DNA Rearrangement Can Account for In Vitro Switching to IgG1

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Summary

During immune responses, B lymphocytes may switch from the expression of immunoglobulin M (IgM) to the expression of another isotype (e.g., IgG, IgE, IgA). In stable hybridomas and myelomas expressing a "switched" (S) isotype, DNA deletions between Sμ and a "downstream" S region (S region recombination) have been found. In primary B cells, studies of the molecular basis of switching have been limited by the ability to sensitively quantitate the amount of DNA deletion; such studies would be of interest because other nondeletional mechanisms (*trans*-splicing, alternative processing of a long transcript) have been proposed to account for isotype switching in certain circumstances. We have applied the digestion-circularization polymerase chain reaction (DC-PCR) technique to measure the amount of S region recombination that occurs in the course of class switching in primary B lymphocytes. Resting B cells were cultured in lipopolysaccharide (LPS) and interleukin 4 (IL-4) to stimulate switching to IgG1. These cells begin to express membrane IgG1 at day 2.5 of culture and reach maximum expression by day 4.5. DNA was prepared from cultured cells and analyzed for Sμ-Sγ1 rearrangement by DC-PCR. Chimeric switch regions, indicating Sμ-Sγ1 recombination, were detected in amounts that, in most cases, correlated with surface expression. Furthermore, when cells were sorted on the basis of surface IgG1 expression, a mean of at least one Sμ-Sγ1 rearrangement per cell was seen in five out of seven experiments. In general, the $IgG1^+$ cells obtained at 4.5 and 5.5 d of culture had close to 2 Sμ-Sγ1 rearrangements per cell. In $IgG1^-$ cells, Sμ–Sγ1 rearrangements were detectable, but at frequencies substantially lower that in $IgG1^+$ cells. Thus, these results indicate that DNA deletion accompanies class switching in normal B cells stimulated with LPS and IL-4.

Immunoglobulin class switching is known to be associated with DNA deletion events resulting in the excision of DNA between the Sμ region and the switch (S)[1] region of a downstream isotype (1). Although it is clear that the majority of myelomas and hybridomas that express "switched" isotypes have undergone a DNA deletion event of this type, there is some doubt as to the general relevance of this process in more physiologic switching circumstances (2). Indeed, evidence has been obtained consistent with alternative splicing of very long transcripts or with trans-splicing in certain circumstances (3–7). It has also been suggested that one of these mechanisms may be an important intermediate in the deletional switching process (8).

DNA deletion has been demonstrated to occur during in vitro switching by the isolation of predicted excision circles (9–11). Efforts to quantitate DNA deletion during in vitro switching, particularly in cells stimulated by LPS and IL-4, a model in which very vigorous switching to IgG1 and IgE occurs in vitro, have been described (12, 13). However, the technique used for these measurements, quantitative Southern blotting, lacks the quantitative power to exclude a major role for nondeletional mechanisms early in development. Because quantitative Southern blotting to measure switch DNA deletion relies on the loss of the germline configuration, it can only measure cases where substantial DNA rearrangement has occurred and would not be useful where a small amount of DNA rearrangement has occurred (e.g., the beginning stages of class switching). For that reason, we have used a newly developed quantitative PCR assay, digestion-circularization PCR (DC-PCR), to measure class switching DNA rearrangement (14). DC-PCR has advantages over other methods of detecting switch rearrangement (Southern blotting, or direct PCR) (15, 16) in that (*a*) a positive signal is produced from switched DNA, (*b*) no signal is produced from germline DNA, and (*c*) a single-size product is produced, which can then be quantitated. To accomplish this, genomic DNA is first digested with restriction enzymes such that the junction of the S regions that have been brought together lie on a DNA fragment with known EcoRI ends. Then, these fragments are circularized by ligation under dilute DNA con-

[1] *Abbreviations used in this paper:* CSB, cold staining buffer; DC-PCR, digestion-circulation PCR; nAChR, nicotinic acetylchlorine receptor B subunit gene; PI, propidium iodide; sIgG1, surface IgG1, S region, switch region.

centrations, favoring intramolecular ligation. Finally, PCR amplification is performed across the ligated EcoRI ends. A uniform-sized fragment is produced regardless of the structure of the S region junction.

For switching to IgG1, quantitation relies on the measurement of the number of copies of Sμ–Sγ1 and of a control gene, the nicotinic acetylcholine receptor β subunit gene (nAChR, nAChRe). Because nAChR DC-PCR produces a signal from unrearranged DNA, it serves as a control for DNA preparation, restriction digestion, and ligation. The amount of Sμ–Sγ1 rearrangement is determined from the ratio of Sμ–Sγ1 and nAChR copies. The measurement of Sμ–Sγ1 and nAChR copies relies on the introduction of known amounts of competitive plasmid templates p4AP and p2A0, respectively (14, 17). Normal B cells induced to switch to IgG1 by LPS and IL-4 were analyzed for surface IgG1 expression and DNA rearrangement (Sμ–Sγ1) at several different time points. At all time points analyzed, sufficient DNA rearrangement had occurred to account for the number of cells expressing surface IgG1. Furthermore, analysis of Sμ–Sγ1 DNA rearrangement from cells sorted on the basis of IgG1 expression showed that most $IgG1^+$ cells had at least one chromosome rearranged and that a small percentage of $IgG1^-$ cells had at least one chromosome rearranged. Thus, our experiments support the notion that DNA rearrangement occurs with expression of the new isotype in normal B cells.

Materials and Methods

Cell Culture. Resting B cells were prepared from spleens of 8–12-wk-old female C57BL/6 mice obtained from the Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD) as described previously (18). Briefly, dispersed splenocytes were depleted of T cells by treatment with anti-T cell antibodies (monoclonal rat anti-mouse anti-Thy 1.2 (HO-13-4-9) (19), anti-CD5 (53-7.313) (20), and anti-CD8 (53.6.7) (20), or (3.155) (21) followed by monoclonal mouse anti-rat antibody (MAR 18.5) (22) plus guinea pig complement (GIBCO BRL, Gaithersburg, MD). From the resulting B cell–enriched fraction, small dense resting cells (66–70% fraction) were isolated by centrifugation through a discontinuous Percoll (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) gradient. These cells were cultured in RPMI 1640 (Biofluids, Rockville, MD) supplemented with 5% fetal bovine serum (Biofluids), 2 mM L-glutamine (Biofluids), 50 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), 1 U/ml penicillin G potassium (Biofluids), 1 μg/ml streptomycin sulfate (Biofluids), plus 20 μg/ml LPS (LPS W *Escherichia coli* O111:B4; Difco Laboratories, Detroit, MI) with or without recombinant mouse IL-4 (10,000 U/ml, ~0.46 nM) (23) at 37°C and 6% CO_2.

