largest fragment marked with a dot in Fig. 7, lane 9. Segments 4 and 5 resulted from deletions of 0.8 and 1.5 kb, relative to segment 2, from the  $S\mu$  region. Segment 6 has deleted 3.1 kb, including sequences 5' to the  $S\mu$  region, but not 3' to it. From the genomic DNA mapping data, it is possible that the  $J_{H4}$  segment has been deleted. This map (6) corresponds to the smallest fragment in Fig. 7, lanes 9 and 24.

black bars (above the restriction enzyme maps) labeled  $J_{H1-2}$ ,  $J_{H3-4}$ , M2-5B,  $\alpha$  (33), and  $\epsilon$  (36). As the expressed  $V_H$  gene has undergone a recombination with the  $J_{H4}$  gene segment, thereby deleting the  $J_{H1-3}$  genes, the probe for the  $J_{H1-2}$  genes will not anneal with the expressed  $VDJ_4$  allele. The  $J_{H1-2}$  probe will, however, anneal with the recombined  $DJ_{H2}$  gene segment from the nonexpressed H chain chromosome (9) (Fig. 6), enabling us to use the  $J_{H1-2}$  probe to distinguish the expressed and nonexpressed alleles.

Fig. 7 shows blots of *Bgl* II fragments of I.29 cell DNA annealed with the IgH chain probes indicated below each blot. The  $J_{H3-4}$  probe detected two rearranged  $J_H-C\mu$  *Bgl* II fragments in the IgM.S and I.29 $\mu$  cells passaged *in vivo* (lanes 3 and 4), the larger of which (16 kb) encodes the expressed  $VDJ_4-C\mu$  gene, and the smaller of which (7.7 kb) encodes the nonexpressed  $DJ_{H2}-C\mu$  allele (9). The fragments detected by the  $J_{H3-4}$  probe, which also annealed with the  $J_{H1-2}$  probe, are indicated by small black dots on the right edge of the bands detected by the  $J_{H3-4}$  probe.

IgA<sup>+</sup> cells from the I.29 tumor, i.e., cells that have switched *in vivo*, of which the cloned cell line BFO.3 is typical (25), contain an expressed  $\alpha$  gene present

