Immunoglobulin Switch Transcript Production In Vivo Related to the Site and Time of Antigen-specific B Cell Activation

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Summary

Immunoglobulin (Ig) class switch recombination is associated with the production and splicing of germline IgC_H messenger RNA transcripts. Levels of $\gamma 1$ transcripts in mouse spleen sections were assessed by semiquantitative analysis of reverse transcriptase polymerase chain reaction (PCR) products during primary and secondary antibody responses to chicken gamma globulin (CGG). This was correlated with the appearance of CGG-specific B cells and their growth and differentiation to plasma cells. After primary immunization with CGG, $\gamma 1$ switch transcripts appeared after 4 d, peaked at a median of six times starting levels between 10 and 18 d after immunization, and returned to background levels before secondary immunization at 5 wk. By contrast, after secondary challenge with CGG, a sevenfold increase in transcripts occurs during the first d. The level again doubles by day 3, when it is six times that which is seen at the peak of the primary response. After day 4, there was a gradual decline over the next 2–3 wk. Within 12 h of secondary immunization, antigen-specific memory B cells appeared in the outer T zone and by 24 h entered S phase, presumably as a result of cognate interaction with primed T cells. Over the next few hours, they migrated to the edge of the red pulp, where they grew exponentially until the fourth day, when they synchronously differentiated to become plasma cells. The same pattern was seen for the migration, growth, and differentiation of virgin hapten-specific B cells when CGG-primed mice were challenged with hapten protein. The continued production of transcripts after day 3 indicates that switching also occurs in germinal centers, but in a relatively small proportion of their B cells. The impressive early production of switch transcripts during T cell–dependent antibody responses occurs in cells that are about to undergo massive clonal expansion. It is argued that Ig class switching at this time, which is associated with cognate T cell–B cell interaction in the T zone, has a major impact on the class and subclasses of Ig produced during the response.

Generally cells producing IgG, IgA, or IgE in the absence of IgM or IgD have undergone switch recombination of the Ig heavy chain genes (1, 2). In this process, genetic material between the rearranged V region genes and the selected IgH constant region genes is deleted (3–5). Several groups have noted a close correlation between switch recombination to a particular IgH isotype and the previous production of switch transcripts (6–10). These are sometimes referred to as sterile or germline IgC_H transcripts. Switch transcripts are derived from the genes that encode the relevant heavy chain constant region isotype together with the flanking switch region and a small exon upstream of the switch region. Deletion of the upstream exon has been found to abolish switching (11, 12). The transcription product is processed with the switch region being deleted and the transcript of the 5′ exon spliced to that of the IgH constant region (13). The spliced out transcripts of the intron are able to form DNA-RNA hybrids with the switch region; it has been suggested that this property may be of functional significance (14). The processing of the transcript is required to induce high levels of switching (15, 16). Recent studies of B cells from transgenic mice indicate that the production and splicing of mouse $\gamma 1$ switch transcripts, but not the production of the unspliced message alone, is sufficient to induce Ig switch recombination (17). Switch transcripts have no or only very small open reading frames and therefore probably do not encode functional proteins (13).

Most studies of the association of Ig isotype-specific switch transcripts have been carried out in vitro, where the ability of cytokines to induce switching to a particular isotype has been assessed. IL-4 classically induces switch to IgG1 and then IgE (18–21). The contrary effect is brought about by IL-2, and IFN-γ – IgG2a synthesis is induced and

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the production of IgG1 and IgE is lowered (18, 22). Cytokines delivered in vivo such as IL-4, IFN-γ, and IL-12 can have a marked effect on the class of Ig produced during an antibody response (23–27). Relatively little work describing the stage(s) during physiological antibody responses where switch recombination occurs has been published. Nonexclusive candidate targets for the induction of switching are: (*a*) B cells that have taken up and processed antigen as they make cognate interaction with primed T cells in the T zones of secondary lymphoid organs (28–30); (*b*) centrocytes making cognate interaction with T cells during selection in germinal centers (31); (*c*) B blasts undergoing clonal expansion in germinal centers or extrafollicular foci (28–30). To address this problem, we have studied the level of γ1 switch transcripts in cryostat sections of the spleen during the response of B10A mice to primary and secondary immunization with chicken gamma globulin (CGG).[1] This has been correlated with the distribution, growth, and differentiation of antigen-specific B cells assessed by immunohistology in serial sections from the same spleen. To compare the behavior of memory and virgin B cells, some groups of CGG-primed mice were challenged with (4-hydroxy-3-nitrophenyl)acetyl (NP) conjugated to CGG. This way, the response of virgin NP-specific B cells was compared with that of memory CGG-specific cells.

Materials and Methods

Mice. Female B10A mice (homozygous $IgC_H{}^b$, $H_2{}^k$) were used in all experiments. These were isolator-bred, specific pathogen-free animals (Harlan UK Ltd., Bicester, Oxford, UK). They were kept in autoclaved filter boxes, and their bedding, food, and water were also autoclaved. These mice have a low background of immune response in their spleens; germinal centers are rarely seen in the spleens of unimmunized mice. Experimental groups were age and weight matched.

Antigens. CGG (Sigma, Poole, Dorset, UK) was used either as a solution in sterile saline or as an alum precipitate (32). NP-CGG and other NP-protein conjugates used in immunohistology and ELISA were prepared as described (33) using the succinimide ester of NP (Cambridge Research Biochemicals, Northwhich, Cheshire, UK). *Bordetella pertussis* vaccine was obtained from Lederle Labs (Gosport, Hants, UK).