Cell Surface Marker Analysis and Sorting. Cell surface IgG1 and IgM was measured by standard flow cytometry techniques (24). Briefly, B cells were washed three times in cold staining buffer (CSB) (3% fetal bovine serum in HBSS without phenol red [Biofluids]) and resuspended at a final concentration of $5-10 \times 10^6$ cells/ml of CSB. One hundred μl of cell suspension was stained with goat anti-mouse anti-IgG1 coupled to FITC and/or anti-IgM coupled to R-PE (Southern Biotechnology Associates, Inc., Birmingham, AL) at a final concentration of 10 μg/ml for 30 min at 4°C. Stained cells were washed three times in CSB, and resuspended in 500 μl CSB plus 10 μl 50 μg/ml propidium iodide (PI) (Sigma Chemical Co.) (in 0.1% sodium citrate [Mallinckrodt, Inc., Paris, KY] in PBS, pH 7.4) (Biofluids) before cytometric analysis by FACScan® or cell sorting by FACStar^{plus}® (both from Becton Dickinson Immunocytometry Systems, San Jose, CA) or Epics 753 (Coulter Electronics, Inc., Hialeah, FL). Dead cells and clusters were removed from analysis or sort by gates on PI staining and on size using forward and side scatter.

DNA. DNA from B cells was prepared by standard methods (25). Briefly, the cells were lysed in 0.5% SDS (National Institutes of Health [NIH] Media Unit, Bethesda, MD), 10 mM Tris-HCl, pH 7.5, (NIH Media Unit), 100 mM NaCl (Mallinckrodt), 50 mM EDTA (NIH Media Unit), and 0.2 mg/ml proteinase K (Sigma Chemical Co.) at 37°C overnight. The lysate was extracted with an equal volume of phenol (Fluka Chemie AG, Buchs, Switzerland) and Sevag (chloroform:isoamyl alcohol [both from Mallinckrodt] [24:1]) and dialyzed in Spectrapor2 membrane tubing (6.4-mm cylindrical diameter, 12,000–14,000 m.w. cutoff) (Spectrum Medical Industries, Inc., Los Angeles, CA) against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The sample was treated with 1 μl 10 mg/ml RNase (Ribonuclease A, Type X-A; Sigma Chemical Co.) at 37°C for several hours and then extracted and dialyzed as before. DNA concentration was determined by UV absorption at 260 and 280 nm.

Precise DNA concentration of competitive plasmid templates, p4AP and p2AO (14), was determined by fluorometry (Hoefer Scientific Instruments, San Francisco, CA).

DNA oligonucleotide primers were prepared on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) and purified through NAP-5 columns (Pharmacia LKB) under conditions suggested by the manufacturer. Primer concentration was determined by UV absorption at 260 and 280 nm. Primers for control DC-PCR of the nicotinic acetylcholine receptor β subunit gene (nAChR) (5′ GGC CGG TCG ACA GGC GCG CAC TGA CAC CAC TAA G and 5′ GCG CCA TCG ATG GAC TGC TGT GGG TTT CAC CCA G) generate a 490-bp PCR product from plasmid p2AO or a 753-bp PCR product from digested and circularized genomic DNA template (14). The 5′ μ primer (5′ GGC CGG TCG ACG GAG ACC AAT AAT CAG AGG GAA G) and 3′ γ1 primer (5′ GCG CCA TCG ATG GAG AGC AGG GTC TCC TGG GTA GG) generate a 265-bp PCR product from p4AP or a 219-bp PCR product from digested and circularized genomic DNA template (14).

DC-PCR. DC-PCR was performed as described previously (14) and is described here in detail.

Genomic DNA (2 μg) was digested in 100 μl with 50 U of restriction endonuclease EcoRI (New England Biolabs, Inc., Beverly, MA) in buffer supplied by the manufacturer at 37°C overnight. EcoRI was subsequently inactivated by incubation at 70°C for 20 min.

Digested DNA samples were diluted to 1.8 μg/ml in standard ligation buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM spermidine, 100 μg/ml BSA, 0.7 mM ATP) (25). For ligation, 2 U of T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD) or 400 U of T4 DNA ligase (New England Biolabs) was added to a final volume of 100 μl and incubated at 16°C overnight. These conditions allow for greater than 90% circularization of digested DNA fragments of size 15 kb or less (26). The Sμ–Sγ1 EcoRI fragment is estimated to be up to 15 kb (27).

To monitor the efficiency of digestion and circularization of each sample, PCR was performed on ligated DNA with nAChR primers. 5 ng of ligated DNA was amplified in 20 μl nAChR PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 μM nAChR primers, 200 μM of each dNTP) (Pharmacia), p2AO (222

copies) and 1.5 U Taq DNA polymerase (Boehringer Mannheim, Corp., Indianapolis, IN) or recombinant Taq DNA polymerase (AmpliTaq; The Perkin-Elmer Corp., Norwalk, CT) overlaid with mineral oil (Sigma Chemical Co.). Amplification was always performed in a DNA Thermal Cycler (Perkin-Elmer Corp.) under the following conditions: 94°C for 6 min, 5 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min; 30 cycles at 94°C for 1 min, 68°C for 1 min, and 72°C for 2 min; 72°C for 7 min.

PCR products were analyzed by standard PAGE (8%) (25). Briefly, 10 μl of sample plus 1 μl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in H_2O) was electrophoresed through 8% polyacrylamide (Bio-Rad Laboratories, Richmond, CA, or National Diagnostics, Atlanta, GA) gels in 1× TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) buffer. After bromophenol blue was electrophoresed to the bottom of the gel, the gel was stained in a 0.5 μg/ml ethidium bromide solution (Sigma Chemical Co.), visualized by a 300-nm UV transilluminator (UVP, San Gabriel, CA), and recorded by photography with Type 57 4 × 5 Instant Sheet Film (Polaroid Corp., Cambridge, MA).

Subsequently, each sample was tested by Sμ–Sγ1 DC-PCR amplification. 5 ng of ligated DNA was amplified as before in Sμ–Sγ1 PCR buffer (2.0 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.5 μM 5′ μ primer, 0.5 μM 3′ γ1 primer, 200 μM of each dNTP) (Pharmacia), p4AP (222 copies) and 1.5 U Taq DNA polymerase.

Quantitative DC-PCR. Initial quantitation of nAChR and Sμ–Sγ1 copy number was done by a competitive substrate method (14, 17). To quantitate nAChR copy number, the competitive plasmid template p2AO was titrated (6,000, 2,000, 667, 222, 74, 25, 8 copies) into a constant amount of digested and circularized genomic template (5 ng). The resulting DNA mixtures were amplified in 20 μl nAChR PCR buffer, 1.5 U Taq DNA polymerase, and 0.1 μl [α-^{32}P]-dCTP (3,000 Ci/mmol) (Amersham Corporation, Arlington Heights, IL). To quantitate Sμ–Sγ1 copy number, the competitive plasmid template p4AP was titrated as above into a constant amount of genomic template. The resulting DNA mixtures were amplified in 20 μl Sμ–Sγ1 PCR buffer, 1.5 U Taq DNA polymerase, and 0.1 μl α-[^{32}P]-dCTP (3,000 Ci/mmol). The competitive plasmid and genomic templates generate different sized PCR products from the same primer set. After resolving PCR products on 8% PAGE, the gels were dried for 1 h at 80°C on a gel dryer (Hoefer or Bio-Rad) and autoradiographed with X-OMAT™ AR diagnostic film (Eastman Kodak Co., Rochester, NY).

Data Analysis. The amount of PCR products generated from plasmid and genomic templates was measured by one of two methods: (*a*) dried gels containing radioactive PCR product were counted on a radioanalytic imaging system (Mark II; AMBIS Systems, San Diego, CA, courtesy of Dr. Ida Owens, National Institute of Child Health and Human Development) or (*b*) autoradiographs were scanned on a computing densitometer (Molecular Dynamics, Inc., Sunnyvale, CA). Bands on the gel or autoradiograph corresponding to plasmid and genomic template PCR product were measured as radioactive counts or arbitrary volumetric units. Background counts or volumes were subtracted. To measure the relative molar amounts of plasmid and genomic template PCR product, the measured counts or volumes were adjusted for the amount of radioactive cytosine incorporated into each product. In nAChR DC-PCR, the measurement of the genomic template PCR product was multiplied by 238/342, because the p2AO generated PCR product contains 238 cytosines and the genomic template generated PCR product contains 342 cytosines. Similarly, in Sμ–Sγ1 DC-PCR, the genomic template generated PCR product measurement was multiplied by 108/89.

The amount of nAChR gene copies in the sample is determined to be the number of copies of p2AO that generate an amount of PCR product equal to the amount generated by the genomic template. The number of Sμ–Sγ1 copies is similarly determined relative to p4AP. The equivalence point where PCR product generated by plasmid and genomic templates are equal was determined graphically. The log(genomic template product/plasmid template product) was calculated and plotted versus the log(plasmid copy number) with the aid of Microsoft Excel (Microsoft Corporation, Redmond, WA) and Cricket Graph (Cricket Software, Malvern, PA) Macintosh programs. A best fit line was used to determine the number of plasmid copies at the equivalence point where log(genomic template product/plasmid template product) = 0.

After determining nAChR and Sμ–Sγ1 copy number for a sample, a value for the amount of Sμ–Sγ1 rearrangement per diploid genome in a sample is given by Sμ–Sγ1 copies/(nAChR copies/2). This number is divided by 1.89, a normalization factor determined from a cell line with known nAChR and Sμ–Sγ1 copy numbers (14), and then expressed as a percentage to yield an accurate measure of Sμ–Sγ1 rearrangement. In theory, this measurement can vary from 0 to 200%, the latter indicating two Sμ–Sγ1 rearrangements per diploid genome.

More accurate quantitation of nAChR and Sμ–Sγ1 copy number in most samples was done by a serial dilution method to ensure that the PCR was performed in the exponential range of the reaction (14, 28). Briefly, each sample, a mixture containing 5 ng digested-circularized genomic DNA plus an amount of plasmid template such that the amount of plasmid was close to the equivalence point, was serially diluted 1:2 four or five times. nAChR and Sμ–Sγ1 DC-PCR amplification was performed on each point of the dilution series in the same solutions and same conditions as described for initial quantitation. PCR products were separated by gel electrophoresis and measured as described above. Calculations to determine the number of nAChR and Sμ–Sγ1 copy numbers are described in the Results in conjunction with Fig. 3.

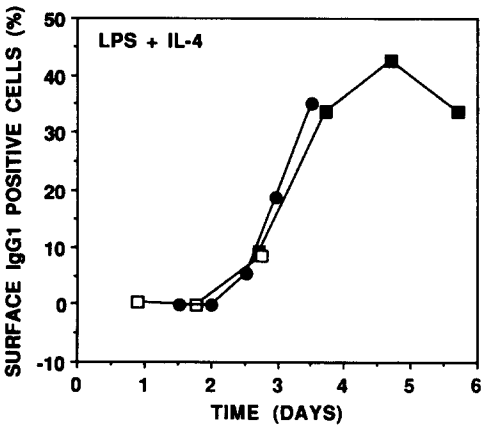

Figure 1. Kinetics of the appearance of IgG1 bearing B cells. B cells were cultured and LPS and IL-4 for the days indicated and IgG1 surface expression was measured by flow cytometry. The results of three experiments are shown. B cells cultured in LPS alone served as the negative control.

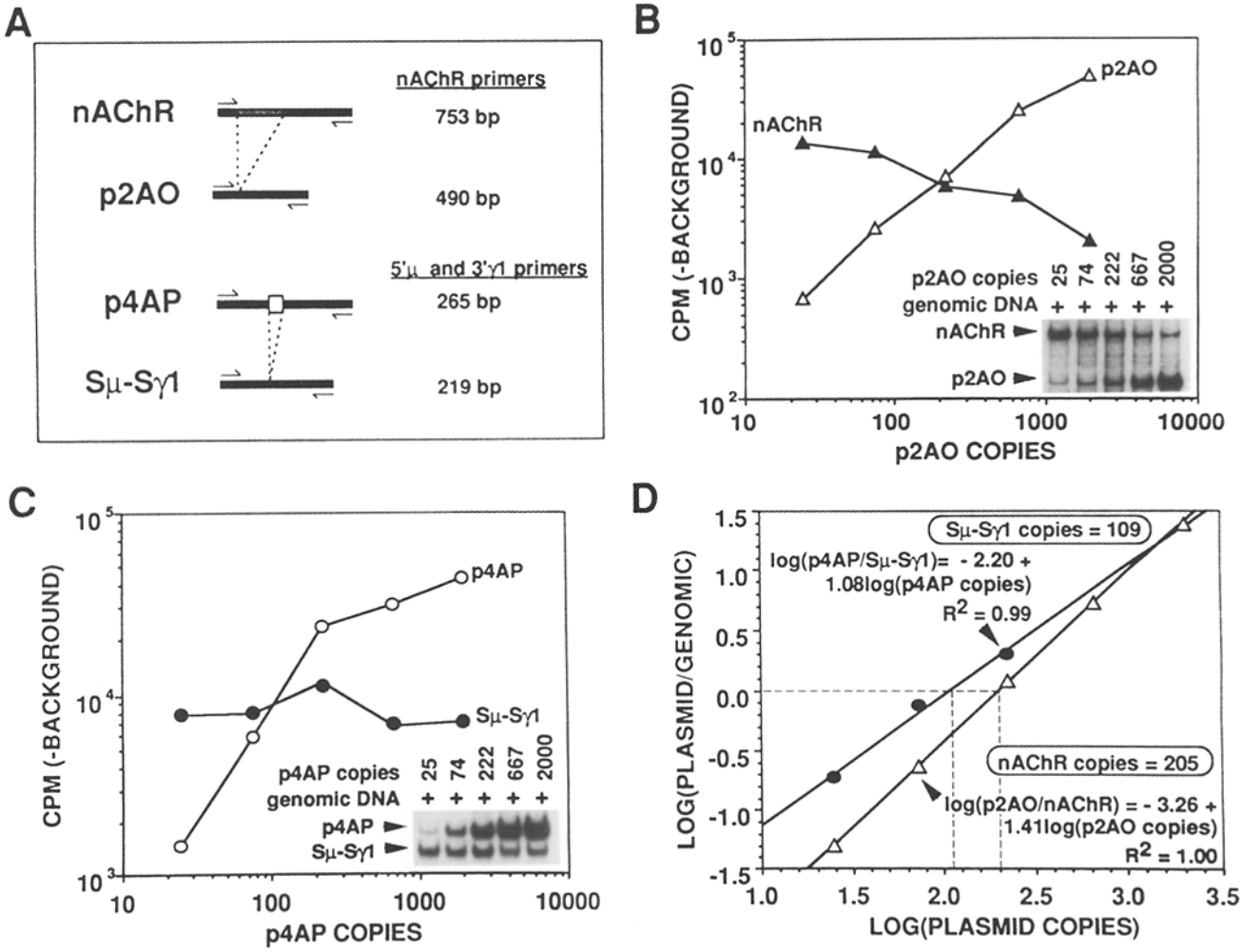

Figure 2. Representative example of quantitation of Sμ–Sγ1 and nAChR copy number by competitive plasmid titration. (*A*) The expected size PCR products and starting templates are shown for nAChR primers and for 5′μ and 3′γ1 primers. Arrows representing primer homologous sequences point in direction of polymerization (5′ to 3′). Thick lines indicate DNA template. nAChR and Sμ–Sγ1 label the genomic template for nAChR and 5′μ and 3′γ1 primers, respectively. Shaded line and connecting vertical dotted lines indicate DNA deleted to make the competitive substrate, p2AO. Open box and connecting vertical dotted lines indicate DNA inserted to make the competitive substrate, p4AP. (*B*) Titration of p2AO into constant amount of digested-circularized genomic template and subsequent PCR on the mixture with nAChR primers is shown. Inset shows autoradiograph of PCR products after separation on 8% PAGE. The number of p2AO copies and the presence of 5 ng genomic template (+) in starting mixture is labeled above each lane. Graph illustrates the gel data after counting. nAChR and p2AO label PCR product from genomic and plasmid template, respectively. (*C*) Same as *B* except to measure Sμ–Sγ1 copies, titration uses p4AP instead of p2AO and PCR uses 5′μ and 3′γ1 primers instead of nAChR primers. (*D*) To calculate nAChR and Sμ–Sγ1 copy number, the results from *B* and *C* are graphed as shown. Copy number is determined from best fit lines where PCR product from plasmid and genomic templates is equal (log[p2AO/nAChR] = 0 and log[p4AP/Sμ–Sγ1] = 0). This sample (IgG1$^+$ B cells obtained by sorting after 2.5 d culture with LPS + IL-4) has 205 nAChR copies and 109 Sμ–Sγ1 copies.

Results

Kinetics of IgG1 Surface Expression in B Cells Treated with LPS and IL-4. Using flow cytometry, the kinetics of IgG1 surface expression on B cells treated with LPS and IL-4 is shown in Fig. 1, which represents data drawn from three separate experiments. No surface IgG1 is detected before 2.5 d of culture. The fraction of cells expressing surface IgG1 reaches maximum at about day 4.5. If IL-4 is omitted from the culture, no surface IgG1 positive cells are detected (data not shown). Based on this, we concentrated on examining DNA from cell populations cultured for 2.5–5.5 d for quantitation of Sμ–Sγ1 DNA rearrangement. We have previously reported that no rearrangement is detectable before 2.5 d (14).

Quantitative DC-PCR Measurement of Sμ–Sγ1 Rearrangements. To measure DNA deletion occurring during Sμ–Sγ1 rearrangement, we utilized the technique of quantitative DC-PCR (14).

Initial quantitation was performed by titration of plasmid into a constant amount of genomic template and performing PCR on the mixtures. A representative example is shown in Fig. 2. The amount of nAChR in the sample is equal to the amount of p2AO, the competitive plasmid template, that

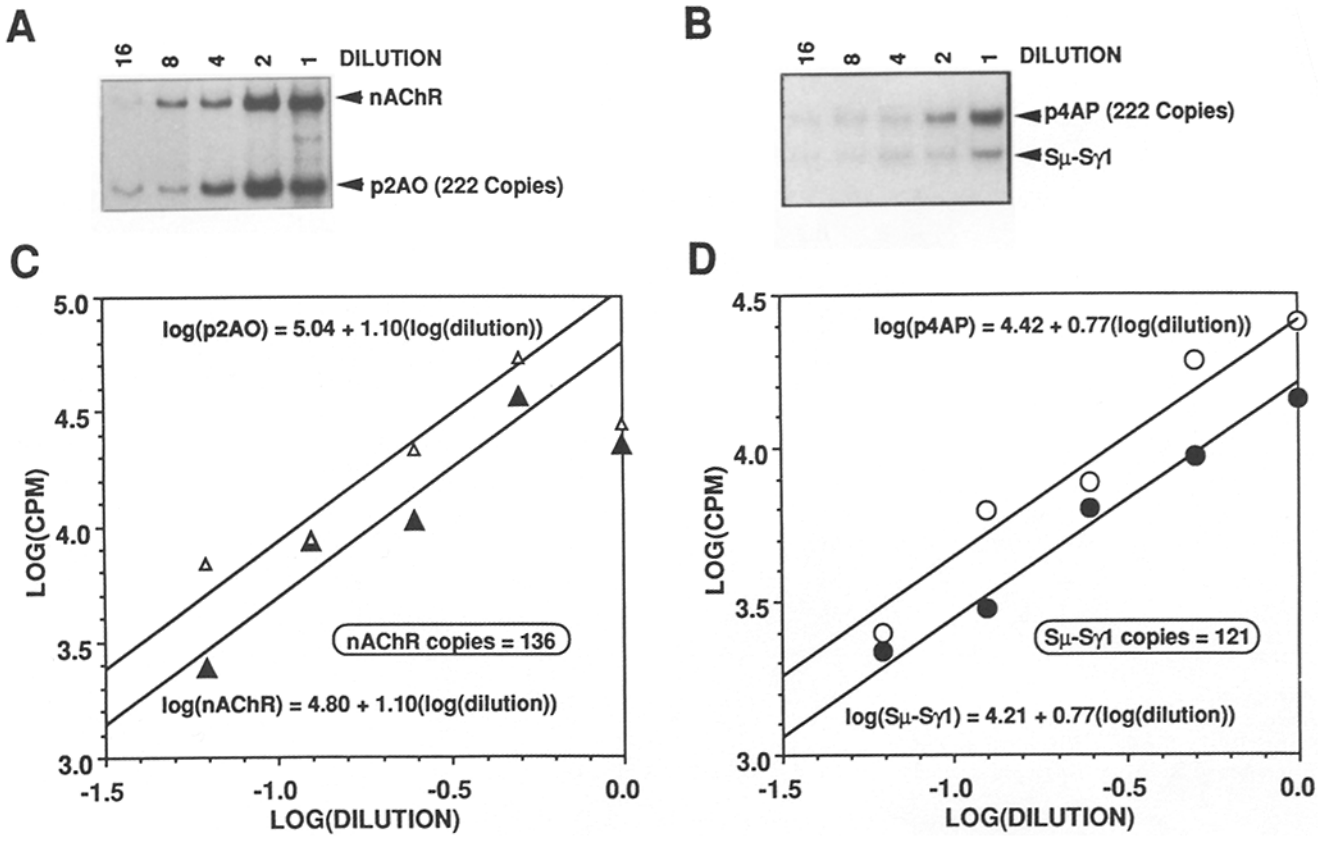

Figure 3. Representative example of further quantitation of Sμ–Sγ1 and nAChR copy number by serial dilution. Genomic template comes from B cells cultured with LPS + IL-4 for 2.5 d and sorted for IgG1$^+$ as in Fig. 2. (*A*) Autoradiograph of PCR products from a reaction using nAChR primers on a serial doubling dilution of a mixture of digested-circularized genomic template (5 ng) and 222 copies of p2AO and subsequently resolved by 8% PAGE. Dilution is shown above each lane. (*B*) Same as *A* with the exception that starting DNA mixture contained 222 copies p4AP instead of p2AO and PCR used 5′μ and 3′γ1 primers instead of nAChR primers. (*C*) nAChR copy number is calculated from data in *A* by determining parallel best fit lines for both nAChR genomic template and p2AO template PCR products with respect to log (dilution). Points where PCR amplification reached a plateau (Dilution 1) were discarded from consideration. The difference in log (dilution) between both lines (p2AO − nAChR) was multiplied by the number of p2AO copies in the starting mixture (222) to determine the number of nAChR copies in the starting mixture. 136 nAChR copies was found in this case. (*D*) Sμ–Sγ1 copy number was determined from the data in B in a similar manner as in *C*. Parallel best fit lines for both Sμ–Sγ1 genomic and p4AP template versus dilution were calculated. The equations were solved as in *C* with the knowledge that the starting mixture contained 222 copies of p4AP, resulting in a calculation of 121 Sμ–Sγ1 copies.

gives an equivalent amount of PCR product. This can be visualized in Fig. 2 *B* as the point where the nAChR and p2AO lines intersect. The amount of Sμ–Sγ1 is determined similarly with reference to the amount of p4AP as shown in Fig. 2 *C*. From these two graphs, the number of nAChR and Sμ–Sγ1 copies is calculated as shown in Fig. 2 *D* to be 205 and 109 copies, respectively. The amount of Sμ–Sγ1 rearrangement is calculated as described in the Materials and Methods to be 109/[(205/2) × 1.89] × 100 or 56.2%.

More precise quantitation was performed by serial dilution of a mixture of plasmid and genomic template near the equivalence point determined from titration experiments as shown in Fig. 3. PCR amplification was performed on serial dilutions of a mixture of genomic template and 222 copies of plasmid template as shown in Fig. 3, *A* and *B*. A negative control containing plasmid template and digested, but unligated genomic DNA resulted in no detectable genomic PCR product indicating that there is no contaminating carryover of previously amplified product in the PCR reaction mix (data not shown). The number of nAChR copies was determined by plotting the log(genomic template product) and log(p2AO product) versus the log(dilution) (Fig. 3 *C*). A best fit line was determined for both genomic and p2AO products. In theory, these lines should be parallel with a slope of one. In a set of 131 experiments, the observed slopes were 1.14 ± 0.39. For each experiment, parallel best fit lines were determined by calculating the average slope, statistically weighted for the number of points in each data set, and the centroid (average values of x and y) for data derived separately from genomic and plasmid templates. The nAChR copy number is determined from these two parallel lines by multiplying the x difference in log(dilution) between the p2AO and the genomic lines times the number of p2AO copies. The number of Sμ–Sγ1 copies is determined similarly by plotting log(genomic template product) and log(p4AP product) versus the log(dilution) (Fig. 3 *D*). The amount of Sμ–Sγ1 rear-

Table 1. *Sμ-Sγ1 Rearrangements in Unsorted B Cells*

Treatment	Time	sIgG1$^+$ cells[§]	Sμ–Sγ1/ nAChR[‖]	Experiment	Method
	d	%	%		
LPS	2.5	0.5	<1.8	E.12-12	Titration
		0.2	<0.8	E.17-1	Titration
	4.5	2.1	<2.7	E.12-12	Titration
		3.6	<1.4	E.17-1	Titration
LPS + IL-4	2.5	4.9	3.8	E.12-12	Dilution
		4.6	2.5 ± 0.2*	E.12-14	Dilution
		8.8	16	E.17-1	Dilution
	3.5	33	65	E.17-4	Dilution
		30	42	E.17-5	Titration
	4.5	29	76 ± 24.1‡	E.12-12	Dilution
		51	84 ± 20.5*	E.12-18	Dilution
		35	103 ± 16.4*	E.17-1	Dilution
	5.5	36	43	E.12-9	Titration

* Average of 2.
‡ Average of 4.
§ Surface IgG1$^+$ B cells as determined by FACS® analysis.
‖ A measure of Sμ-Sγl rearrangement determined by (Sμ–Sγl copies)/[(nAChR copies/2) × 1.89] × 100.

rangement per diploid genome in a sample is calculated from nAChR and Sμ–Sγ1 copy numbers as above. The nAChR and Sμ–Sγ1 copy numbers are calculated from this data to be 136 and 121 copies, respectively, as shown in Fig. 3, *C* and *D*. Thus, the amount of Sμ–Sγ1 rearrangement is calculated to be 121/[(136/2) × 1.89] or 94.1%. In this example, the estimate of the frequency of Sμ–Sγ1 rearrangement by dilution is 1.6-fold greater than that estimated by titration, illustrating the importance of performing serial dilution quantitation. However, the values from the two procedures are usually substantially closer, unless there is a significant plateau in the PCR amplification during the initial quantitation as seen in Figs. 2 *C* and 3, *A* and *C*. Thus, for all experiments we used both titration and dilution quantitation wherever possible.

Switch Rearrangement Can Account for Surface Expression in B Cells. Mouse B cells were cultured in LPS with or without IL-4 for 2.5–5.5 d and assayed for IgG1 surface expression by FACS® and Sμ–Sγ1 rearrangement by quantitative DC-PCR as shown in Table 1. The day 2.5 time point was chosen because that is when cells are just beginning to become IgG1 surface positive (Fig. 1) and because no Sμ–Sγ1 rearrangement is observed before this time (14). The day 4.5 time point was chosen because that is when the maximum number of B cells are surface IgG1 positive (Fig. 1). B cells cultured with LPS alone express no detectable surface IgG1 at 2.5 d, as shown for two experiments in Table 1. A low percent of surface positive cells (2.1 and 3.6%) in two experiments at 4.5 d is close to the detection limit of FACS® analysis with our reagents. B cells cultured in LPS only fail to produce detectable Sμ–Sγ1 rearrangement at 2.5 or 4.5 d; the limits of sensitivity of the assays ranged from 0.8 to 2.7% (Table 1).

The percentage of B cells cultured in LPS and IL-4 that become surface IgG1$^+$ varies from a minimum of 4.6% on day 2.5 to a maximum of 51% on day 4.5 as shown in Table 1 for seven experiments at four time points. In most experiments, Sμ–Sγ1 rearrangements as measured by DC-PCR occurred at percentages above those observed for IgG1$^+$ surface expression except for two cases at day 2.5. In these cases, we obtained values of 2.5% Sμ–Sγ1 rearrangement with 4.6% surface IgG1$^+$ and 3.8% rearrangement and 4.9% surface IgG1$^+$. The discrepancies could reflect measurement inaccuracy, particularly since estimates of both surface IgG1 expression and Sμ–Sγ1 rearrangement are near their limit of sensitivity. Alternatively, these cells may have been "caught" at a slightly earlier point in the differentiation process when DNA rearrangement may not yet have occurred in some of the cells that already express surface IgG1.

Because in most cases, the percent Sμ–Sγ1 rearrangement exceeds the percentage of IgG1$^+$ cells (see Table 1), there must be either more than one chromosome rearranged in some of the IgG1$^+$ cells or some of the IgG1$^-$ cells must contain an Sμ–Sγ1 rearrangement. To test this, we sorted cells on the basis of IgG1 surface expression as shown in Fig. 4. After sorting, purity was determined by reanalysis of IgG1$^+$ surface expression on the sorted cell populations. DNA was prepared from these cells and quantitative DC-PCR was performed to determine the amount of Sμ–Sγ1 rearrangement.

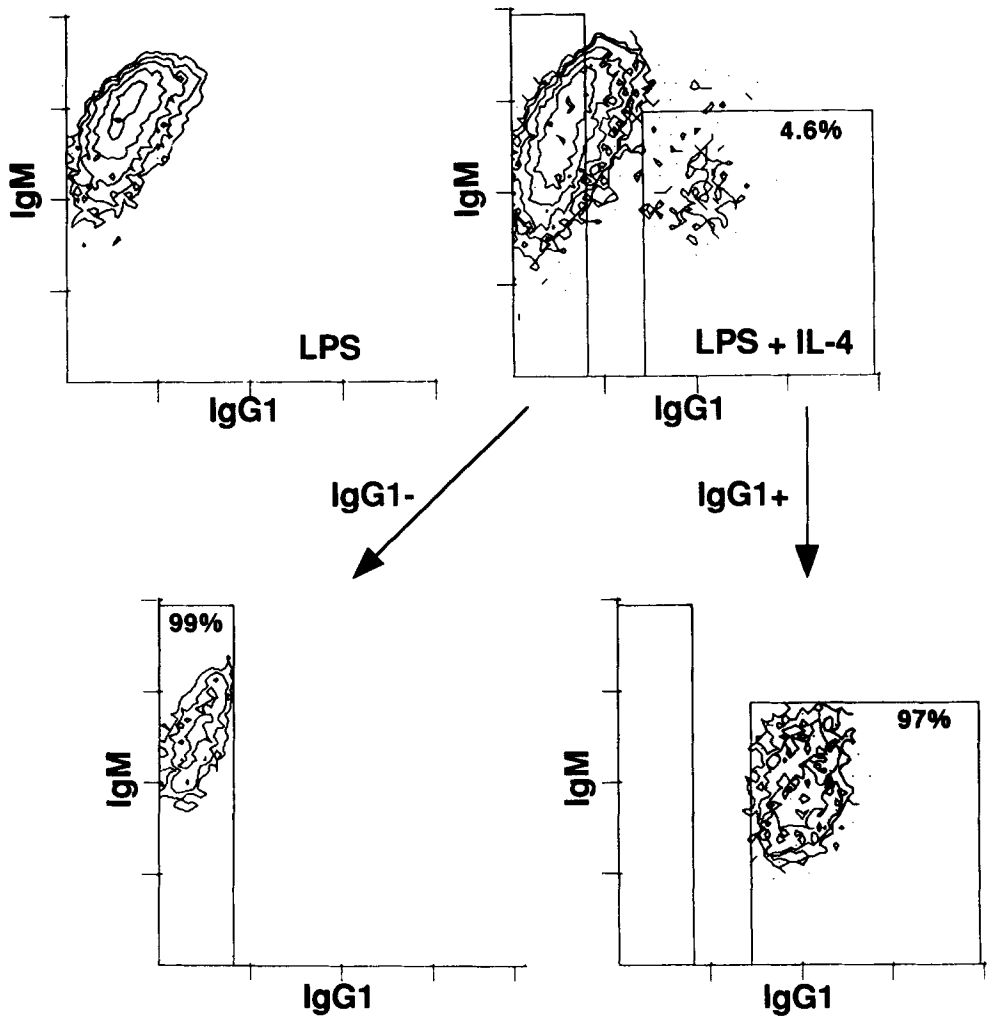

Figure 4. Representative example of sorting B cells on the basis of IgG1 expression. B cells cultured with LPS +/− IL-4 for 2.5 d were stained with anti-IgM-PE and anti-IgG1-FITC. Stained cells were analyzed and sorted on a FACStar^{plus}. Flow cytometric two-dimensional contour plots are shown with fluorescence intensity of anti-IgG1-FITC staining on the x axis and of anti-IgM-PE staining on the y axis. The upper two plots show the pre-sort analysis of cells cultured in LPS only or LPS + IL-4 in the left and right, respectively. The two boxes in the upper right plot indicate the sorting gates for IgG1$^-$ and IgG1$^+$ cells in the left and right, respectively. 4.6% is the percentage of IgG1$^+$ cells in the starting population. The lower two plots show the post-sort analysis of IgG1$^-$ and IgG1$^+$ cells from the LPS + IL-4 culture in the left and right, respectively. The boxes in the lower plots are the sorting gates. The percentages shown in the lower plots are the percentage of cells in the IgG1$^-$ and IgG1$^+$ sorted populations that are within the IgG1$^-$ and IgG1$^+$ sort gates, respectively (purity of sort). In this example, 3.2×10^6 and 9.7×10^4 IgG1$^-$ and IgG1$^+$ cells were collected, respectively. Typically, $>10^5$ cells were collected to insure that sufficient DNA could be prepared for the DC-PCR assay.

The results of analysis of IgG1$^+$ cells in seven cases at three time points are summarized in Table 2. The purity of these sorted cells (percent sIgG1$^+$) ranged from 72 to 97%. The measured Sμ–Sγ1 rearrangement in these cells is greater than the percent sIgG1$^+$ in five of these cases. Four measurements are greater than 100%, implying that some cells have two Sμ–Sγ1 rearrangements. Percentages >200 are theoretically impossible and most probably reflect error in the

Table 2. *Sμ–Sγ1 Rearrangements in Sorted B Cells from LPS + IL-4 Cultures: sIgG1$^+$ Cells*

Time	sIgG1$^+$ cells‡	Sμ-Sγ1/nAChR§	Experiment	Method
d	%	%		
2.5	97	86	E.12-14	Dilution
	81	94	E.17-1	Dilution
	72	61	E.17-2	Dilution
4.5	87	249	E.12-12	Dilution
	81	139 ± 80.4*	E.12-18	Dilution
	97	241 ± 12.6*	E.17-1	Dilution
5.5	95	147	E.12-9	Titration

* Average of 2.
‡ Surface IgG1$^+$ B cells as determined by FACS® analysis.
§ A measure of Sμ-Sγ1 rearrangement determined by (Sμ–Sγ1 copies)/[(nAChR copies/2) × 1.89] × 100.

Table 3. *Sμ–Sγ1 Rearrangements in Sorted B Cells from LPS + IL-4 Cultures: sIgG1⁻ Cells*

Time	sIgG1⁺ cells*	Sμ-Sγ1/nAChR‡	Experiment	Method
d	%	%		
2.5	2.7	4.4	E.12-12	Dilution
	1.0	4.0	E.12-14	Dilution
3.5	1.3	38	E.17-4	Dilution
	0.1	16	E.17-5	Titration
4.5	0.0	12	E.12-12	Dilution
	0.3	6.9	E.17-1	Dilution
5.5	0.0	6.6	E.12-9	Titration

* Surface IgG1⁺ B cells as determined by FACS® analysis.
‡ A measure of Sμ–Sγ1 rearrangement determined by (Sμ–Sγ1 copies)/[nAChR copies/2) × 1.89] × 100.

DC-PCR measurements. However, in such cases it seems very likely that virtually all the surface IgG1⁺ cells have undergone two Sμ–Sγ1 rearrangements. Two out of the seven Sμ–Sγ1 rearrangement measurements are less than the observed percent sIgG1⁺: 61 versus 72% and 86 versus 97% in the day 2.5 samples. These discrepancies may reflect measurement inaccuracy or a subpopulation of surface IgG1⁺ cells that do not contain Sμ–Sγ1 rearrangements.

The analysis of surface IgG1⁻ cells from seven cases at four time points is summarized in Table 3. The purity of the sorted populations was quite good, with surface IgG1⁺ cells ranging for 0–2.7%. In all seven measurements, some Sμ–Sγ1 rearrangement is detectable and always exceeds the level that could be accounted for by contaminating IgG1⁺ cells. Interestingly, the two highest values for Sμ–Sγ1 rearrangement in surface IgG1⁻ cells are observed at day 3.5 and are substantially greater than the values seen on days 4.5 or 5.5. The Sμ–Sγ1 rearrangement found in IgG1⁻ cells could be due to rearrangement on the unexpressed chromosome or to rearrangement on the expressed chromosome in cells that have not yet expressed surface IgG1.

Discussion

To determine if deletional mechanisms could quantitatively account for class switching in normal lymphocytes, we analyzed switching to IgG1 in B cells cultured in LPS and IL-4 by flow cytometry and by the newly developed DC-PCR assay. This allows us to measure surface IgG1 and Sμ–Sγ1 rearrangement, respectively.

DC-PCR is well suited to measure switch events, particularly those that occur in a small fraction of the cells under study, since the rearrangement is detected by the appearance of unique products (i.e., the DNA fragment containing the chimeric Sμ–Sγ1 region and the ligation circularized DNA and PCR products derived from it). By contrast, other techniques that attempt quantitation of DNA deletional switch events usually rely on the loss of the germline configuration of Cγ1, which is accurate only when the fraction of cells undergoing the switch event is substantial, since it depends upon subtracting the frequency of "unswitched" chromosomes in the experimental condition from that in the control condition. Nonetheless, it should be noted that Radbruch et al. and Kepron et al. were able to show that among B cells derived from cultures stimulated with LPS and IL-4, (13) a substantial degree of loss of unrearranged Cγ1 occurred in surface IgM-negative cells (12) and surface IgG1-positive cells, implying that DNA deletion accounted for much of the switching events in these cells.

The results we obtained with DC-PCR are also consistent with the deletional model of class switching. Indeed, DNA deletion in unsorted B cells stimulated with LPS and IL-4 was sufficient in seven of nine cases to account for surface IgG1 expression. In the remaining two cases, a small measurement inaccuracy could account for the difference. Among IgG1⁺ cells obtained by cell sorting, sufficient DNA deletion to account for IgG1 surface expression was observed in five out of seven cases. Again, measurement inaccuracy could account for the small differences (86 versus 97% and 61 versus 72%) in the remaining two cases. These results thus indicate that at all times throughout the culture, even at the earliest time that sIgG1⁺ cells could be detected (2.5 d), sufficient switch recombination exists to explain the expression of the switched isotype.

In IgG1⁻ cells purified from cultures stimulated with LPS plus IL-4, DNA deletion was detected above the level of IgG1⁺ cell contamination in all cases. This is particularly striking since B cells cultured with LPS alone failed to display detectable Sμ–Sγ1 rearrangement, under conditions in which such events could have been detected in from 0.8 to 2.7% of the cells, depending on the individual experiment. The observation that sIgG1⁻ cell populations invariably express evidence of some Sμ–Sγ1 rearrangement is consistent either with rearrangement occurring before surface expression of IgG1 (i.e., the cells have been "caught" after the DNA rearrangement event but prior to expression of cell surface IgG1) or with Sμ–Sγ1 rearrangement occurring to a substantial degree on the non-expressed chromosome in cells whose expressed chromosome has not undergone such rearrangement. Although we can not unequivocally distinguish between these two possibilities, the apparent accumulation, with time, of switch events in IgG1⁺ cells, as reflected by the increase in the frequency of chromosomes that have undergone rearrangement in IgG1⁺ cells from ~1 (i.e., percent of switched chromosomes divided by percent of IgG1⁺ cells) at day 2.5 to ~1.8 at days 4.5 and 5.5 indicates that the majority of the unexpressed chromosomes in IgG1⁺ cells will eventually undergo switch events to Sγ1. This is consistent with results from the analysis of loss of germline Cγ1 in LPS plus IL-4 stimulated cells (12, 13) and from the work of others who demonstrated that among hybridomas, switch recombination to the non-expressed C region of the same isotype as that on the productively rearranged chromosome occurred with a considerable frequency (29–31).

The finding that the frequency of Sμ–Sγ1 rearranged chromosomes was greater in IgG1$^-$ cells at day 3.5 (38 and 16% in two experiments) than in IgG1$^-$ cells at days 4.5 and 5.5 (12, 7, and 7%) suggests that IgG1$^-$ cells in which deletional switch events occurred often became IgG1$^+$. This could be explained by an initial switch event on the unexpressed chromosome followed by switching on the expressed chromosome and subsequent membrane IgG1 expression. Thus, early in culture, when cells have rearranged only one of their chromosomes, a substantial fraction of cells that will eventually switch will still be surface IgG1$^-$ but later, when such cells have rearranged both chromosomes, they will be transferred to the surface IgG1$^+$ category. This assumes that the entry of new cells into the switching process has diminished or ceased by day 4.5. Indeed, if we assume that few cells enter the switching process after 3.5 d, then one could also explain the fall in percent Sμ–Sγ1 rearrangement among surface IgG1$^-$ cells by assuming that some cells had been caught between the rearrangement event on the expressed chromosome and the membrane expression of IgG1 derived from that chromosome.

It is interesting to note that the maximum frequency of cells that express membrane IgG1 in these experiments was ~50%, consistent with a large body of experience in our laboratory of B cells stimulated with LPS and IL-4. The fact that IgG1$^-$ cells at days 4.5 and 5.5 have relatively low levels of Sμ–Sγ1 rearrangement implies that a substantial fraction of B cells cultured under these conditions do not undergo switch recombination. Indeed, even at days 4.5 and 5.5, a major fraction of the B cells continue to express substantial amounts of sIgM, implying that they have not switched to another isotype. These cells may be ones that would have "switched" had the culture been capable of sustaining switch events for a longer period. Alternatively, they may either have failed to receive a competent switch stimulus or, alternatively, may reflect cells that are incapable of switching in response to LPS plus IL-4. We cannot exclude the possibility that the initial B cell population is a heterogenous one that includes cells incapable of switching, at least to the set of stimuli that we have used.

Our results do not eliminate the possibility that nondeletional mechanisms of class switching do occur in a physiologically meaningful manner. Since we do not measure switch recombination events in individual cells, it is possible that the population of IgG1$^+$ cells at 2.5 d, for example, includes some cells that have undergone Sμ–Sγ1 deletional rearrangement on both chromosomes plus others that have not undergone switch recombination, but are expressing IgG1 by one of the mechanisms mentioned above. Furthermore, if measurement inaccuracy is not the explanation for the few cases where DNA deletion was insufficient to account for IgG1 surface expression, then a small percentage of cells (up to 15%) may be producing IgG1 as a result of nondeletional mechanisms. Nonetheless it does appear that the weight of our evidence is consistent with deletional switching being the dominant mechanism even at the earliest time that expression of switched isotype can be observed. If nondeletional mechanisms are obligatory precursors to deletional switch recombination, they must occur before membrane IgG1 expression and must have largely been replaced by deletional mechanisms by the time surface IgG1 is expressed since under all conditions the majority of surface IgG1$^+$ cells do express Sμ–Sγ1 rearrangement.

Finally, it must be pointed out that stimulation of Ig class switching by other stimuli could, conceivably, rely more heavily on nondeletional mechanisms, although there is no a priori reason why this should be so. Efforts to examine in vivo switch events as well as switching in response to other stimuli are planned to address this issue.

We thank Dr. David Alling for aid in statistical analysis; Cyndy Watson and Jane Hu-Li for excellent technical assistance; Joe Ritter for aid with the AMBIS; Jeff Hooley and Susan Barbieri for FACS® sorting; Randy Ribaudo for aid with the computing densitometer; the Laboratory of Immunology for aid with FACS® analysis and helpful discussion; and Shirley Starnes for preparation of this manuscript.

C. C. Chu was supported by grant PF-3299 from the American Cancer Society.

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Received for publication 21 May 1993.

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