Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3954–3957, June 1986
Immunology

Class switch recombination is IgG1 specific on active and inactive IgH loci of IgG1-secreting B-cell blasts

(immunoglobulin class switch/B-cell differentiation factor/IgG1 allotypes/polyclonal stimulation)

ANDREAS RADBRUCH, WERNER MÜLLER, AND KLAUS RAJEWSKY

Institute for Genetics, University of Cologne, Weyertal 121, D-5000 Cologne 41, Federal Republic of Germany

Communicated by Walter Gilbert, January 2, 1986

ABSTRACT **Mouse B lymphocytes can be activated polyclonally by bacterial lipopolysaccharide (LPS) to secrete Ig and perform Ig class switch. In the presence of the T-cell lymphokine B-cell differentiation factor, the frequency of IgG1-secreting cells is drastically enhanced. We show here that IgG1-secreting B cells isolated from such cultures have undergone a similar DNA rearrangement of the switch regions (S_μ, $S_\gamma 1$) of the Ig heavy chain constant region genes C_μ and $C_\gamma 1$ on both active and inactive IgH loci. This result argues against a stochastic model of class switch recombination and suggests programmed class-specific switch recombination in the case of the switch to IgG1. In accord with this notion, cells expressing IgM but not IgG on the surface have not deleted or rearranged C_μ or $S_\gamma 1$ on either chromosome.**

Upon mitogenic activation, most murine B lymphocytes switch from the expression of IgM to that of another Ig isotype (1). Previously we had analyzed the Ig heavy chain (IgH) gene rearrangements accompanying class switching in B cells stimulated polyclonally with bacterial lipopolysaccharide (LPS) and found a rapid deletion of the previously expressed constant region (C_H) genes in the switched population (2, 3). The representation of Ig isotypes in the population of LPS-activated B cells is not random in that most of the cells switch to the expression of $C_\gamma 3$ or $C_\gamma 2b$ but few switch to $C_\gamma 1$. This pattern can be changed to the predominant expression of $C_\gamma 1$ by the addition of T-cell culture supernatants containing B-cell differentiation factor γ ($BCDF_\gamma$) (4, 5). In the present paper we analyze the active and inactive IgH loci of IgG1- and IgM-secreting B-cell blasts in BCDF- and LPS-containing cell cultures for the occurrence of switch recombination.

MATERIALS AND METHODS

Animals. (C57BL/6 × BALB/c)F_1 mice were bred in our animal colony.

Cell Culture and Cell Sorting. F_1 spleen cells (1–2 × 10^6 per ml) were cultured in RPMI 1640 medium with 10% fetal calf serum, antibiotics, 40 μg of LPS per ml, and 10% culture supernatant of induced EL4 cells (6) containing $BCDF_\gamma$ in 250-ml flasks. The cells were fed and/or diluted every 3–4 days. Before sorting, dead cells and debris were eliminated by repeated centrifugation of the cells over Ficoll-Paque (7).

For selection of $IgG1^a$-positive cells, the cells were then fixed in ethanol/acetic acid (8), stained first with a control antibody conjugated to Texas red (Ac146; IgG1, κ) and then with fluoresceinated monoclonal antibody to $IgG1^a$ [ref. 9; Ig(4a) 10.9.6, gift of L. A. Herzenberg; fluorescein-conjugated and affinity-purified by us; 3.4 fluorescein molecules per Ig molecule], and then sorted as described (2, 10). The purity of the sorted cells was determined by fluorescence microscopy. Only fluorescein-labeled cells with a regular shape in phase-contrast and no red counterstain were scored positive.

Table 1. Percentages of IgM-, IgG3-, and IgG1-expressing cells among spleen cells activated by LPS and EL4 culture supernatant

	% of IgM-, IgG3-, and IgG1-expressing cells*						
Day	S_μ	$S_\gamma 1$	C_μ	$C_\gamma 1$	$S_\gamma 3$	$C_\gamma 3$	$C_\gamma 1 + C_\mu$
0	38	0.1					
2	71	14†	10	≤1	≤1	≤1	—
5	57	14	43	8	≤1	≤1	1
7	22	16	26	30	2	≤1	1

S, surface Ig; C, cytoplasmic Ig. Staining was done with fluoresceinated and rhodaminated goat anti-isotype antibodies, some self-made and some from Southern Biotechnology Associates (Birmingham, AL). Cells were first stained on the surface and then were spun on a slide in a cytocentrifuge, fixed, and stained for cytoplasmic Ig. They were analyzed in a Leitz fluorescence microscope.
*Percentage of positive and intact (according to phase-contrast) cells in the case of single isotypes and percentage of cells expressing $C_\gamma 1$ of cells expressing C_μ in the case of double-producing cells.
†Very weak staining of doubtful significance.

For selection of surface IgM-positive cells, F_1 spleen cells were stimulated with LPS and EL4 supernatant (10% of culture volume) for 8 days and then stained for surface IgM with a fluoresceinated goat anti-mouse IgM (2, 8) and a biotinylated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL); staining in a second step was with avidin-Texas red (10). In the presence of propidium iodide (1–10 μg/ml) the scatter-positive cells were sorted against red and for green fluorescence. The deflected cells were >95% pure.

Quantitative Restriction Analysis. The DNA was prepared from >10^6 cells each, digested, and hybridized to 3′ C_μ, $S_\gamma 1$ and $S_\gamma 2b$, or T1.2 probe successively according to standard methods and as described (2). Briefly, the DNA was cut with *Eco*RI or *Hin*dIII (Boehringer Mannheim), separated on an agarose gel, and blotted to a nitrocellulose filter. This filter was hybridized first to the 3′ C_μ probe and, after the autoradiographs had been obtained, to the $S_\gamma 1$ probe. Again, autoradiographs were produced and the filters were hybridized subsequently either to the $S_\gamma 2b$ or the T1.2 probe.

The relative peak heights were determined by scanning the autoradiographs shown in Fig. 2 with a Quick Scan (DESAGA, Heidelberg, F.R.G.) as described (2), except that the standard curve was determined with graded amounts of DNA. All values in the tables are within the linear dose range, covered by the standard curve.

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Abbreviations: BCDF, B-cell differentiation factor; LPS, lipopolysaccharide; C, constant.

Table 2. Quantitative restriction analysis of $S_\gamma 1$ rearrangement in IgG1^a-positive blasts

Cells	Haplotype	$S_\gamma 1$ relative to $S_\gamma 2b$		Purity of sorted cells,* %
		a Haplotype	*b* Haplotype	
C57BL/6 liver	*b*	—	1.18	
BALB/c liver	*a*	1.25	—	
F_1 spleen	*a* × *b*	1.00	1.00	
F_1 blasts				
Day 8	*a* × *b*	1.16	1.75†	
IgG1^a-positive blasts				
Day 6	*a* × *b*	0.3	0.2	88
Day 8	*a* × *b*	0.1	0.1	85
Day 12	*a* × *b*	ND	0.1	85

Quantification of the blot that is shown in Fig. 2. The assay was standardized by setting the ratio of unrearranged $S_\gamma 1$ to $S_\gamma 2b$ to 1.00 for one of the controls. ND, not determined.
*Purity was scored as described in the text.
†We think that the deviation of this value from the expected one (1.00) is due to a technical artifact.

RESULTS AND DISCUSSION

IgG1-Expressing B-Cell Blasts. We activated (C57BL/6 × BALB/c)F_1 B cells (carrying IgH loci of the *a* and *b* allotype) by LPS in the presence of $BCDF_\gamma$ (6). Analysis of the frequencies of the μ, $\gamma 3$, and $\gamma 1$ isotypes in such a culture by fluorescence microscopy (Table 1) confirmed earlier results (4–6) in that the frequency of IgG1-expressing cells is high, whereas that of IgG3-expressing cells is low. After 6–12 days (see Table 2), B-cell blasts secreting IgG1 of the *a* allotype were purified by cell sorting from (a × b)F_1 cultures. Briefly, the cells were fixed and stained with a rhodaminated control antibody and a fluoresceinated monoclonal anti-IgG1^a antibody [Ig(4a)10.9.6; ref. 9], and IgG1^a-positive cells were sorted out in a modified two-laser/two-color FACS I (2, 8, 10). The anti-IgG1^a stained 5–15% of the (a × b)F_1 blast cells stimulated with LPS and BCDF for 6–12 days. This is about 45% of the cells expressing IgG1 in the cytoplasm at that time when evaluated by double-staining with Ig(4a)-conjugated fluorescein isothiocyanate and goat anti-IgG1-conjugated Texas red isothiocyanate (data not shown). After cell sorting, >85% of the deflected cells stained only and brightly for cytoplasmic IgG1^a but not for the control antibody Ac146. The contaminating cells were mostly unstained and attached

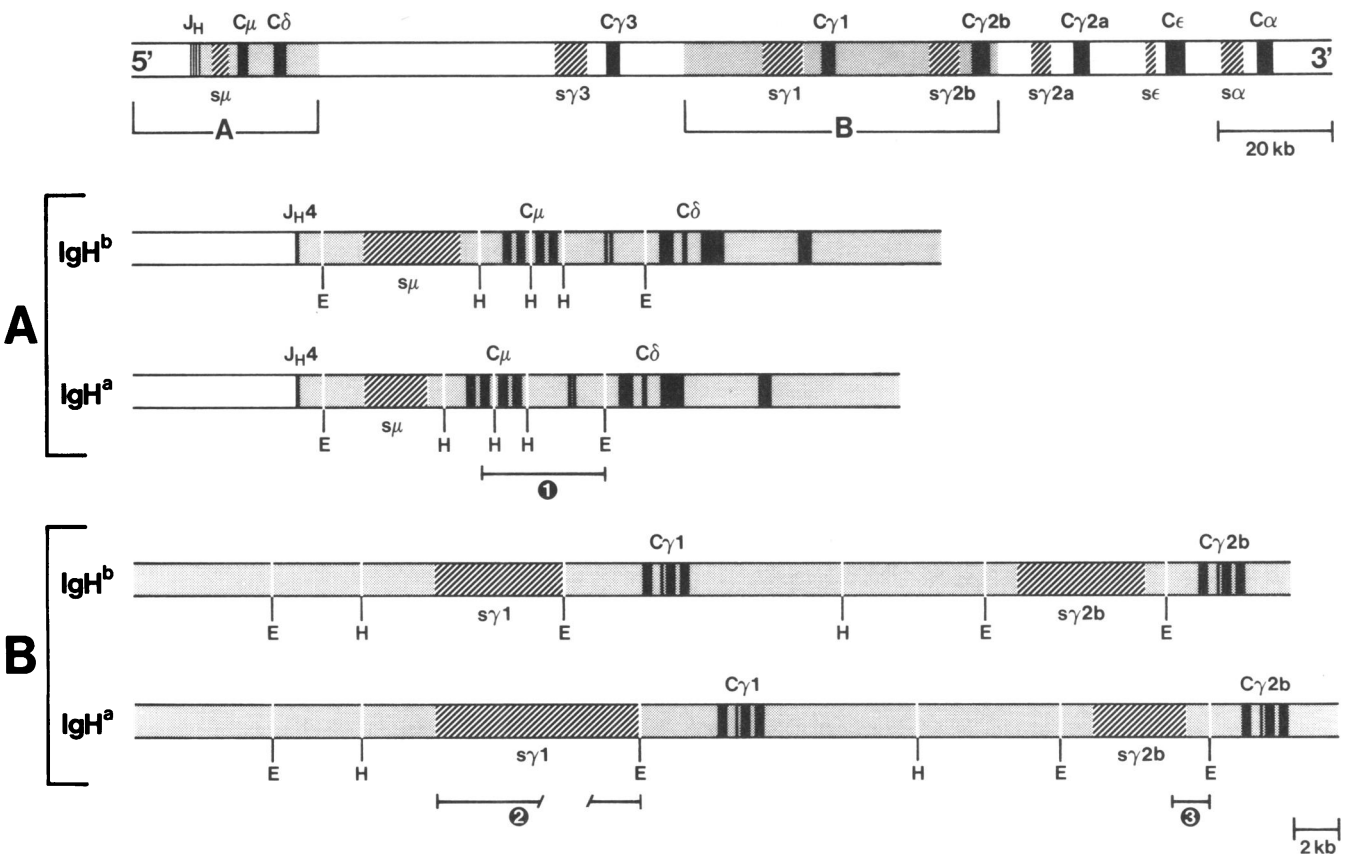

FIG. 1. Strategy of restriction analysis of IgG1^a-positive blast cells. The murine IgH locus is shown on the top line according to ref. 11. The relevant areas are stippled. The switch regions (S_μ, etc.) are shaded and the joining (J) region and C region genes (C_μ, etc.) are in solid black. The *a* and *b* allotypes of the IgH locus in its germ line configuration are compared for restriction polymorphisms in the S_μ (*A*) and the $S_\gamma 1$ and $S_\gamma 2b$ regions (*B*) (11, 12). This polymorphism changes the length of the corresponding *Eco*RI restriction fragments. The restriction fragments are detected by probes specific for C_μ (3′ C_μ, gift of A. L. M. Bothwell and F. Sablitzky) (1), $S_\gamma 1$ (gift of T. Honjo) (2), and $S_\gamma 2b$ (pBR1.4, gift of K. Marcu) (3). Only the *Hin*dIII (H) and *Eco*RI (E) sites, relevant for the present analysis, are shown. kb, Kilobases.

to stained cells probably by fixation (Table 2). DNA was prepared from the sorted purified IgG1^a-positive cells and control cells, cut with *Eco*RI, and analyzed by the Southern blotting technique. The selection of probes and the *Eco*RI and *Hin*dIII sites of the relevant part of the mouse IgH locus are shown in Fig. 1. The (active) IgH^a and (inactive) IgH^b loci of the sorted cells exhibit restriction polymorphism of the C_μ, $S_\gamma 1$, and $S_\gamma 2b$ regions (refs. 11, 12; Fig. 1) and therefore can be analyzed separately. We determined the relative amount of the germ line C_μ, $S_\gamma 1$, and $S_\gamma 2b$ fragments in the DNA of the sorted cell populations. Absence of a fragment corresponds to rearrangement or deletion of the respective part of the IgH locus. The DNA rearrangement is heterogeneous if no new fragments appear. The Southern blot of the IgG1^a-positive cells is shown in Fig. 2 and its quantitative evaluation is given in Table 2. The intensity of the band hybridizing to the $S_\gamma 2b$ probe gives the amount of unrearranged DNA downstream of $C_\gamma 1$. This is about the same for all cell populations analyzed and, moreover, for both IgH alleles (Fig. 1, Table 2).

Since the $S_\gamma 2b^b$ germ line bands are not weaker than the $S_\gamma 2b^a$ bands for any of the cell populations selected for IgG1^a expression (Table 2), the inactive chromosome is in the majority of the cells not rearranged beyond $S_\gamma 2b$. Indicative for rearranged DNA upstream of $C_\gamma 1$, the $S_\gamma 1$ probe shows a considerable reduction (up to 90%) of germ line fragments of the *a* and the *b* haplotypes for cells expressing IgG1^a. The few remaining $S_\gamma 1$ germ line fragments (overexposed in Fig. 2) are probably due to contaminating IgG1^a-negative cells (at most, 15%; Table 2) in the sorted population, most of which are probably IgM-positive plasma blasts. Thus, although selected for the expression of IgG1 of the *a* allotype, the *a* and *b* IgH loci are rearranged equally frequently—namely, in >80% of the IgG1^a-positive cells (Table 2).

This is accompanied by rearrangement or deletion of the C_μ genes on both chromosomes (Fig. 2 *Top*). In this case, weak remaining C_μ bands would have escaped our attention because of background problems. Taken together, these results indicate that in the IgG1^a-expressing cells the DNA is deleted on both chromosomes presumably from S_μ to $S_\gamma 1$. Switch recombination on both chromosomes had been shown earlier for LPS blasts that had switched to IgG3 (2) and for a variety of hybridoma and myeloma cells expressing IgG or IgA. The latter cells, however, had often shown switch recombination to different classes on the active and inactive chromosome, and only some cell lines had switched to the same C_H gene on both IgH loci (e.g., two of four IgA-expressing plasmacytomas described in ref. 13, neither an IgG3- nor an IgG1-expressing hybridoma that underwent switch recombination on both chromosomes, ref. 14). Since these cells had been analyzed many generations after the class switch event, it is possible that the rearrangements of the inactive IgH locus are partly secondary. In our previous analysis of LPS-activated B cells (2, 3), active and inactive chromosomes of the IgG3-expressing blasts could not be analyzed separately because no serological polymorphism is known for IgG3. Still, it is likely that also in late IgG3-positive LPS blasts, the switch recombination took place mostly between S_μ and $S_\gamma 3$ on both chromosomes, since we used $S_\gamma 1$ (just downstream of $C_\gamma 3$) for standardization of DNA amounts and found no indication for $S_\gamma 1$ rearrangement (2). However, an independent comparative DNA quantification is lacking in that experiment. In the present experiment, the situation is clear since we looked at active and inactive chromosomes of the same cells and both behaved similarly.

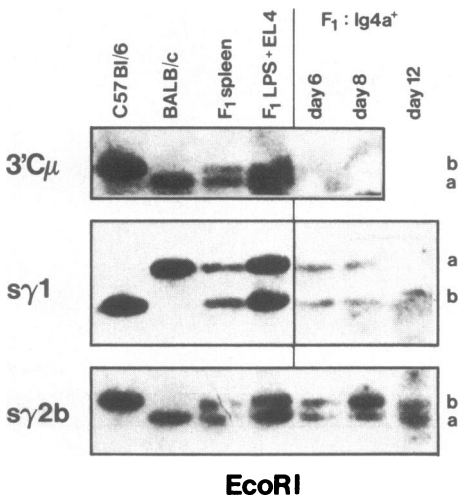

FIG. 2. C_μ and $S_\gamma 1$ rearrangement in IgG1^a-positive LPS blasts induced with BCDF. Southern blot of *Eco*RI-treated DNA from C57BL/6 liver, BALB/c liver, (C57BL/6 × BALB/c)F_1 spleen, F_1 blasts stimulated with LPS in the presence of EL4 supernatant containing BCDF for 8 days, and sorted IgG1^a-positive F_1 blasts generated under the same conditions 6, 8, and 12 days after initiation of the culture. The probes are indicated on the left side (see Fig. 1). The $S_\gamma 1$ autoradiograph of sorted IgG1^a-positive cells is overexposed compared to the controls in order to show the faint $S_\gamma 1$ germ line bands. The purity of the sorted cells was evaluated by fluorescence microscopy of an aliquot of sorted cells, scoring as positive those cells that clearly were stained with Ig(4a)-conjugated fluorescein isothiocyanate but not with Ac146-conjugated Texas red and showed intact morphology.

IgM-Expressing B-Cell Blasts. In contrast to the IgG1-expressing cells, B cells expressing surface IgM but not IgG from day 8 of such a culture show little, if any, rearrangement of C_μ ($C_\mu/C_\tau\alpha$ ratio in Table 3) and no deletion of C_μ genes (Fig. 3, Table 3). This result contrasts at first sight with our previous demonstration of C_μ gene deletion to ≈50% in LPS-activated B cells bearing IgM on the surface (2). Since in this case the cells had not been counterselected against IgG expression, we attribute, in the light of the present results, the reduction in C_μ content to a subpopulation of cells that had already switched to IgG but still carried IgM on the surface, together with the new isotype.

The present data do not exclude an intermediate stage of class switching at which the cells have undergone site-

Table 3. Quantitative restriction analysis of IgH rearrangement in IgM$^+$/G$^-$ blasts

Source of DNA	*Eco*RI C_μ relative to $C_\tau\alpha$	*Eco*RI $S_\gamma 1$ relative to $C_\tau\alpha$	*Hin*dIII C_μ (3′ end) relative to T1.2
C57BL/6	—	—	1.3
BALB/c	0.76	0.87	1.3
F_1 spleen	1	1	1
F_1 LPS/EL4 blasts* (IgM$^+$/G$^-$)	0.81	1.06	1.14
F_1 spleen	0.97	1.04	0.95
F_1 LPS/EL4 blasts* (not sorted)	—	—	0.93

Quantification of the *Eco*RI- and *Hin*dIII-digested DNA shown in Fig. 3. For *Eco*RI-digested DNA, the peak heights of the C_μ and $C_\gamma 1$ alleles were added and compared to the peak height of the upper T1.2 band (3.8 kilobases corresponding to $C_\tau\alpha$; refs. 15, 16). For *Hin*dIII-digested DNA, the peak height of the lower C_μ band, representing the 1.3-kilobase 3′ end of the C_μ gene, was compared to the peak height of the smallest T1.2 band shown (≈3.2 kilobases) corresponding to V_α or J_α germ-line fragment. *Eco*RI-digested DNA of C57BL/6 is not included because of apparent loss of this DNA from the filter before the T1.2 hybridization (Fig. 3). The C_μ to $S_\gamma 1$ ratio of it is, however, comparable to those of the other DNAs (data not shown).
*In the presence of LPS and EL4 supernatant.

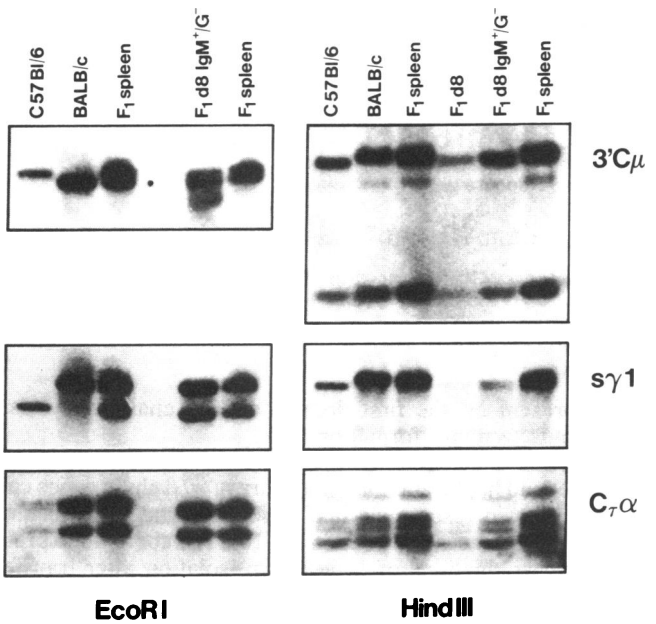

FIG. 3. C_μ and $S_\gamma 1$ rearrangement in IgM^+/G^- blasts. Southern blot of *Eco*RI- and *Hind*III-digested DNA from C57BL/6 liver, BALB/c liver, (C57BL/6 × BALB/c)F_1 spleen, IgM^+/G^- F_1 blasts from day 8 of culture in the presence of LPS and EL4 supernatant (see Fig. 2), and, again, F_1 spleen cells. The DNA was hybridized successively to the 3' C_μ, $S_\gamma 1$, and a probe detecting the $V_{T\alpha}$, $J_{T\alpha}$, and $C_T\alpha$ genes (genes of the α chain of the T-cell receptor; probe T1.2; gift of M. Steinmetz and T. Tokuhisa; ref. 17) for standardization of DNA amounts.

specific deletion on only one of the two chromosomes (2, 14, 18). However, such a stage of differentiation, if it exists, must be short lived, since the cells essentially complete the deletion process within 6 days after mitogenic stimulation (Tables 1 and 2).

With respect to the possibility of coexpression of IgM and IgG1 in the absence of deletion (19), we can only say that cells *secreting* Ig of both isotypes are rare since we find essentially no coexpression of cytoplasmic (indicative for secreted) IgM and IgG1 at any stage of the culture (Table 1).

Programmed Class Switch Recombination. Taken together, our data argue against the previously proposed stochastic model of class switching (3), at least in the case of the switch to IgG1. In the stochastic model, the cells go through a stage of differentiation in which they randomly delete C_H genes on expressed and nonexpressed IgH loci. In contrast, a B cell switching to IgG1 is programmed to do so by a mechanism that directs site-specific recombination to the $S_\gamma 1$ region. It is possible that this mechanism makes use of the $S_\gamma 1$-specific nucleotide sequences identified recently (20, 21) and is triggered by the lymphokine BCDF (5). Alternatively, the latter could expand switch precursors and/or IgG1-expressing cells (5).

Class switching to other isotypes, for some of which specific lymphokines have also been described (22–25), may follow a similar mechanism. Furthermore, the preferential usage not only of certain C_H but also of heavy chain variable (V_H) and diversity (D) elements by certain Abelson virus-transformed pre-B-cell lines (26) suggests that, in general, the selection of genetic elements in Ig gene rearrangements may be programmed to a larger extent than previously anticipated.

We thank E. Severinson, A. Coutinho, S. Klein, F. Sablitzky, U. Krawinkel, and H. Tesch for stimulating discussions, S. Irlenbusch for expert technical help, U. Ringeisen for the illustrations, and Å. Böhm for help with the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 74, the Bundesministerium für Forschung und Technologie, and the FAZIT Foundation.

1. Coutinho, A. & Forni, L. (1982) *EMBO J.* **1**, 1251–1257.
2. Radbruch, A. & Sablitzky, F. (1983) *EMBO J.* **2**, 1929–1935.
3. Radbruch, A., Sablitzky, F. & Rajewsky, K. (1984) in *Progress in Immunology V*, eds. Yamamura, Y. & Tada, T. (Academic, Tokyo), pp. 657–660.
4. Isakson, P. C., Pure, E., Vitetta, E. S. & Krammer, P. H. (1982) *J. Exp. Med.* **155**, 734–741.
5. Layton, J. E., Vitetta, E. S., Uhr, J. W. & Krammer, P. H. (1984) *J. Exp. Med.* **160**, 1850–1863.
6. Müller, W., Kühn, R., Goldmann, W., Tesch, H., Smith, F. I., Radbruch, A. & Rajewsky, K. (1985) *J. Immunol.* **135**, 1213–1216.
7. Radbruch, A., in *Handbook of Experimental Immunology*, eds. Weir, D. M., *et al.* (Blackwell, Edinburgh, Scotland), in press.
8. Klein, S., Sablitzky, F. & Radbruch, A. (1984) *EMBO J.* **3**, 2473–2476.
9. Huang, C., Parsons, M., Oi, V., Huang, H. & Herzenberg, L. A. (1983) *Immunogenetics (N.Y.)* **18**, 311–317.
10. Weichel, W., Liesegang, B., Gehrke, K., Göttlinger, C., Holtkamp, B., Radbruch, A., Stackhouse, T. K. & Rajewsky, K. (1985) *Cytometry* **6**, 116–123.
11. Shimizu, A., Takahashi, N., Yaoita, Y. & Honjo, T. (1982) *Cell* **28**, 499–506.
12. Marcu, K. B., Banerji, J., Penncavage, N. A., Lang, R. & Arnheim, N. (1980) *Cell* **22**, 187–196.
13. Coleclough, C., Cooper, D. & Perry, R. P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1422–1426.
14. Hurwitz, J. L., Coleclough, C. & Cebra, J. J. (1980) *Cell* **22**, 349–359.
15. Hayday, A. C., Diamond, D. J., Tanigawa, G., Heilig, J. S., Folsom, V., Saito, H. & Tongawa, S. (1985) *Nature (London)* **316**, 828–832.
16. Winoto, A., Mjolsness, S. & Hood, L. (1985) *Nature (London)* **316**, 832–836.
17. Dembic, Z., Bannwarth, W., Taylor, B. A. & Steinmetz, M. (1985) *Nature (London)* **314**, 271–273.
18. Yaoita, Y. & Honjo, T. (1980) *Nature (London)* **286**, 850–853.
19. Perlmutter, A. P. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7189–7193.
20. Davis, M. L., Kim, S. K. & Hood, L. E. (1980) *Science* **209**, 1360–1365.
21. Mowatt, M., Dery, C. & Dunnick, W. (1985) *Nucleic Acids Res.* **13**, 225–237.
22. Elson, C. O., Heck, J. A. & Strober, W. (1979) *J. Exp. Med.* **149**, 632–643.
23. Kishimoto, T. & Ishizaka, K. (1973) *J. Immunol.* **111**, 1194–1205.
24. Kawanishi, H., Saltzman, L.-E. & Strober, W. (1982) *J. Immunol.* **129**, 475–480.
25. Hirashima, M., Yodoi, T. & Ishizaka, K. (1981) *J. Immunol.* **121**, 838–842.
26. Alt, F. W., Blackwell, T. K., De Pinho, R. A., Reth, M. G. & Yancopoulos, G. D. (1986) *Immunol. Rev.* **89**, 5–30.