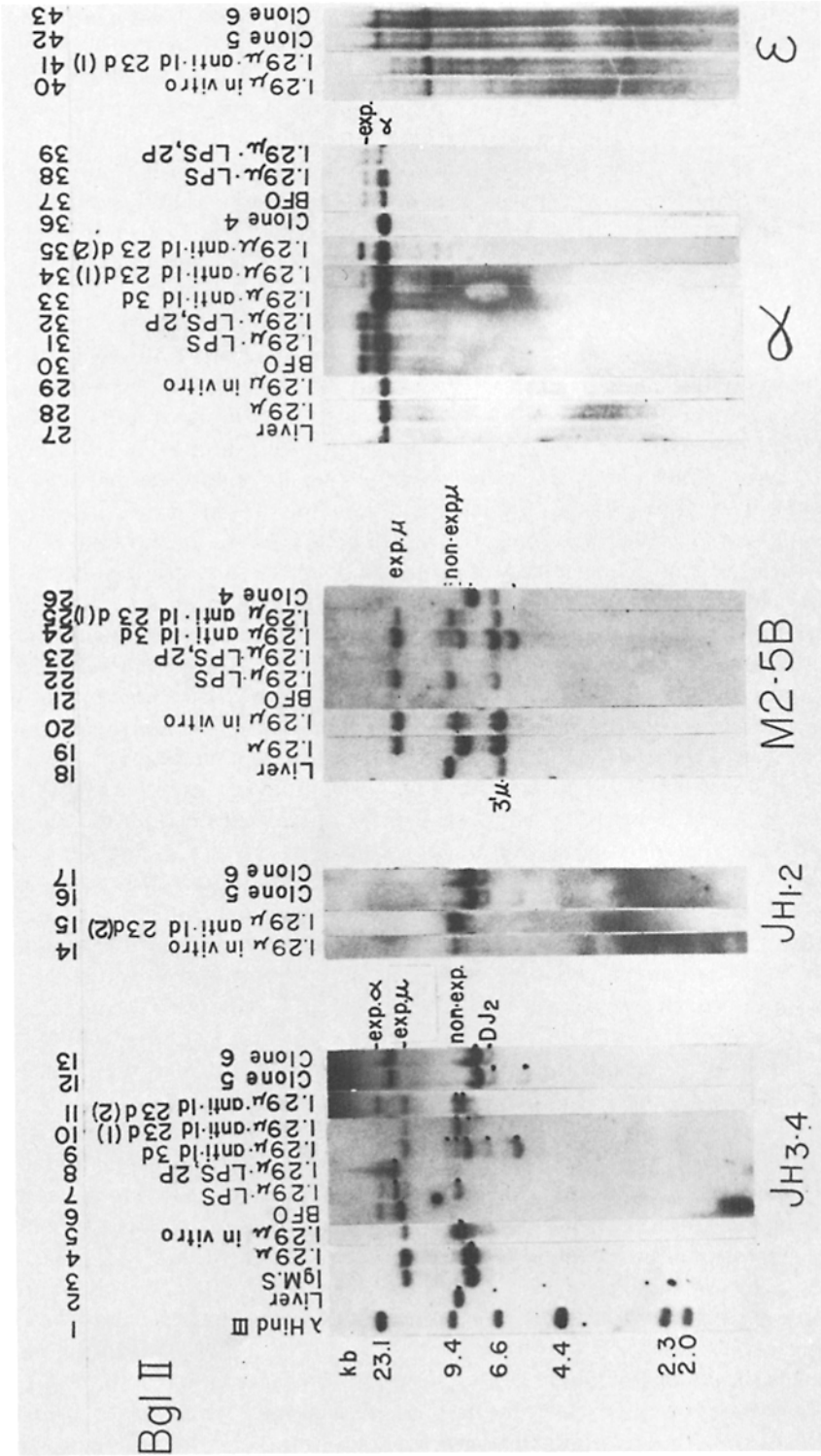


FIGURE 7. Blots of 1.29 cell DNA digested with Bgl II. Sizes of the Hind III fragments of phage λ DNA, end-labeled with 32P using the Klenow fraction of *E. coli* DNA polymerase (lane 1), and electrophoresed in a parallel lane, are given in kilobases, on the left. Sources of the DNA are described in the text. Probes used are indicated below each blot. Bgl II fragments from the expressed VDJ<sub>α</sub> segments are larger than those from the nonexpressed DJ<sub>α</sub>-C<sub>μ</sub> segments. The positions of the Bgl II fragments encoding the expressed α (exp. α), expressed μ (exp. μ) and nonexpressed DJ<sub>α</sub>-C<sub>μ</sub> segments are indicated.

on a large Bgl II fragment (25.5 kb in BFO.3 cells), which anneals with both the  $J_{H3-4}$  and  $\alpha$  probes (lanes 6 and 30), but not with the  $J_{H1-2}$  probe (9). This assignment has been verified by analyses of cloned  $\alpha$  genes and of  $\alpha$  RNA in I.29 cells (data not shown). The map of this expressed  $\alpha$  gene segment is given in Fig. 5 B, 6. The nonexpressed  $DJ_{H2}$  segment in BFO.3 cells (the 17 kb Bgl II fragment in lane 6, marked with a dot) has recombined with the  $\gamma_3$  gene, as demonstrated by the fact that BFO.3 cells contain one rearranged  $\gamma_3$  gene (and no germline  $\gamma_3$  gene), which comigrates with the nonexpressed  $DJ_{H2}$  segment using two different restriction enzymes, and by analysis of a  $DJ_2-C\gamma_3$  segment cloned from BFO.3 cells (data not shown).

*S $\mu$  Regions Undergo Deletions and Expansions When the IgM<sup>+</sup> Cells Are Adapted to Culture.* The expressed and nonexpressed  $\mu$  gene segments differed in size between the in vivo and in vitro lines of I.29 $\mu$  and IgM.S cells. The expressed  $\mu$  fragment has undergone a small (~0.3 kb) deletion in the  $S\mu$  region in both the I.29 $\mu$  and IgM.S in vitro cell lines relative to the in vivo lines. This was not apparent in Bgl II digests, but was obvious with other enzymes (see maps; Fig. 5 A, 2 and 3). The nonexpressed  $DJ_H-C\mu$  fragment was 0.7 kb larger in three independently produced in vitro lines (two I.29 $\mu$  lines and one IgM.S line) than in the in vivo lines from which they were derived. (Fig. 7, compare lanes 4 and 5; see also maps of nonexpressed  $DJ-C\mu$  segments in Fig. 6, 1 and 2.) The nonexpressed  $DJ_H-C\mu$  fragments in the in vitro I.29 $\mu$  cells were heterogeneous in size, as indicated by the fuzzy edges of the 8.4-kb Bgl II band detected by the  $J_{H3-4}$ ,  $J_{H1-2}$ , and M2-5B probes (Fig. 7, lanes 5, 14, and 20). The tandemly repeated  $S\mu$  sequences lie between the Sst I and Hind III sites located 5' to the  $C\mu$  gene segment (indicated on the map of the germline  $C\mu$  gene, Fig. 5 A, 1 and on the map of the in vivo  $DJ-C\mu$  segment, Fig. 6, 1), although they may extend beyond these sites, as their exact borders have not been defined (7). Our mapping data indicate that the increase in size of the nonexpressed  $DJ-C\mu$  fragment in cultured IgM<sup>+</sup> cells was due to an increase in size of the  $S\mu$  region (Fig. 6, 1 and 2), whereas regions 3' to  $S\mu$  remained unaltered. The blot in Fig. 7, which was annealed with the M2-5B probe, contains a band marked 3'  $\mu$  that corresponds to the 6-kb Bgl II fragment encoding the  $C\mu$  gene and 3' flanking sequences. This band is identical in size in liver and I.29 cells (Fig. 7, lanes 18–20 and 22–26), except it is absent from cells that have deleted both  $\mu$  genes, e.g., BFO.3 (lane 21). Thus, the alterations in size of the nonexpressed  $DJ-C\mu$  segment were localized to the 5' side of the  $C\mu$  gene.