Immunizations. Mice received primary immunization when aged between 7 and 10 wk. This was with a 50-μg alum precipitate of either CGG or NP-CGG given intraperitoneally together with 10^9 chemically killed *B. pertussis*. Secondary immunizations were given intravenously 35 d later as either 50 μg soluble CGG or 50 μg soluble NP-CGG.

Tissue Preparation. Mice were given 2 mg of the thymidine analogue 5′-bromo-2-deoxyuridine (BrdUrd; Sigma) intraperitoneally to label cells in the S phase of the cell cycle 2 h before they were killed by CO_2 asphyxiation when their spleens were removed for immunohistology. The middle third of the spleens was individually wrapped in aluminium foil and snap frozen by sequential dipping in liquid nitrogen. They were then stored in sealed polythene bags at −70°C. 5-μm sections of the spleens cut from the convex to concave surface were either mounted on four spot glass slides for immunohistology or placed in a 1.5-ml polypropylene microfuge tube for mRNA extraction. The glass-mounted sections were air dried for 1 h and then fixed in acetone at 4°C for 20 min. They were again dried for 10 min before sealing in polythene bags and were stored at −20°C until used.

Immunohistology. CGG-binding cells were marked in tissue sections using biotinylated CGG prepared using biotin succinimide ester (34). The cells binding CGG-biotin were then detected using the streptavidin alkaline phosphatase reagent, StreptABComplex/AP (Dako, High Wycombe, Bucks). NP-binding cells were identified using NP-conjugated rabbit IgG followed with biotinylated goat anti–rabbit IgG (Dako) and then StreptABComplex/AP. When NP-binding cells were identified together with CGG-binding cells, NP-conjugated sheep IgG was used, followed by donkey anti–sheep IgG peroxidase conjugate (The Binding Site, Birmingham, UK). Cells expressing IgD, IgG, IgA, and IgM were identified using the appropriate sheep IgG anti–mouse Ig-isotype (The Binding Site) and donkey anti–sheep IgG peroxidase conjugate. CD3-expressing cells were identified using a rat IgG2a mAb KT3 specific for mouse CD3 (Serotec, Kidlington, Oxford, UK) followed by biotinylated rabbit anti–rat IgG (Dako) and StreptABComplex/AP. All reagents were used at dilutions that have been predetermined to give the best specific staining with minimal background. Where paired staining was carried out, the two first reagents were added for 1 h. The reagents were then absorbed onto tissue paper before the slides were washed in Tris-buffered saline, pH 7.6. The two secondary reagents were then added to each section for a further hour, before their removal and washing, as described before. Where a biotinylated second reagent was used, StreptABComplex/AP was added for 30 min before washing, as described before. Peroxidase activity was developed using freshly prepared and filtered diaminobenzidine, one 10-mg tablet (Sigma) in 15 ml Tris-buffered saline, pH 7.6, with 1 drop of 30% wt/wt H_2O_2. Alkaline phosphatase activity was developed using 1 mg/ml Fast Blue BB salt (Sigma) with 0.4 mg/ml naphthol AS-MX phosphate (Sigma) containing 0.8 mg/ml levamisole to block endogenous alkaline phosphatase activity. The staining for CGG- and NP-binding cells was considered to be specific in that no or minimal cell staining was seen when tissues from nonimmunized mice were examined. In addition, when simultaneous staining for NP- and CGG-binding cells was carried out, no double-positive cells were seen, while the immune complex bound to follicular dendritic cells (FDC) gave mixed staining. To distinguish NP-specific cells in follicles from NP-specific immune complex on FDC, double staining was done for IgA and NP. In this situation, the immune complex stained black and the NP-specific cells stained blue. The same means were used to distinguish between CGG-specific B blasts and CGG-specific immune complex in the first 7 d of the responses to NP-CGG in CGG-primed mice.

BrdUrd uptake was detected after the first two staining procedures had been completed. The sections were treated for 20 min with 1 M HCl at 60°C. This renders the epitopes on DNA containing BrdUrd accessible while destroying the activity of previously bound immunohistological reagents but leaving the blue and yellow/brown colored precipitates intact. The slides were washed in two changes of distilled water and then Tris-buffered saline. DNA with BrdUrd was then detected using mouse anti-BrdUrd mAb clone BU20a (Dako), secondary reagent goat anti–mouse biotin and final reagent streptavidin ABC. These were applied as for the immunoalkaline phosphatase staining described

[1] *Abbreviations used in this paper:* BrdUrd, 5′-bromo-2-deoxyuridine; CGG, chicken gamma globulin; FDC, follicular dendritic cell; NP, (4-hydroxy-3-nitrophenyl)acetyl.

above. The alkaline phosphatase activity was developed using Fast Red TR (Sigma) with naphthol ASMX. The slides were mounted in glycerol gelatin (BDH, Poole, Dorset, UK).

Assessment of Germinal Center Size. Sections stained to identify CD3, IgD, and BrdUrd uptake were used. Germinal centers were identified as IgD^- areas surrounded by IgD^+ follicular mantle; germinal centers were distinguished from the T zone by the much larger concentration of $CD3^+$ cells in the latter. The germinal center areas were assessed using the point counting technique of Weibel (35).

Quantitation of Extrafollicular Antigen-specific B Cells and Plasma Cells and Assessment of Germinal Center Size. For this purpose, we used slides that carried three serial sections stained, respectively, to show (*a*) CD3, IgD, and BrdUrd uptake; (*b*) CGG-binding cells, IgD and BrdUrd uptake, and (*c*) NP-binding cells, IgD and BrdUrd uptake. These sections were examined as described above using the point counting system. The concentration of these extrafollicular antigen-specific cells was expressed as the number of cells counted per unit area of spleen section screened. The proportion of CGG- or NP-specific cells that had taken up BrdUrd in the 2 h before the spleen was removed was also determined.

Quantitation of Switch Transcripts. Oligo-TdR–coated magnetic beads (36) were used for cDNA preparation. This was prepared from each spleen from a 5-μm cryostat section taken from the tissue block immediately after those used for immunohistology. Each section was placed into a sterile 1.5-ml polypropylene tube and lysed in 250 μl cold lysis buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% Li-dodecylsulfate, 5 mM dithiothreitol) and homogenized by repeatedly passing through a 21-gauge needle. The lysate was centrifuged at 12,000 *g* for 2 min, and the supernatant was incubated with 250 μg oligo-TdR Dynabeads (Dynal, Wirral, UK) at room temperature for 5 min. The tubes were inserted into a magnet. After 2 min, the supernatant was discarded and the beads were washed twice in cold washing buffer (10 mM Tris-HCl, pH 8.0, 150 mM LiCl, 1 mM EDTA, 0.1% Li-dodecylsulfate) and three times with the same washing buffer without Li-dodecylsulfate. After the last wash, 10 μl 2 mM EDTA, pH 8.0, was added and the tubes were heated to 65°C for 2 min. The tubes were placed in the magnet. 10 μl supernatant was transferred to a new tube, and 1 μl $TdR_{12\text{-}18}$ (500 μg/ml; Pharmacia, Uppsala, Sweden) added. The mixture was heated to 70°C for 10 min and quickly chilled on ice; 4 μl first-strand buffer, 2 μl dithiothreitol (100 mM), 1 μl dNTP (10 mM), 1 μl RNAse inhibitor (RNAguard; Pharmacia), and 0.5 μl reverse transcriptase (M-MLV; Life Technologies, Paisley, UK) were added and the mixture incubated for 2 h at 42°C. The reverse transcriptase was then inactivated by heating to 90°C for 10 min, and the samples were diluted to 50 μl with water and stored at 4°C.

Switch transcripts were detected by intron-spanning reverse transcription (RT)-PCR. Intron-spanning primers were designed using OLIGO version 4.1 (National Biosciences, Plymouth, MA) with the sequences for IgG1 (GenBank accession no. M12389 and J00453). The following primers were used for amplification of γ1 switch transcript (GGG AAC TAG CCC TTG ACC AGG CAT C) and (CCT CCT AGA CAA GCA CAG GCA TGT AGA). Primers were obtained from Pharmacia. Mouse β-actin–specific primers were obtained from Stratagene (Cambridge, UK).

1 μl of each cDNA preparation was amplified by PCR over 26 cycles for β-actin as described below. 6 μl of the resulting PCR product was analyzed on an ethidium bromide gel to obtain an estimate of its cDNA content. This gel contained a standard β-actin amplificate and three threefold dilutions of this. These were used to estimate the concentration of cDNA template in each original cDNA preparation. A volume between 0.3 and 3 μl cDNA template yielding signals of the same density for all β-actin–specific PCR was used for the PCR amplification of switch mRNA. This concentration fell comfortably within the logarithmic range for β-actin amplification with the conditions used. PCR was performed in 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 1 mM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 0.5 U Taq polymerase (Promega, Madison, WI) in a volume of 20 μl. Samples were cycled for 30 s at 94°C, 30 s at an annealing temperature of 65°C for IgG1, 60°C for β-actin, and 1 min plus 2 s for every cycle at 72°C. PCR for β-actin was performed for 26 cycles, and PCR for the switch mRNAs was performed for 34 cycles. The PCR products were separated on a 2% agarose gel containing 0.15 μg/ml ethidium bromide with a 100-bp ladder (Life Technologies) as a molecular weight standard. Preliminary experiments with different cycle numbers followed by dot blot quantitation ensured that amplifications using these cycle numbers were still in their logarithmic phase.

The amplified product was quantified by dot blot analysis; 2 μl of the PCR reaction product was diluted 1/40, 000 with 0.4 M NaOH and 25 mM EDTA; 200 μl of this were transferred onto a prewetted Hybond N^+ membrane (Amersham International, Little Chalfont, UK) using a dot blot apparatus (Bio Rad, Hemel Hempstead, Hertfordshire, UK). The membrane was washed in 2× SSC and incubated overnight at 65°C in hybridization solution (2× SSC, 1% SDS, 0.5% fat-free milk, 10% dextransulfate); 50 ng PCR product from an earlier experiment was used as a probe. The specificity of this probe for the amplificate was shown by Southern blotting a gel of an earlier PCR specific for IgG1 switch transcript and hybridizing under the same conditions as for the dot blot. The probe was labeled with [^{32}P]dCTP (ICN, Thame, Oxfordshire, UK) using an oligolabeling kit (Pharmacia). Hybridization was performed at 65°C in hybridization solution including 100 μg/ml salmon testis DNA (Sigma). Blots were washed in 2× SSC with 1% SDS for 20 min at room temperature and then sequentially in 1×, 0.5×, and 0.1× SSC with 1% SDS for 5 min at 68°C, which resulted in negligible background hybridization. Blots were imaged and quantified using a phosphorimager (Molecular Dynamics, Kent, UK). To account for variabilities in the starting cDNA concentration, all values obtained for the amount of switch RNA amplification product were normalized to β-actin expression.

A further check was made that γ1 switch transcripts were not being underestimated through the starting cDNA concentration being too high to yield logarithimic amplification. This was carried out on the five samples that yielded the greatest amount of amplified product of cDNA of the γ1 switch transcripts. For these samples with high levels of γ1 switch transcript, further PCR for both β-actin and γ1 switch transcript was carried out on three fourfold dilutions of each cDNA preparation. These further studies showed that all of these cDNA samples produced a logarithmic increase in the amount of cDNA over the 34 PCR cycles used.

Results

The Rapid Appearance of γ1 Switch Transcripts during the Secondary Response to CGG. Mice were primed intraperitoneally with alum-precipitated CGG plus *B. pertussis* and

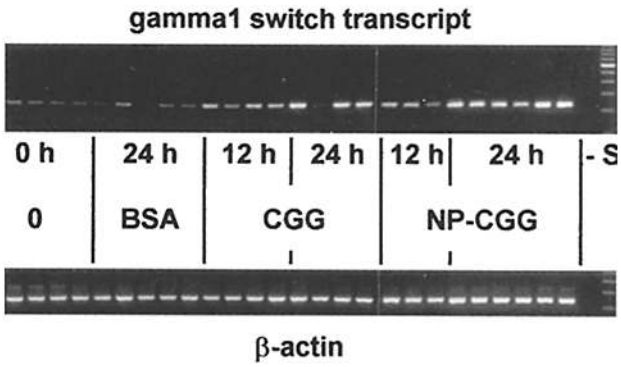

Figure 1. Rapid induction of γ1 switch transcripts in CGG-primed mice after challenge with CGG, NP-CGG. Control mice were primed with CGG and challenged with BSA. Each track corresponds to one animal. The one animal, which did not produce switch transcripts 24 h after challenge, did not have CGG-specific B cells in adjacent spleen sections. *S*, molecular weight marker; –, buffer control.

5 wk later were challenged intravenously with soluble CGG or NP-CGG. Tissue taken from frozen sections of spleens before and at 12 and 24 h after the secondary challenge were processed by RT-PCR to detect the appearance of γ1 switch transcripts. The results of this experiment are shown in Fig. 1. Already by 12 h after challenge, there was a marked increase in the amount of γ1 switch transcript recovered from the spleens, and this had increased substantially by 24 h. There was no obvious difference between the increase in transcripts in mice challenged with CGG alone or with NP-CGG, suggesting that much of the early switching occurred in memory B cells. Control mice that had been primed with CGG and challenged with BSA showed no early change from the low level of switch transcripts that were seen 5 wk after priming.

The Relative Levels of γ1 Switch Transcripts Produced during Primary and Secondary Responses to CGG and NP-CGG. The levels of γ1 switch transcripts recovered during the primary response (Fig. 2 *a*) were consistently lower than

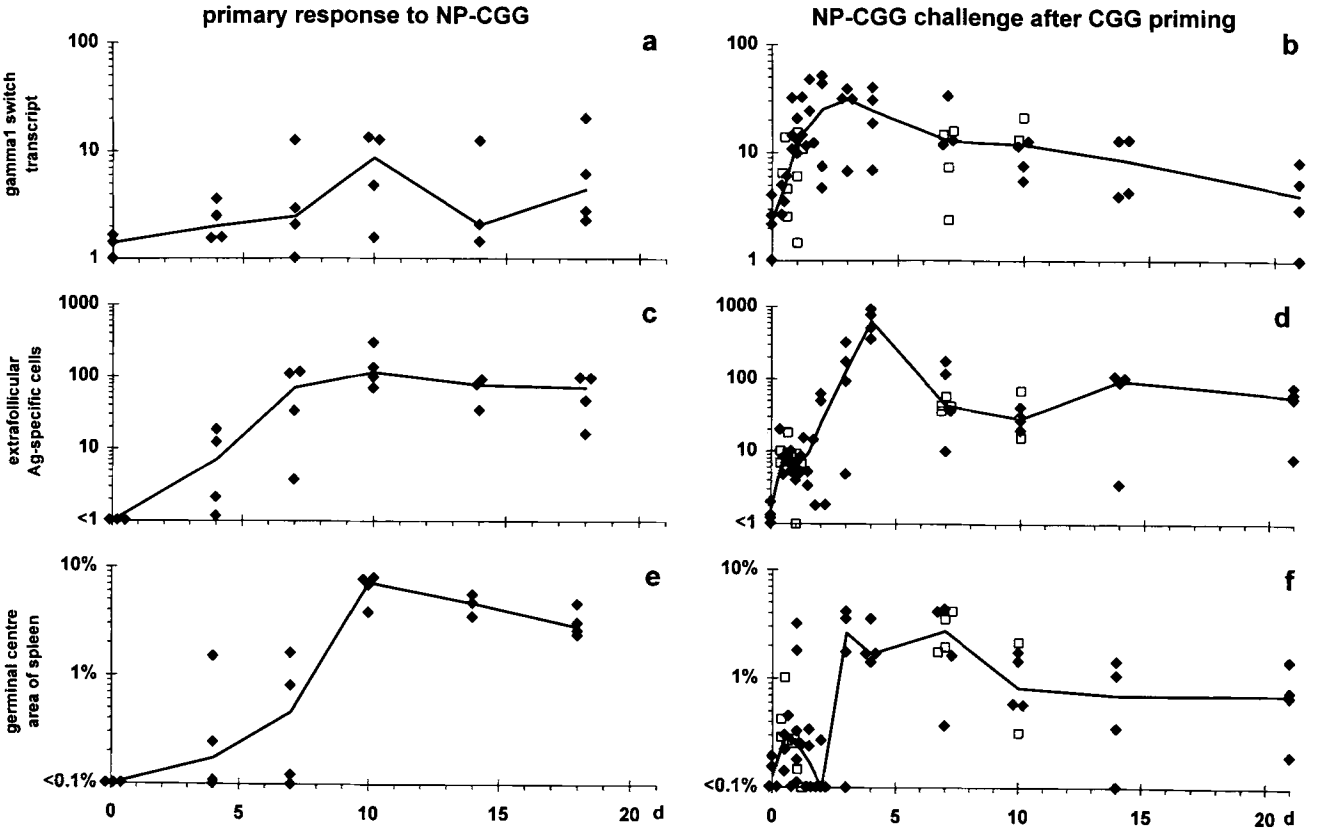

Figure 2. Correlation between γ1 switch transcript production and the site and time of antigen-specific B cell activation. Results for the primary response to NP-CGG are shown in *a*, *c*, and *e*; those for the response of CGG-primed mice to CGG or NP-CGG are shown in *b*, *d*, and *f*. The levels of γ1 switch transcripts recovered are shown in *a* and *b*; the number of extrafollicular Ag-specific B cells or plasma cells are shown in *c* and *d*, and the area of sections occupied by germinal centres are shown in *e* and *f*. The lines are drawn through the median values at each time point. Two mice on day 2 and one mouse on day 3 of the secondary reponse yielded low levels of switch transcript (*b*). These mice also had small numbers of antigen-specific cells (*d*). ◆, mice immunized with NP-CGG; □, mice that underwent a secondary response to CGG only. The semiquantitative analysis of switch transcription is described in Materials and Methods. The values for extrafollicular antigen-specific cells show the number of cells per square millimeter of section, CGG-specific plasma cells in the red pulp of the spleen before and for the first 2 d after challenge are excluded from the number of antigen-specific cells, since they relate to previous immunization rather than the responses to secondary exposure to CGG. Germinal center areas are plotted as the percentage of the section area they occupy.

those seen in the secondary response (Fig. 2 *b*). The peak levels of transcript during the primary response were only 18% of the highest levels which were found during the secondary response. A rapid early rise in the level of switch transcripts was again seen in the secondary response, with the median levels increasing sevenfold in the first 24 h after immunization. The median level again doubles to a peak at day 3 followed by a gradual decline over the next 2–3 wk.

The Location, Growth and Differentiation of Antigen-specific B Cells in the Response of CGG-primed Mice to Intravenous Challenge with Soluble CGG or NP-CGG. Before secondary challenge, antigen-specific B cells in the spleens of primed animals were mainly located in the marginal zone (Fig. 3 *a*). 12 h after intravenous challenge with CGG, antigen-specific cells were visible in the junction of the T zone and follicles, but were no longer detectable in the marginal zone, suggesting antigen-induced migration from the marginal zone to the T zone. At this stage, the number of CGG-specific cells at the junction between the T zone and the follicles was four times that which was found in the marginal zone before challenge, indicating that antigen-specific cells had also been recruited from outside the spleen. By 24 h, the cells at the junction of the T zone with the follicles had entered S phase, as shown by the uptake of BrdUrd given 2 h before the spleens were removed (Fig. 3 *b*). After the first day, CGG-specific cells were mainly identified in those parts of the red pulp adjacent to the T zone (Fig. 3 *c*). There they increased in cell number exponentially over 60–72 h when the median increase in cell number was 90-fold, indicating that they had gone through six or seven cell cycles (Fig. 2 *b*). By day 4 after immunization, almost all the cells in these extrafollicular foci had come out of cell cycle and differentiated to become plasma cells. This synchronous differentiation occurs both in the NP- and CGG-specific cells in animals challenged with NP-CGG. On the sixth day after challenge 90% of these plasma cells disappear and there is morphological evidence that this occurs as the result of apoptosis in situ. The synchronous events occurring in the extrafollicular response suggests that a single cohort of memory and virgin cells is recruited into

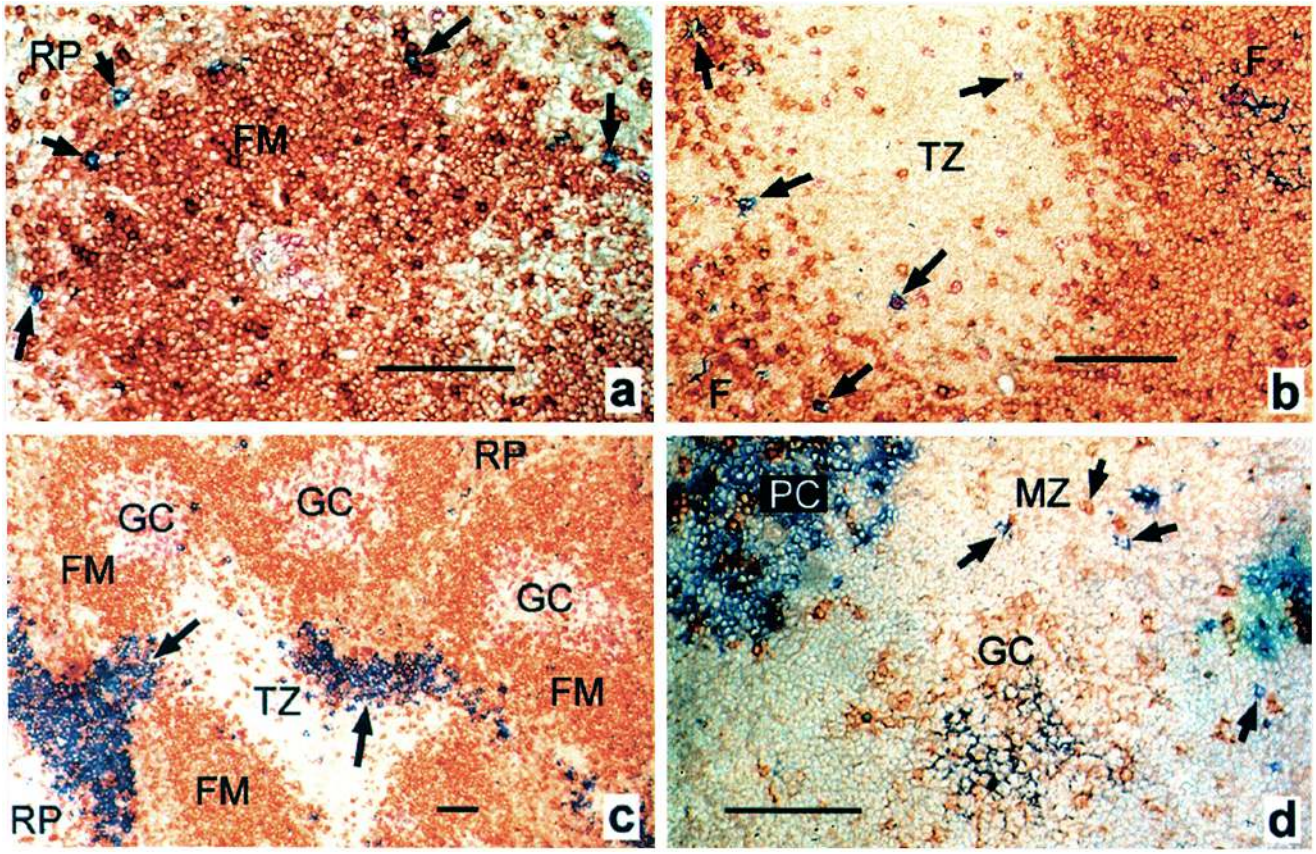

Figure 3. Photomicrographs of antigen-specific cells in the spleens of CGG-primed mice before and after challenge with NP-CGG: (*a*) CGG-specific memory B cells are shown in blue (*arrows*) in the marginal zone 5 wk after priming, IgD^+ follicular mantle (*FM*) B cells are stained brown. *RP*, red pulp. (*b*) CGG-specific B blasts (*arrows*) in the area near the junction of the T zone (*TZ*) and follicle (*F*) 24 h after challenge; these are stained blue, and those with red nuclei took up BrdUrd in the 2 h before the spleen was taken. CGG-containing immune complex (*black*) is seen localized on FDC (*top right*). (*c*) large foci (*arrows*) of CGG-specific plasma cells (*blue*) in those parts of the red pulp adjacent to T zones 4 d after challenge. Well-developed germinal centers (*GC*) are identifiable by their lack of brown IgD^+ cells and the presence of cells that had taken up BrdUrd (*red*). (*D*) The same spleen as in *c* stained to show CGG-specific cells (*blue*) and NP-specific cells (*brown*). The edge of a red pulp focus of plasma cells (*PC*) is shown top left, in the center is a germinal center with NP-specific B cells and NP- and CGG-specific immune complex (*black*) on FDC, memory cells are arrowed in the marginal zone (*MZ*). Bars, 100 μm.

the responses during the first few hours after intravenous challenge. It seems likely that recruitment is curtailed by the removal of free antigen as it complexes with preformed antibody either by phagocytes or by its transport to FDC (Fig. 3, *b* and *c*).

After CGG-primed mice were challenged with NP-CGG, germinal centers were seen developing from day 3 (Figs. 2 *f* and Fig. 3 *d*). Even at their largest, they were about half the peak size of germinal centers in the primary response (Fig. 2 *e*). Groups of mice were studied 7 and 10 d after secondary immunization with soluble CGG; at these limited time points, there was no obvious difference between the size of germinal centers in the NP-CGG– and CGG-challenged mice.

The Primary Response to CGG and NP-CGG. In the primary response, extrafollicular recruitment and clonal expansion of antigen-specific B cells was seen to start on day 4 after immunization (Fig. 2 *c*). The histological picture suggests an extrafollicular process similar to that which is seen in the secondary response, but with recruitment of antigen-specific cells taking place over several days rather than a few hours. It seems likely that this occurs from the time primed T cells first appear and continues until the onset of specific antibody production 7 d after immunization, and it might be extended further as the antigen is alum-precipitated.

Signs of germinal center formation were first seen 4 d after primary immunization with NP-CGG. By day 10, well-developed germinal centers were apparent. These reached peak size by day 14 when they occupied a median of 7% of the splenic section area (Fig. 2 *e*). Their size declined during the third and fourth weeks after immunization.

The Relative Frequency of CGG- and NP-specific Virgin B Cells and CGG-specific Memory B cells in Carrier-primed Mice. The recruitment of both NP- and CGG-specific B cells seems to start most likely at the same time during the primary response to NP-CGG, i.e., when primed T cells that recognize CGG-derived peptides become available. Consequently, the relative number of virgin NP- and CGG-specific cells recruited into the primary response to NP-CGG can be deduced from the respective numbers of these cells observed during their log phase of growth. Fig. 4 shows that the median number of NP-specific cells is 30 times that of CGG-specific cells during this phase. In the response of CGG-primed mice to NP-CGG, the recruitment of NP- and CGG-specific cells was confined to the first few hours after the intravenous challenge. The relative number of cells recruited during this period can be deduced from the observation that six times more CGG-specific plasma cells than NP-specific plasma cells are generated by day 4 after immunization (Fig. 3 *d*). Taken together, these observations suggest that during the response to NP-CGG in CGG-primed mice, 180 CGG-specific memory cells are recruited for each CGG-specific virgin cell.

The Ig Classes Produced by CGG- and NP-CGG–specific Short-lived and Persistent Plasma Cells in the Splenic Red Pulp. By double immunoenzyme staining, it has been possible to

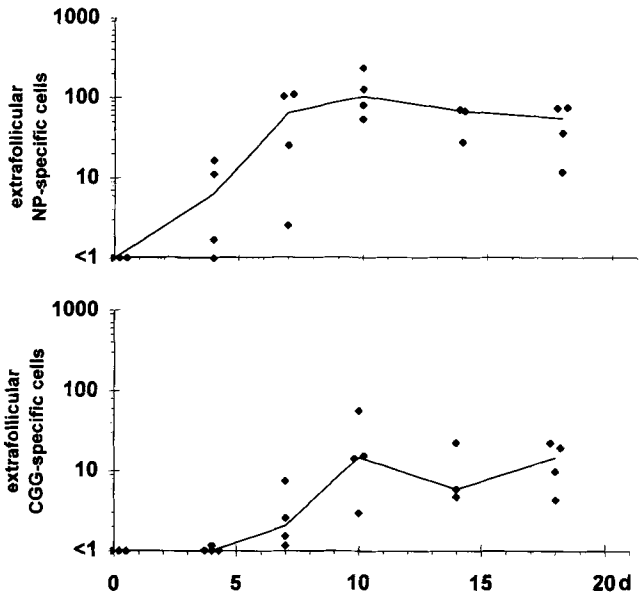

Figure 4. The primary response to NP-CGG is dominated by NP-specific cells. The graphs are showing the numbers of extrafollicular NP-specific and CGG-specific B cells and plasma cells per square millimeter of spleen area. Each diamond corresponds to one animal. The lines are drawn between the median values for each time point.

determine the class of Ig produced by antigen-specific plasma cells. This was carried out at three time points: 5 wk after priming with CGG, and 4 and 14 d after CGG-primed animals had been challenged with NP-CGG (Fig. 5). At each time point, the proportion of switched CGG-specific plasma cells was greater than that of nonswitched plasma cells, but interestingly, significant numbers of IgM-producing, CGG-specific plasma cells were present even 14 d after secondary challenge, indicating a persistent nonswitched population. At 4 d after immunization, 14% of NP-specific plasma cells were switched. This had risen to 64% by day 14.

Discussion

Switch transcripts have been identified in isolated mouse and human tissues (37, 38), but the search for switch tran-

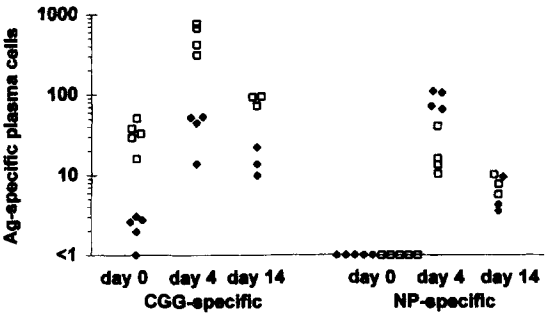

Figure 5. Ig class produced by CGG- and NP-specific plasma cells in the spleen during the response to NP-CGG in CGG-primed animals. Adjacent spleen sections were each stained to identify plasma cells specific for NP or CGG and one of the Ig classes; □ the number of antigen-specific IgG-producing plasma cells per square millimeter of spleen section; ◆ the number of antigen-specific IgM-producing plasma cells.

scripts in tissue sections using in situ hybridization has been unsuccessful (39). None of these studies correlated the presence of switch transcripts with the location and phenotype of the B cells that were producing the transcripts. The present study indicates that switch recombination to the γ1 constant region occurs at different times during responses to NP-CGG. It also shows that the sites of B cell activation vary during the response. The phenotype of the antigen-specific cells within and outside follicles varies (40) as does that of the neighboring cells (41, 42), and consequently, signals inducing switch recombination are likely to vary. It is striking that switch transcript production starts within 12 h of secondary challenge with CGG and increases six-fold before antigen-specific B cells complete their first antigen-driven cell cycle. At this time, the number of antigen-specific cells is small and the amount of switch transcript produced per antigen-specific cell in extrafollicular foci is 100 times what is produced on day 4, when exponential growth of these cells stops (Fig. 6). The early switch activity seems likely to be associated with cognate interaction between nonswitched B cells and T cells in the outer T zone; production of transcripts during cognate interactions in vitro has been noted (43). The antigen-specific B cells that are activated during the first 2 d after immunization undergo extensive proliferation either in the extrafollicular foci or as part of a germinal center reaction. Switching at this stage will have a far greater influence on the relative amounts of each class of antibody that are produced during a response than a switch event that occurs in a cell that is about to differentiate into a plasma cell. The lower levels of switch transcript found after day 3 are likely to be derived from germinal centers as extrafollicular proliferation of antigen-specific B cells was not apparent at that time. Perhaps these late switching events are occurring at the time of centrocyte selection by cognate interaction with T cells in the light zone (44–46). There is a lack of correlation between the level of switch transcript produced and the size of germinal centers; germinal centers were larger in the primary response than they were in the secondary response, but they were associated with lower levels of switch transcript. The reason for this finding is not clear and deserves further investigation.

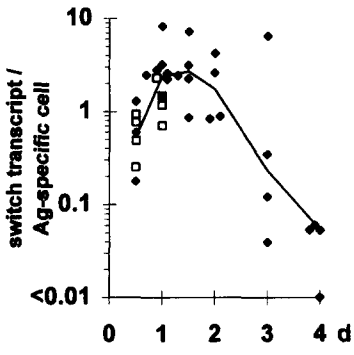

Figure 6. The level of switch transcript isolated during the response of CGG-primed mice to NP-CGG or CGG, expressed as a function of the number of Ag-specific B cells that were detected. The values were calculated by dividing the amount of switch transcript isolated from each spleen (shown in Fig. 2 *b*) by the number of Ag-specific B cells from the same spleen (shown in Fig. 2 *d*).

There is no direct evidence from the present study to indicate the relative contribution of nonswitched memory B cells and virgin B cells to the production of switch transcripts. Several points suggest that switching occurs in memory cells as well as in virgin cells. First, the amount of switch transcript produced after CGG-primed animals were boosted with CGG was as great as that after boosting with NP-CGG; it was calculated that >99% of the CGG-specific B cells recruited in the response to NP-CGG in CGG-primed mice were memory cells. Some of these must have been nonswitched, since 8% of the CGG-specific plasma cells found 4 d after challenge were nonswitched. There also seems to be a persistence of a minority population of nonswitched CGG-specific cells, since 13% of CGG-specific plasma cells in the red pulp 14 d after secondary challenge were IgM^+.

Nonswitched memory B cells are well recognized; these have been isolated from the human spleen (47, 48), blood (49), and palatine tonsil (50). The present study indicates that these cells have the potential to undergo switch recombination on recruitment into recall responses. It seems possible that these nonswitched memory cells are the targets of neoplastic transformation in multiple myeloma, perhaps at the time they are induced to undergo switch recombination. This disease almost always manifests itself as a neoplasm of switched plasma cells, but several groups have identified nonswitched memory B cells in patients that have the same CDR3 structure and V region mutations as the neoplastic cells (51–53). This consistent finding suggests that the neoplasm appears in a cell that has undergone switch recombination after leaving a germinal center.

Considerable progress has been made by studying the ability of cytokines to drive class switching. Much of this research has been carried out in vitro (13, 20, 54–56). A limited number of experiments in vivo have shown that administered cytokines or a cytokine encoded by a transgene can markedly modify the class of antibody produced during an immune response: examples include IL-4 (24, 25) and IL-12 (26). Mice that lack functional genes for IL-4 do not switch to IgE (57), and anti–IL-4 treatment inhibits IgE responses in normal mice (58). IFN- γ reduces in vivo IgG1 and IgE upon anti-IgD injection and enhances the IgG2a response (27).

The relative importance of the different signals that can induce IgH switch recombination is going to be difficult to define unless the cells where this process occurs are identified, and their phenotype plus the phenotype of the surrounding cells are determined. The present study makes a start toward this direction, but its precision would be greatly increased if analyses of small areas of tissue sections are made. The technology for identifying genomic DNA has been refined down to the single cell level for the analysis of cells that have been identified by immunohistology (59, 60). Identification of Ig class switching at the DNA level using the PCR technology is hampered by the size of Ig

switch regions. These span several 1,000 bp, and the site of recombination can occur at almost any point along this region (61). Direct PCR amplification over the recombination site in the genome is impractical because of the variability of the splicing point, but techniques have been developed to detect switching using inverse PCR (62). These techniques have not proved to be sensitive enough for very small cell numbers, and they only indicate that switching has taken place, not when it has occurred. Although the presence of switch circles is an indication of recent switch recombination, the same restrictions apply to their detection by PCR that spans the switch recombination site within the switch circle (63). RT-PCR from intact isolated single cells that have been sorted on the basis of their surface molecules is well established (64–66), but immunohistology in aqueous solutions carried out on cryostat tissue sections where the cell contents are exposed inevitably leads to degradation of the exposed mRNA. Pilot experiments in our laboratory have shown that it may be possible to detect switch transcripts by RT-PCR from RNA isolated from small areas of cryostat tissue sections that have been fixed in ethanol. