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Immunoglobulin switch circular DNA in the mouse infected with *Nippostrongylus brasiliensis*: Evidence for successive class switching from μ to ε via $\gamma 1$

(switch recombination/parasite infection/interleukin 4/IgE/IgG1)

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ABSTRACT **We have characterized immunoglobulin switch circular DNA in mice infected with the nematode parasite *Nippostrongylus brasiliensis*. Two kinds of circular DNA were identified in the lymph nodes as excision products of switch recombination of immunoglobulin heavy-chain constant region (C_H) genes. One is a recombinant between C_μ and $C_{\gamma 1}$ ($\gamma 1$ circle), and the other is a recombinant between $C_{\gamma 1}$ and C_ε (ε circle). In the ε circle, a short piece of switch μ (S_μ) sequence was inserted between S_ε and $S_{\gamma 1}$ sequences. The inserted S_μ sequence could be a trace of the preceding switch from C_μ to $C_{\gamma 1}$. These findings indicate that parasitic infection can induce class switch recombinations in a successive manner, first from C_μ to $C_{\gamma 1}$, and then from $C_{\gamma 1}$ to C_ε.**

Immunoglobulin switch recombination is an important molecular mechanism which changes biological effector functions of antibody molecules (1, 2). As B lymphocytes differentiate into memory or plasma cells, the isotype of immunoglobulin heavy (H) chains changes from μ to one of the other isotypes, $\gamma 1$, $\gamma 2a$, $\gamma 2b$, $\gamma 3$, ε, and α in the mouse (3, 4). During the process of class switching structures of variable (V) regions remain unchanged, thus the antigen specificity is not affected (5). In the switching event, constant regions of heavy chain genes (C_H) are replaced through a site-specific DNA recombination process (6–9) which takes place between two switch (S) regions located upstream from the C_H genes. Usually, S regions are rich in repetitive sequences ranging 2–10 kilobases (kb) long (10, 11), and recombination appears to take place at "promiscuous" sites in the S region. Although reciprocity remains to be established, switch recombination is assumed to be accompanied by intramolecular DNA deletion (12–14). Switch recombination generates the switched immunoglobulin H chain gene on the chromosome, and the reciprocal recombinant on the excised circular DNA.

Infection by the larvae of the nematode parasite *Nippostrongylus brasiliensis* causes an approximately 100-fold increase in serum IgE concentration (15, 16). IgG1 production is also greatly increased. These observations indicate that the parasite can trigger a series of events responsible for the isotype switch. The lymphokine interleukin 4 (IL-4) is known to be one of the key molecules which mediates class switching in the parasite-infected mouse. Finkelman *et al.* (17) observed that monoclonal anti-IL-4 antibody could suppress the polyclonal IgE responses in the infected mouse. In culture IL-4 was shown to promote switching from μ to $\gamma 1$ or to ε when splenic B cells were stimulated by a mitogenic lipopolysaccharide (LPS) (18–20). However, it was not clear whether the switch to ε occurred directly from μ or successively via $\gamma 1$. With the sequence data from switched immunoglobulin genes on the chromosome, the possibility of successive switching has been discussed. There are some examples of DNA insertions between two recombined S sequences, which could be explained by a successive deletion model (9, 21–23), but it was difficult to determine whether the inserted sequence was indeed due to successive switching. This is because the S sequence is highly repetitive and homologous to other S sequences, and because the secondary deletion is often found within the S region in myelomas and cell lines.

In *N. brasiliensis*-infected mice it is important to determine whether the already switched IgE-positive cells are selected to produce the high level of IgE or whether the switch recombination is activated for ε. To test these possibilities, we have analyzed extrachromosomal circular DNA in lymph node cells from mice infected with the parasite. If the switch recombination is directed to $\gamma 1$ and ε, switch circular DNA of these classes should be generated in the infected mouse (12–14). In this report, we characterize two kinds of switch circular DNA. One is an excision product of the switch recombination between C_μ and $C_{\gamma 1}$, and the other is a product between $C_{\gamma 1}$ and C_ε. These data suggest that switch recombination is directed in a progressive manner first from C_μ to $C_{\gamma 1}$ and then from $C_{\gamma 1}$ to C_ε.

MATERIALS AND METHODS

Animals. Female BALB/c mice (10 weeks old) were obtained from The Jackson Laboratory. For each experiment 10 mice were infected with 700 larvae of *N. brasiliensis* as described (15). Cells were obtained from mesenteric lymph nodes 8, 10, or 12 days after inoculation.

Cell Culture. For the *in vitro* switch experiment, splenocytes were obtained from BALB/c mice (6-week-old females) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and antibiotics. Cells were stimulated with *Salmonella typhosa* LPS (Difco) at 40 μg/ml, and recombinant IL-4 (Genzyme) at 250 or 1000 units/ml. After 3 days of culture, IL-4-stimulated cells were recovered and examined for their surface immunoglobulin expression (12). In brief, cells were washed three times and then incubated at 4°C for 30 min with fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse heavy chain ($\gamma 3$, $\gamma 1$, $\gamma 2b$, $\gamma 2a$, or α; The Binding Site, Birmingham, U.K.). To detect surface ε molecules, FITC-labeled rat monoclonal anti-ε antibody was used (Bioproducts for Science, India-

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Abbreviations: IL-4, interleukin 4; LPS, lipopolysaccharide; C, constant region; S, switch region.

napolis). After incubation, cells were washed twice and examined by fluorescence microscopy.

Isolation of Circular DNA. Circular DNA was prepared from the alkaline lysate of total cells as described (12, 24). To eliminate chromosomal DNA, the sample was treated with ATP-dependent DNase from *Micrococcus luteus* (United States Biochemical).

Phage Libraries. Circular DNA samples were digested with restriction endonuclease *Eco*RI or *Xho* I, and the digests were ligated with the phage arm DNA of λDashII (Stratagene). Recombinant phage were screened with various 5′- and 3′-switch region probes by plaque hybridization. The probes used were 3′-S_μ (*Hin*dIII–*Hin*dIII, 2.1 kb), 5′-$S_{\gamma 3}$ (*Xba* I–*Bgl* II, 1.9 kb), 5′-$S_{\gamma 1}$ (*Bgl* II–*Bgl* II, 1.5 kb), 3′-$S_{\gamma 1}$ (*Bgl* II–*Eco*RI, 7.5 kb), 5′-S_ε (*Xho* I–*Bgl* II, 1.3 kb), and 3′-S_ε (*Hin*dIII–*Xho* I, 2.0 kb) (for restriction map, see ref. 25).

Other Methods. Methods used for the DNA analysis were all standard procedures (26). Nucleotide sequences were determined by the Sanger method (27) using Sequenase version 2.0 (United States Biochemical).

RESULTS

Switch Circular DNA in the Parasite-Infected Mouse. For the study of switch circular DNA, extrachromosomal DNA was obtained from mesenteric lymph node cells by the alkaline lysis method as described (12, 24). To eliminate residual chromosomal DNA, the sample was treated with ATP-dependent linear DNase (28, 29). Since the serum IgG1 and IgE start to appear 10 days after the infection, samples were obtained on days 8, 10, and 12. To isolate circular DNA clones, 2–4 × 10^6 recombinant phage were examined with the 5′- and 3′-S-region probes of various immunoglobulin C_H genes. As summarized in Table 1, two types of clones were identified in the mouse infected with the parasite. One type was the γ1 circle, a prospective excision product of switch recombination between the C_μ and $C_{\gamma 1}$ genes. Clones of this type were positive with both 5′-$S_{\gamma 1}$ and 3′-S_μ probes. The other type was the ε circle, which hybridized with both 5′-S_ε and 3′-$S_{\gamma 1}$ probes and represented a possible excision product of switch recombination between $C_{\gamma 1}$ and C_ε. In the phage library made from parasite-free control mice, no switch-probe-positive clones were identified. It should be noted that prospective clones were not isolated for the switch from C_μ to C_ε. These clones, if present, should have been positive with both 5′-S_ε and 3′-S_μ probes. The lack of clones indicative of switching from μ to ε is not due to technical limitations such as inadequate sizes of restriction fragments for cloning. The recombined *Eco*RI fragments for the switch from μ to ε were too large for efficient phage packaging. To address this difficulty, *Xho* I was used for the library construction.

Table 1. Circular DNA clones identified in lymph nodes from parasite-infected mice

S region sequences		Predicted switch	Frequency of switch circular DNA clones			Library used
			Day	Day	Day	
5′-S	3′-S		8	10	12	
γ1	μ	μ → γ1	1.7	4.0	1.3	*Eco*RI
ε	γ1	γ1 → ε	0.6	0.5	0	*Eco*RI
ε	μ	μ → ε	0	0	0	*Xho* I

Circular DNA was prepared from lymph nodes of parasite-infected mice on days 8, 10, and 12. To make the phage library, DNA was digested with *Eco*RI or *Xho* I and ligated with arms of λDashII. For each experiment, two animals were used and 2–4 × 10^6 recombinant phage were obtained. Libraries were screened with various 5′- and 3′-S probes by plaque hybridization. Numbers of clones isolated per 10^6 phage are shown.

Table 2. Circular DNA clones identified in the *in vitro* culture with IL-4

S region sequences		Predicted switch	Frequency of switch circular DNA clones			Library used
			0	250	1000	
5′-S	3′-S		U/ml	U/ml	U/ml	
γ3	μ	μ → γ3	44.0	4.2	0.7	*Eco*RI
γ1	μ	μ → γ1	1.0	19.7	30.6	*Eco*RI
ε	γ1	γ1 → ε	0	0.3	1.7	*Eco*RI
ε	μ	μ → ε	0	0	0	*Xho* I

Circular DNA was prepared from *in vitro* cultured splenic cells stimulated with LPS (40 μg/ml) and IL-4 [0, 250, or 1000 units (U)/ml]. After 3 days of culture, cells (≈1 × 10^8) were lysed with alkali and circular DNA was prepared as described by Griffin *et al.* (12, 24). DNA was digested with *Eco*RI or *Xho* I and ligated with phage arms of λDashII. About 1–7 × 10^4 recombinant phage were generated. Libraries were screened with various S-region probes by plaque hybridization. Numbers of clones isolated per 10^5 phage are shown.

Switch Circular DNA in Cultured Splenocytes. Switch circular DNA from cultured splenocytes was also studied. IL-4-stimulated splenic cells were analyzed because the

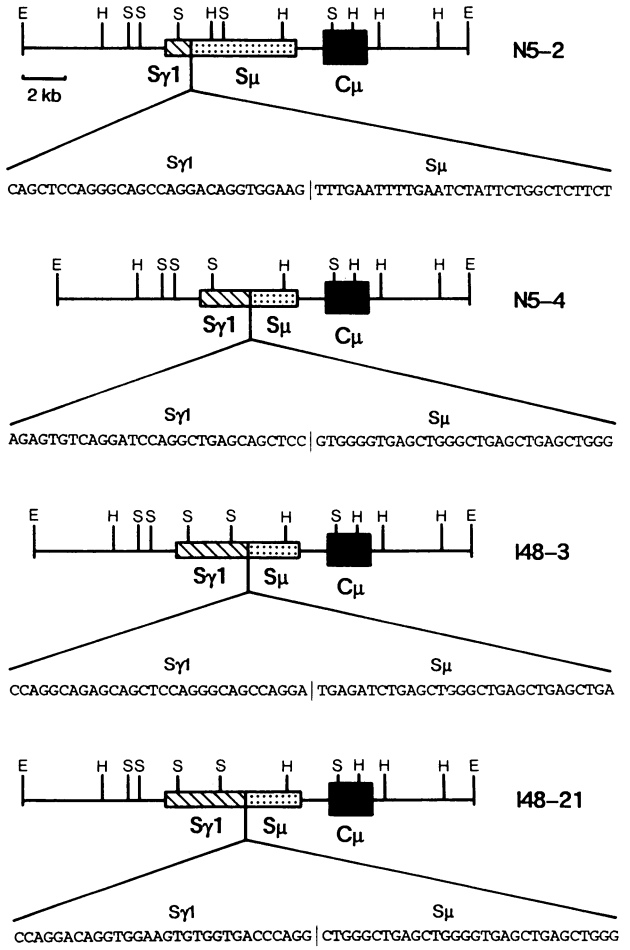

FIG. 1. Circular DNA clones for the switch from C_μ to $C_{\gamma 1}$. Four clones hybridizing with 5′-$S_{\gamma 1}$ and 3′-S_μ probes were analyzed by restriction mapping and DNA sequencing. Clones N5-2 and N5-4 were isolated from lymph nodes of mice infected with *N. brasiliensis*. Clones I48-3 and I48-21 were from splenocytes cultured with LPS and IL-4. Restriction sites are indicated: E, *Eco*RI; H, *Hin*dIII; and S, *Sac* I. Filled boxes represent C_μ genes. S regions are represented by stippled and hatched boxes. To identify the precise recombination site, the sequence was compared with the germ-line S_μ (11) and $S_{\gamma 1}$ (10) sequences. Recombination sites are indicated by vertical lines.

switch in the parasite-infected mouse appears to be mediated by the lymphokine IL-4 (18). A total of 5×10^8 cells were obtained from BALB/c mice (6-week-old females) and cultured with the mitogen LPS (40 μg/ml) and IL-4 (250 or 1000 units/ml). When treated with IL-4 (250 units/ml), 24% of cells became surface γ1-positive, while ε-positive cells were less than 0.1%. With a higher concentration of IL-4 (1000 units/ml), 1.5% of cells were positive for surface ε. As in the *in vivo* experiments, two types of switch circle clones were identified in the IL-4 culture (Table 2). One type was 5′-$S_{\gamma 1}$/3′-S_μ-positive (γ1 circle), and the other was 5′-S_ε/3′-$S_{\gamma 1}$-positive (ε circle). As described elsewhere (12), $S_{\gamma 3}$ circles (5′-$S_{\gamma 3}$/3′-S_μ-positive) were the major switch products in the LPS-stimulated cells. However, when IL-4 was added, the formation of $S_{\gamma 3}$ circles was suppressed, and the generation of γ1 circles was largely enhanced. Clones of ε circles (5′-S_ε/3′-$S_{\gamma 1}$-positive) were also isolated from the IL-4-treated splenic cells. As in the *in vivo* experiment, ε circles were all from the switch from $C_{\gamma 1}$ to C_ε, and no circles for the switch from C_μ to C_ε were identified in the IL-4 culture.

Sequence Analysis of Circular DNA Clones. To confirm that isolated clones were indeed recombinants between two different C_H genes, they were further analyzed by restriction mapping and DNA sequencing. Fig. 1 illustrates four examples of γ1 circles positive with both 5′-$S_{\gamma 1}$ and 3′-S_μ probes. Two clones (N5-2 and N5-4) were from the *in vivo* experiment

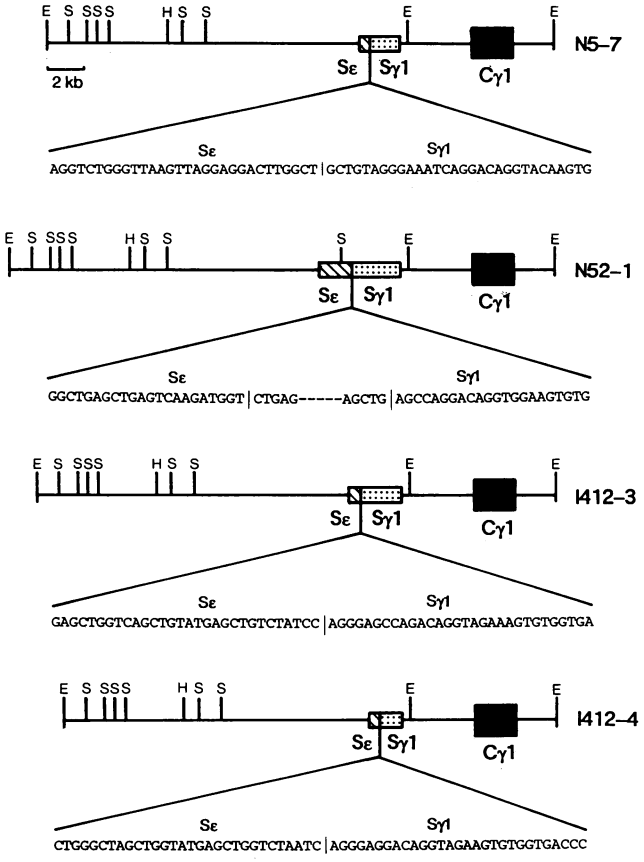

FIG. 2. Circular DNA clones for the switch from $C_{\gamma 1}$ to C_ε. Four clones hybridizing with 5′-S_ε and 3′-$S_{\gamma 1}$ probes were analyzed by restriction mapping and DNA sequencing. Clones N5-7 and N52-1 were isolated from lymph nodes of mice infected with *N. brasiliensis*, while I412-3 and I412-4 were from splenocytes cultured with LPS and IL-4. Nucleotide sequences were compared with those of germ-line $S_{\gamma 1}$ (10) and S_ε. The germ-line S_ε sequence was determined in this study. Restriction sites are indicated: E, *Eco*RI; H, *Hin*dIII; and S, *Sac* I. Filled boxes represent $C_{\gamma 1}$ genes. Switch regions are represented by stippled and hatched boxes. Recombination break points are indicated by vertical lines.

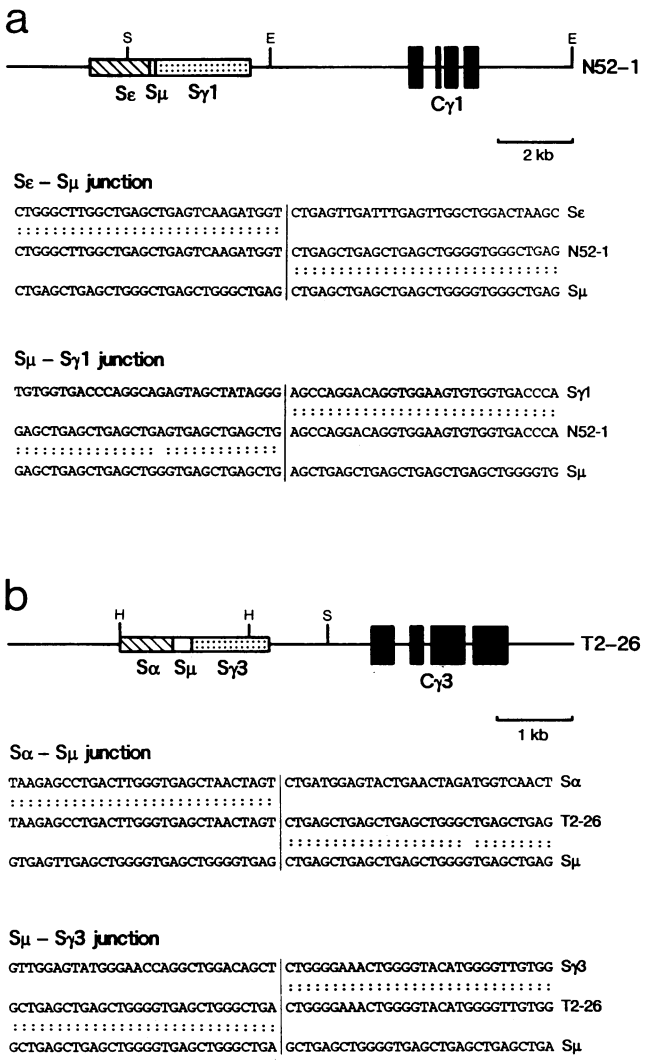

FIG. 3. Circular DNA clones containing three recombined S-region sequences. Two clones, N52-1 (*a*) and T2-26 (*b*), were analyzed by restriction mapping and DNA sequencing. N52-1 was isolated from a parasite-infected mouse on day 10. The S region was composed of S_ε, S_μ, and $S_{\gamma 1}$ in this order, followed by the $C_{\gamma 1}$ gene. T2-26 was from LPS-stimulated splenic cells cultured with the cytokine transforming growth factor β (12). It contained three S-region sequences, S_α, S_μ, and $S_{\gamma 3}$, in this order followed by $C_{\gamma 3}$. To identify precise recombination sites, the recombined S-region sequences were compared with those of germ-line S regions. Germ-line sequences for S_ε and S_α were determined in this study. Sequences for S_μ (11), $S_{\gamma 3}$ (30), and $S_{\gamma 1}$ (10) were from the literature. Recombination sites are indicated by vertical lines. Switch regions are shown in shaded boxes. Filled boxes represent C_H exons. Restriction sites are indicated: E, *Eco*RI; H, *Hin*dIII; S, *Sac* I. A : indicates identical residues.

(*N. brasiliensis*-infected), and the other two (I48-3 and I48-21) were from the *in vitro* culture (IL-4-treated). By comparing restriction maps of the clones with those of the germ-line C_μ and $C_{\gamma 1}$ genes, these clones were shown to be recombinants between S_μ and $S_{\gamma 1}$ regions. Recombination breakpoints were all identified within the S regions. Nucleotide sequence determination further confirmed that the two switch sequences, $S_{\gamma 1}$ and S_μ, were joined in the tandem orientation.

Similarly, four S_ε-positive clones were analyzed (Fig. 2). Two (N5-7 and N52-1) were from the infected mouse, and two others (I412-3 and I412-4) were from the IL-4 culture. These clones contained the 5′-S_ε sequence connected to 3′-$S_{\gamma 1}$, representing possible excision products of the switch from

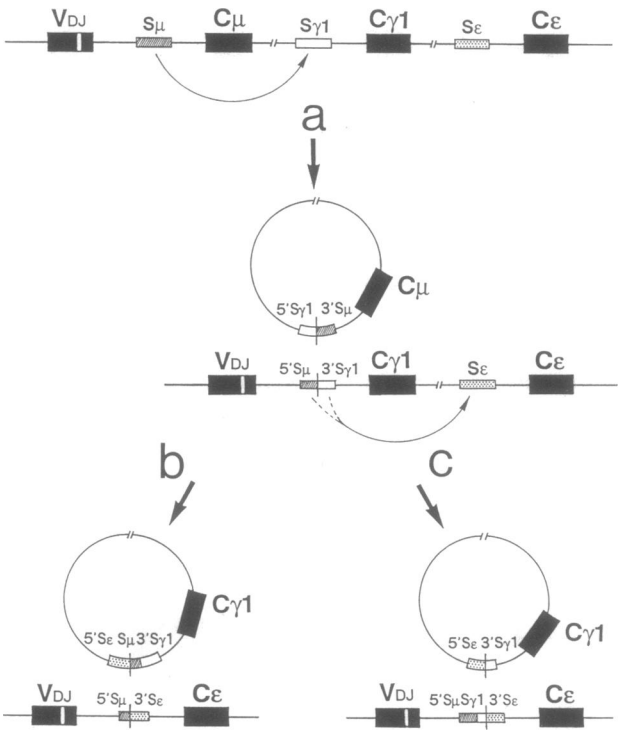

FIG. 4. Schematic diagram of successive switch recombination. In the parasite-infected mouse or in the IL-4-treated splenic cells, successive switching occurs, first from μ to $\gamma 1$ (*a*), and then from $\gamma 1$ to ε (*b* or *c*). Two kinds of switch circular DNA are generated; one is from the recombination between C_μ and $C_{\gamma 1}$ (clones in Fig. 1), and the other is between $C_{\gamma 1}$ and C_ε (clones in Fig. 2). In the switch from $\gamma 1$ to ε, S_μ sequence should be inserted between S_ε and $S_{\gamma 1}$ on the switch circle if the S_μ sequence in the $\gamma 1$ gene were used for the recombination with S_ε (*b*). If the $S_{\gamma 1}$ sequence were recombined with S_ε (*c*), no S_μ sequence would be retained on the excision product. Shaded and open boxes represent S regions. Filled boxes represent C_H genes. Recombination sites are indicated by vertical lines. V_{DJ}, variable region genes assembled with diversity and joining segments.

$C_{\gamma 1}$ to C_ε. Interestingly, in the clone N52-1 a short stretch of sequence was inserted at the joint, which was not accounted for by either the S_ε or the $S_{\gamma 1}$ sequence. As shown in Fig. 3, a sequence search of GenBank (MUSIGCD09) revealed that the unaccounted sequence was derived from the S_μ region. The presence of the S_μ insert at the breakpoint is strong evidence for a successive switch from μ to ε via $\gamma 1$ (Fig. 4). The S_μ insert could be interpreted as a trace of the preceding recombination between S_μ and $S_{\gamma 1}$ prior to the switch to ε. In the switch from μ to $\gamma 1$, a hybrid S region composed of 5'-S_μ and 3'-$S_{\gamma 1}$ is generated in the $\gamma 1$ gene (Fig. 4*a*). In the secondary switch from $\gamma 1$ to ε, a part of the S_μ sequence would be inherited in the ε gene, if the recombination occurred with the 5'-S_μ region in the $\gamma 1$ gene (Fig. 4*b*). In contrast, if the recombination occurred with the 3'-$S_{\gamma 1}$ region, the S_μ sequence would not be retained (Fig. 4*c*). A similar example of the S_μ insertion was also found for the successive switch from μ to α via $\gamma 3$ induced with the cytokine transforming growth factor β (12) (Fig. 3*b*).

DISCUSSION

In the present study we have characterized switch circular DNA in the mouse infected with the parasite *N. brasiliensis* (Table 1). Splenic cells cultured with IL-4 were also studied (Table 2). Two types of switch circular DNA were identified in these switch systems. One type is the $\gamma 1$ circle (5'-$S_{\gamma 1}$-positive), and the other is the ε circle (5'-S_ε-positive). Since the 5'-$S_{\gamma 1}$-positive clones were also positive with the 3'-S_μ probe, they could represent excision products of a switch from C_μ to $C_{\gamma 1}$ (Figs. 1 and 4*a*). Interestingly, the 5'-S_ε-positive clones did not contain the 3'-S_μ sequence but contained 3'-$S_{\gamma 1}$ instead (Fig. 2). This observation suggests that these ε clones are the excision product of the switch from $C_{\gamma 1}$ to C_ε (Fig. 4 *b* and *c*). In our libraries, no ε circles were identified for the switch from C_μ to C_ε. In the IL-4-mediated class switch, switch recombination appears to occur in a successive manner, first from C_μ to $C_{\gamma 1}$ and then from $C_{\gamma 1}$ to C_ε (Fig. 4). This suggests an interesting possibility, that the IgG1-positive cells could be immediate precursors to the IgE-producing cells. Sequential switching probably explains Finkelman's observations (17) that anti-IL-4 is very effective in inhibiting a secondary IgE response. It is yet to be determined, however, whether the switch to ε always occurs via $\gamma 1$. More examples of 5'-S_ε clones have to be analyzed to address the question. In the present study, switch circles started to appear 1–2 days before the production of IgG1 and IgE in the infected mouse (Table 1). Detection of switch circles supports the notion that IL-4 triggers the switch recombination rather than selects the preswitched cells for ε.

During the process of switching to ε, many cells express both IgG1 and IgE (20). Differential splicing of a large RNA transcript has been one of the explanations for the simultaneous expression of multiple isotypes (31, 32). Trans-splicing of two separate RNA transcripts is an alternative explanation. However, the present experiments suggest that successive DNA deletion is another explanation for simultaneous expression of $\gamma 1$ and ε, if we can assume that the $\gamma 1$ mRNA is relatively stable and functional even after the switch to ε. The IL-4 system will continue to provide useful tools for the study of switch mechanisms not only at the DNA level but at the RNA level as well.

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