*S $\mu$  Regions On the Nonexpressed Allele Underwent Deletions and Further Expansion Shortly After Induction.* When ammonium sulfate-precipitated anti-Id was added to I.29 cells for 3 d, most of the nonexpressed  $DJ-C\mu$  fragments, but not the expressed  $VDJ-C\mu$  fragments, underwent a variety of deletion events to yield three different predominant smaller fragments (Fig. 7, lanes 9 and 24). Maps obtained by digestion of I.29 cell DNA with Sst I, Hind III, Pst I, Eco RI, and Bam HI, and by hybridization with the  $J_H$  and M2-5B probes indicate that these deletions occurred within and 5' to the  $S\mu$  region (Fig. 6, 4–6). The fragment mapped in Fig. 6 has undergone a 3.1-kb deletion that appears to include  $J_{H4}$ , according to our genomic DNA mapping data. At this early time after adding anti-Id, some of the  $DJ_2-C\mu$  fragments have not undergone deletion, but instead

have enlarged by 0.6 kb, as indicated by the presence of a faint 9-kb fragment (the largest fragment marked by a dot in Fig. 7, lane 9).

The large variety of nonexpressed DJ-C $\mu$  fragments that were observed after 3 d of treatment were not observed at later times after induction, but rather such cultures usually contained only two Bgl II fragments from the nonexpressed allele (Fig. 7, cf. lanes 7, 10, 11, 15, and 25). Surprisingly, the smaller DJ-C $\mu$  Bgl II fragments present after 3 d of induction were not present at later times. We did not detect recombination of any other C $_H$  gene with the nonexpressed DJ segment in these cultures, except with the C $\alpha$  gene in one experiment (I.29 $\mu$ -LPS, Fig. 7, lanes 7 and 38). In this culture of I.29 $\mu$  cells that had been treated with LPS 31 d earlier, the nonexpressed DJ $_{H2}$  segment was present on two different Bgl II fragments detected by the J $_{H3-4}$  probe (marked by dots in lane 7). The smaller fragment (8.4 kb) annealed with the M2-5B probe (lane 22), whereas the larger fragment (18 kb) annealed with the  $\alpha$  probe (lane 38). Note that the smaller  $\alpha$  band in I.29 $\mu$ -LPS cells appears to be a doublet consisting of a germline  $\alpha$  gene and a slightly larger  $\alpha$  fragment.

These LPS-treated cells were subsequently passaged in mice two times. This resulted in an increase of the IgA $^+$  cells from 30% before passage in vivo to 73% after passage (lanes 8, 23, and 39). The expressed  $\mu$  gene and the 8.3-kb Bgl II fragment that encoded the nonexpressed DJ $_{H2}$ -C $\mu$  fragment have mostly disappeared, but the larger DJ $_{H2}$ -C $\alpha$  fragment (18 kb) remained (lanes 8 and 39).

The IgE $^+$  clones contained nonexpressed DJ $_2$ -C $\mu$  fragments that differed in size from the DJ $_2$ -C $\mu$  fragments in the LPS- or anti-Id-treated cultures (Fig. 7, compare lanes 7 and 11 with 12 and 13), which further attests to the heterogeneity in the sites of recombination on the nonexpressed chromosome.

*Switch Regions of Nonrearranged  $\alpha$  Genes Have Undergone Deletions 3 d After Addition of Anti-Id.* Within 3 d after the addition of ammonium sulfate-precipitated anti-Id, some of the DNA fragments encoding the nonrearranged C $\alpha$  gene had undergone a 0.9-kb deletion. The shortened  $\alpha$  fragment can be seen in a blot of Bam HI-digested DNA as a band migrating slightly faster than the germline  $\alpha$  fragment (Fig. 8 A, compare lanes 1 and 7-9 with 5). The shortened  $\alpha$  fragment did not anneal with the J $_{H3-4}$  probe (data not shown). The map of this fragment indicates that the deletion occurred within the S $\alpha$  region (Fig. 5 B, 5). This 0.9-kb deletion was observed in four of five different cultures of I.29 $\mu$  cells that had been treated with anti-Id for 3 d (Fig. 8 A, lanes 1 and 7-9). The one culture that did not exhibit this deletion (Fig. 8 A, lane 6) did not subsequently undergo switching. Cultures that had undergone large amounts of switching to IgA expression (>30% IgA $^+$ ) did not display the shortened  $\alpha$  fragment (lanes 3 and 10-15), whereas one culture that was 30% IgA $^+$  did have this fragment (lane 2).

*Switching to IgA Is Effected by S $\mu$ -S $\alpha$  Recombination.* 3 d after treatment of I.29 $\mu$  cells with ammonium sulfate-precipitated anti-Id, no rearranged expressed  $\alpha$  genes were detected (Fig. 7, lane 33; Fig. 8, lanes 6-9), probably because the proportion of cells containing the expressed  $\alpha$  gene fragment was too small (0-3% of the cells were IgA $^+$ ).

Rearranged, expressed  $\alpha$  gene fragments in amounts that approximately correlated with the proportion of IgA $^+$  cells were apparent in cultures of I.29 $\mu$



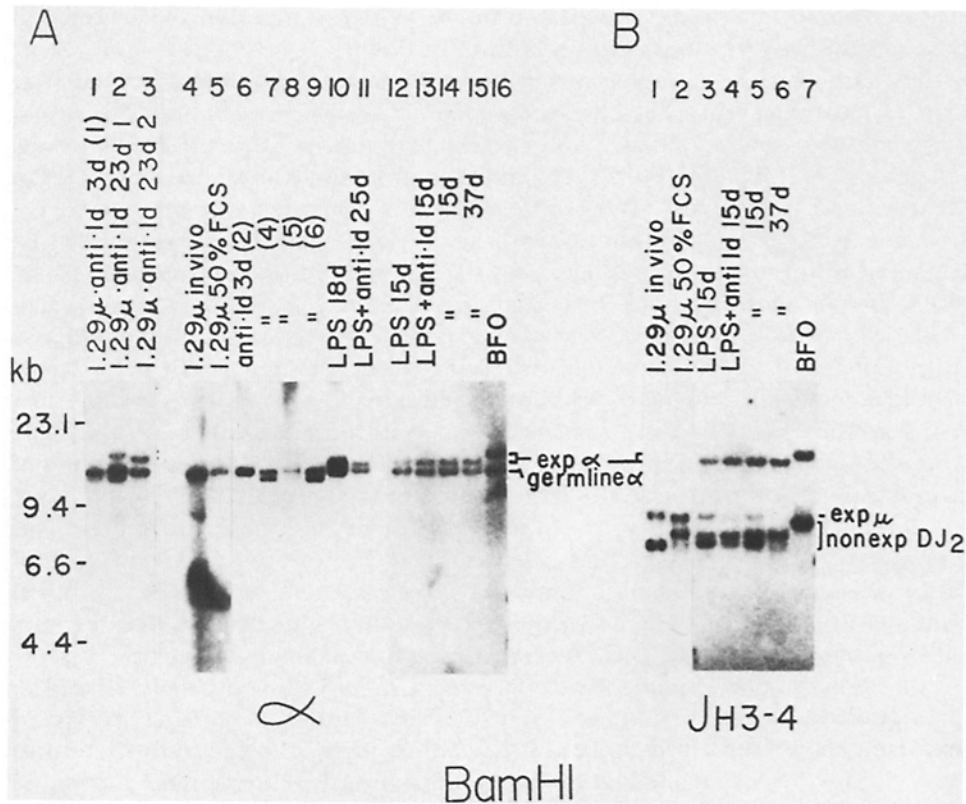


FIGURE 8. Blots of I.29 cell DNA digested with Bam HI. DNA were prepared from untreated I.29  $\mu$  or BFO.3 cells, or from cultures of I.29  $\mu$  cells that had been treated with the indicated reagents. Blots were annealed with the  $\alpha$  (A) or JH3-4 (B) probes. (A) The Bam HI fragments (12.6–15 kb) labeled expressed ["(exp)  $\alpha$ "] in A, lanes 2 and 3 and 10–16, co-migrated with bands detected by the JH3-4 probes (B, lanes 3–7). (JH3-4 data for lanes 2 and 3 in A are not shown.) The germline  $\alpha$  band did not comigrate with bands detected by the JH3-4 probe. Smaller  $\alpha$  gene fragments present in lanes 1 and 2 and 7–9 in A also did not comigrate with bands detected with the JH3-4 probe. (B) The JH3-4 probe detected: (1) the expressed  $\alpha$  segment (12.6–15 kb), (2) the expressed  $\mu$  segment, which was slightly larger (8.3 kb) in the in vivo line of I.29  $\mu$  cells (lane 1) than in the in vitro lines (8 kb) (lanes 2–5), and (3) smaller fragments from the nonexpressed DJ<sub>2</sub>-C $\mu$  segment, which comigrated with fragments detected by the M2-5B probe (not shown), except in BFO cell DNA (lane 7), where the nonexpressed DJ segment comigrated with a fragment detected by the  $\gamma_3$  probe (not shown). The percent of the cells that were IgA<sup>+</sup> in the cultures analyzed in A were: (1) 3, (2) 30, (3) 50, (4–7) 0, (8) 1.1, (9) 2, (10) 27, (11) 99, (12) 32, (13) 74, (14) 58, (15) 100, and (16) 100 cloned IgA<sup>+</sup> cell line. The DNAs present in lanes 12–16 in A were identical to those present in lanes 3–7 in B.

cells that had been treated 23 d previously with the ammonium sulfate-precipitated anti-Id (Fig. 7, lanes 34 and 35), or 15 d earlier with LPS (Fig. 8 A, lane 12), or 15 d earlier with LPS plus anti-Id antibody (Fig. 8 A, lanes 13 and 14). Expressed  $\alpha$  genes were detected in cultures that contained at least 12% IgA<sup>+</sup> cells. The restriction enzyme maps of these rearranged, expressed  $\alpha$  genes indicate that the DNA recombinations that effect switching from IgM to IgA in cultured I.29 cells occur within or immediately 5' to the S $\mu$  and S $\alpha$  regions (Fig. 5 B, 7–9).

*IgE<sup>+</sup> Cells Contain a Rearranged, Expressed  $\epsilon$  Gene.* Southern blots were also performed on genomic DNA from the IgE<sup>+</sup> clones. These clones (clones 4, 5, and 6) contained one germline  $\epsilon$  gene and one rearranged  $\epsilon$  gene, which migrated at the same position as the expressed V<sub>H</sub>DJ gene segment when the DNA was digested with Bgl II (Fig. 7, lanes 12, 13, 49, and 43). The maps of the germline  $\epsilon$  gene and the predominant expressed  $\epsilon$  gene segment in I.29 cells are given in Fig. 5 C, 10 and 11. The DNA recombination between the expressed  $\mu$  and germline  $\epsilon$  genes that effected the switch to IgE expression occurred within or very close to the S $\mu$  and S $\epsilon$  regions, as revealed by the positions of the Sst I and Hind III sites located 5' to the expressed  $\epsilon$  gene (Fig. 5 C, 11).

*Do the Sites of Switch Recombination Differ Among Different Expressed  $\alpha$  Genes?* Maps of three rearranged, expressed  $\alpha$  genes present in three different cultures of cells that had switched in vitro (Fig. 5 B, 7–9) indicate that these fragments were produced either by the use of different sites of S $\mu$ -S $\alpha$  recombination or by different DNA deletion events 5' to the site of switch recombination. The fragments bearing the expressed  $\alpha$  genes mapped in Fig. 5 B, 7–9, are from DNA shown in Fig. 8 A, lanes 2 and 3 and in Fig. 7, lanes 31, 34, and 35, respectively (lanes 34 and 35 are from different gels). The presence of three different expressed  $\alpha$  fragments in these three populations suggests that these cultures were each dominated by a different clone of IgA cells. Consistent with this hypothesis is the fact that the IgA<sup>+</sup> cells in these cultures did not increase significantly in number until late, 15 d, after addition of the ammonium sulfate-precipitated anti-Id antibody or 23 d after addition of LPS. By contrast, in experiments in which cells were treated with LPS or LPS plus anti-Id, and in which switching occurred more rapidly, e.g., in the experiment presented in Table I, the sizes of the Bam HI fragments containing expressed  $\alpha$  genes were much more similar among different cultures (Fig. 8 A, lanes 10–15; Fig. 8 B, lanes 3–6, which contain the same DNA as in 8 A, lanes 12–15, annealed with the J<sub>H3-4</sub> probe). Because the sizes of the Bam HI fragments bearing rearranged, expressed  $\alpha$  genes are very similar among these different cultures, it is probable that the apparent homogeneity, seen in Southern blots, of the expressed  $\alpha$  fragments within these cultures was not due primarily to clonal outgrowth, but to a limited variation in the sites of switch recombination. This hypothesis will be examined by analyses of cloned DNA fragments containing the sites of S $\mu$ -S $\alpha$  recombination.

## Discussion

*IgM<sup>+</sup> Cells From the I.29 Lymphoma Can Be Induced to Undergo Isotype Switching in Culture.* Two types of evidence proved that IgM<sup>+</sup> cells from the I.29 lymphoma can undergo H chain switching in vitro. (a) Cells containing both IgM and IgA within their cytoplasm appeared in cultures treated with LPS or anti-Id plus LPS. The fact that the double-stained cells appeared at early times after induction (4–11 d) and disappeared at later times argues that the double-stained cells were cells that had recently switched to IgA synthesis. (b) Cloned IgM<sup>+</sup> cells, derived from the I.29 $\mu$  cell line, could also be induced to switch to IgA.

Although a low amount of switching occurred in cells cultured in the absence of exogenous inducers, much more switching was induced by treatment with

LPS and even more by the addition of both LPS and a crude or a purified monoclonal anti-Id antibody. The fact that, in cultures treated for 4 d with LPS or LPS plus anti-Id, a much greater proportion of the IgA<sup>+</sup> cells also contained IgM than in cultures that did not receive these inducers argues that these reagents actually induced switching, rather than simply permitting the preferential outgrowth of previously existing IgA<sup>+</sup> cells.

The viability of LPS-treated cultures was only slightly lower than that of untreated cultures at day 4, although by 8 d after LPS treatment the cell viability was very low. By 11–15 d, the viability again increased. Thus, the accumulation of IgA<sup>+</sup> cells, which began after 4 d but before 8 d, started before the general increase in cell viability. Since the percent of cells that were IgA<sup>+</sup> 4–11 d after induction was similar whether only live or both live and dead cells were counted, the accumulation of IgA<sup>+</sup> cells was not due simply to their preferential viability after LPS treatment. However, it is possible that a portion of the increase in IgA<sup>+</sup> cells could be due to more rapid proliferation after LPS treatment.

Anti-Id and LPS appeared to be synergistic because the purified anti-Id by itself did not induce any switching, nor did an increase in the amount of LPS added produce a greater proportion of IgA<sup>+</sup> cells. It is possible that the monoclonal anti-Id antibody by itself did not induce switching because it could not sufficiently cross-link the Ig receptors on the cell surface. Because anti-Id and LPS presumably interact with different receptors (49, 50), they could, potentially, stimulate synergistically.

Hamano and Asofsky (51) have described an IgM<sup>+</sup> B cell hybridoma that could be induced by anti-IgM in the absence of LPS to switch to IgG2a expression. Thus far, three different anti-IgM antibodies have failed to reproducibly induce I.29 $\mu$  cells to switch: one monoclonal rat anti-mouse IgM, and two goat anti-mouse IgM antibodies, one of which had been shown to be effective in inducing Ig secretion when added to B cells in the presence of T cell factors (31, 52). The rat monoclonal anti-IgM did induce a small amount of switching in one experiment (not shown). Anti-IgM in the presence of LPS killed I.29 $\mu$  cells and did not induce switching.

The role of T cells in the induction of switching is problematic. The evidence presented here and that of Mongini et al. (19, 20) demonstrates that switching can occur in the absence of T cells. It has also been shown, however, that T cells or T cell factors can enhance the switching to certain isotypes by antigen- or LPS-stimulated B cells (18–20, 53). It is possible that the addition of T cells to LPS- or anti-Id-stimulated I.29 $\mu$  cells would augment the number of cells that have undergone isotype switching and/or would influence the choice of isotype.

*Specificity of Isotype Switching.* A number of experiments (1, 20–23) indicate that splenic IgM<sup>+</sup> B cells are not committed to switch to a specific isotype. Under our conditions, however, I.29 cells switch most frequently to IgA, and less frequently to IgE or IgG2, but not at all to IgG3 or IgG1. Furthermore, in the IgM<sup>+</sup> cells from the I.29 lymphoma, the state of activation, as assayed by level of methylation and by transcription, of the nonrearranged  $\alpha$  and  $\epsilon$  genes, differs from that of the  $\gamma_1$  genes (44; J. Stavnezer and S. Sirlin, manuscript in preparation). Thus, I.29 cells may represent a differentiated state of B cells predetermined to switch only to certain isotypes. Further evidence for precommitment

of subpopulations of B cells to certain isotypes is found in the work of Kawanishi et al. (18). These authors showed that a higher proportion of IgM<sup>+</sup> B cells from Peyer's patches than from spleen would switch to IgA when stimulated with LPS in the presence of cloned IgA-switch helper T cells. The data indicate that, in the absence of T cells, B cell clones are not restricted to switching to only one isotype, nor can they switch to all isotypes. Instead, they appear to be capable of switching to a subset of isotypes. Furthermore, our data support that of others (20–23, 54) indicating that the precursors for IgG<sup>+</sup>, IgA<sup>+</sup>, and IgE<sup>+</sup> cells are not distinct.

From an examination of  $\gamma$  mRNA in I.29 cells treated with LPS, we concluded that switching to IgG2, but not to IgG3 or IgG1, occurred in some cultures. This result is inconsistent with the hypothesis that the frequency of switching to the various  $\gamma$  subclasses in the absence of T cells is determined simply by the order of C<sub>H</sub> genes on the chromosome, with genes located 5' being favored (8, 19). These data also argue against the hypothesis that switching occurs successively among  $\gamma$  subclasses (8, 19, 34). Similarly, although the appearance of IgE<sup>+</sup> cells among I.29 $\mu$  cells that have been treated with LPS and/or anti-Id was usually transient, it does not appear likely that IgA<sup>+</sup> cells were usually derived from IgE<sup>+</sup> cells, because among 74 IgE<sup>+</sup> cells examined in cultures that were actively switching to IgE and IgA, no cells were observed that stained with both anti-IgE and anti-IgA simultaneously.

*DNA Rearrangements Occur Within 3 d After Induction of Isotype Switching.* By 3 d after the addition of the ammonium sulfate-precipitated anti-Id to I.29 $\mu$  cells, two types of DNA rearrangements were observed. (a) The S $\alpha$  region associated with some of the nonrearranged  $\alpha$  gene fragments underwent a 0.9-kb deletion. Because this shortened  $\alpha$  gene fragment disappeared from cultures that contained a large proportion of IgA<sup>+</sup> cells, it is possible that this deletion event is an intermediate in the process of switching. This could be the consequence of the binding of the switch recombinase enzyme to the S $\alpha$  region, which results in a recombination of sequences within the S $\alpha$  region, independently of a recombination with the S $\mu$  region. This is consistent with the fact that the occurrence of deletions within the S $\alpha$  region was not necessarily followed by switching, as a number of the IgM<sup>+</sup> cell lines cloned from I.29 $\mu$  cells have  $\alpha$  genes bearing the shortened S $\alpha$  region (data not shown). This deletion may be part of an aborted attempt to switch. It is likely that the deletion occurred only on the expressed H chain chromosome, because in cultures that had switched extensively, the shortened  $\alpha$  gene was not detected. Instead, a normal-sized germline  $\alpha$  gene fragment was present, which must be from the nonexpressed H chain chromosome. The cloned IgE<sup>+</sup> cell lines have also suffered a deletion within the S $\alpha$  region of one of their two  $\alpha$  genes, but of 0.6 kb rather than of 0.9 kb. (b) The other type of recombination event detected within 3 d after induction involved S $\mu$  sequences and the region 5' to the S $\mu$  sequences on the nonexpressed chromosome only. From an examination of the intensity of bands produced by annealing blots with the J<sub>H1-2</sub> and M2-5B probes, one can conclude that ~90% of the DJ<sub>H2</sub>-C $\mu$  fragments from the nonexpressed chromosome have undergone recombination that resulted in either an increase in size or a deletion of sequences (Fig. 7, lanes 9 and 24). The rapidity with which recombinations

occurred and the variety of recombination events that were detected was surprising, especially since no recombination events involving the  $S\mu$  region of the expressed chromosome were detected at this early time. It is possible that recombination events between  $S\mu$  and  $S\alpha$  sequences on the expressed chromosome occur much less frequently or more slowly because the  $S\mu$  and  $S\alpha$  regions, which are located  $\sim 200$  kb apart, must first be juxtaposed. To explain why no deletion events were detected involving only the  $S\mu$  region of the expressed  $\mu$  gene, we suggest that DNA recombination events on the expressed chromosome are limited in their variety, perhaps because they are more specifically controlled. It is possible that the two alleles could be distinguished by the recombination machinery because the expressed allele may be transcribed at a higher rate than the nonexpressed allele. The steady state level of RNA in IgM.S cells from the expressed allele is  $\sim 100$  times greater than the amount of RNA from the nonexpressed allele, as judged by hybridization with the  $J_{H3-4}$  and  $J_{H1-2}$  probes, respectively (not shown). These probes each hybridize to approximately the same number of nucleotides in spliced RNA in I.29 cells.

We have not detected a rearranged expressed  $\alpha$  gene at very early times after the addition of anti-Id. However, it is obvious, from the occurrence of DNA recombinations within cells that had been treated for 3 d with anti-Id, that a DNA recombinase able to recombine switch sequences has been activated in these cells. It is likely that we have not detected a rearranged expressed  $\alpha$  gene fragment at this early time because only a small percent of the cells have undergone switch recombination ( $<3\%$  of the cells in the culture after 3 d of induction expressed IgA). By 15 d after the addition of LPS or anti-Id plus LPS to I.29 $\mu$  cells, predominant rearranged Bam HI fragments bearing expressed  $\alpha$  genes were detected (Fig. 8, lanes 12–14). These data are consistent with the hypothesis that switching in I.29 cells is effected by DNA recombinations.

The results of Hurwitz and Cebra (10) and Radbruch and Sablitsky (11) suggest that DNA recombinations that effect switching occur at early times after the addition of switch-inducers to normal B cells. These authors treated spleen cells with LPS and, after 4 or 6 d, isolated LPS blasts that did not express IgM, or that had membrane-bound IgG3. Both groups found that  $\sim 50\%$  of the  $C\mu$  genes had been deleted from these cells. It was not possible, however, to determine whether the  $C\mu$  genes had been deleted from the expressed and/or nonexpressed H chain chromosome, since the cells were not clonal in origin.

*Specificity of Recombination Sites.* Examination of independent switching events in a cloned cell line may lead to an understanding of the specificity of the DNA sequences recombined during switch recombination. Although the overall size of the fragments containing the sites of  $S\mu$ - $S\alpha$  recombination appeared identical among several cultures of I.29 cells that had switched in vitro, it is likely that the actual sites of recombination differ. The size of Sac I fragments bearing the rearranged expressed  $\alpha$  gene in IgA<sup>+</sup> cells from the noncloned tumor also appeared identical in genomic Southern blots (9). However, the sites of  $S\mu$ - $S\alpha$  recombination among six different expressed  $\alpha$  genes cloned from IgA<sup>+</sup> cells in the tumor all appeared to differ from each other when examined by mapping with restriction enzymes that recognize four basepair sequences within the Sac I fragments (J. Stavnezer, unpublished data). These differences were produced by

independent switch recombination events, because the sites of  $S\mu$ - $S\alpha$  recombination in two independent clones of the expressed  $\alpha$  gene present in a long-term culture of the cloned  $IgA^+$  cell line (BFO.3), appeared identical by the same criterion (D. Klein and J. Stavnezer, manuscript in preparation). The lack of specificity as to the precise sites recombined is as expected from an examination of myeloma cell DNA (7, 55).

*I.29 as a Model for H Chain Switching.* The I.29 cell line differs from other cell lines capable of undergoing isotype switching in vitro, i.e., pre-B cell lines (56–58) and various myeloma and hybridomas (59), as switching in these systems occurs spontaneously at a low frequency and cannot be induced by LPS, a reagent that induces switching in cultured normal B cells (15, 60). In myeloma and hybridoma cells that switch in vitro, DNA recombinations do not occur within or near the tandemly repeated switch sequences (59, 61), whereas in I.29 cells and in cells that have switched in vivo, DNA recombinations have occurred within or immediately 5' to the S sequences (7, 46, 55). Finally, switching by myelomas and hybridomas generally occurs to the  $C_H$  gene located immediately 3' to the gene being expressed (59, 62), whereas, like I.29 cells, single clones of normal B cells appear to be capable of switching from IgM directly to IgA or to IgE (20, 21, 54, 63). Therefore, the I.29 cell line provides a unique and apparently physiological model for isotype switching by normal mature B cells.

### Summary

The murine B cell lymphoma I.29 contains cells expressing surface IgM or IgA with identical heavy chain variable regions (9, 25, and D. Klein and J. Stavnezer, unpublished data). Purified  $IgM^+$  cells from the lymphoma have been adapted to culture and induced to switch to IgA, IgE, or IgG2 by treatment with lipopolysaccharide (LPS) or by treatment with a monoclonal anti-I.29 antiidiotype plus LPS. Clones of  $IgM^+$  cells have been obtained and induced to switch. Under optimal conditions, 30% of the cells in the culture expressed IgA 8 d after the inducers were added, and by 15 d 90% of the cells were  $IgA^+$ . In actively switching cultures, up to 50% of the cells whose cytoplasm stained positively with anti-IgA stained simultaneously with anti-IgM, which indicates that the appearance of  $IgA^+$  cells in the cultures was due to isotype switching and not to clonal outgrowth. Examination by Southern blotting experiments of the Ig heavy chain genes in I.29 cells before and after switching revealed that isotype switching was accompanied by DNA recombinations that occurred within or immediately 5' to the tandemly repeated switch sequences. Within 3 d after the addition of inducers of switching, the nonexpressed chromosome underwent a variety of deletions or expansions within the  $S\mu$  region, and a portion of the  $S\alpha$  regions had undergone a 0.9-kb deletion. In cultures that contained at least 12%  $IgA^+$  cells, rearranged, expressed  $\alpha$  genes, produced by recombination between the  $S\mu$  region within the expressed  $\mu$  gene and the  $S\alpha$  region, were detected.

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*Note added in proof:* By hybridizing RNA blots with DNA probes specific for either the  $\gamma 2b$  or  $\gamma 2a$  genes (encoding the  $C_{H3}$  domains, given by Drs. S. Tilley and B. Birshstein, Albert Einstein Medical College, NY), we have established that I.29 $\mu$  cells switch to IgG2a but not to IgG2b.

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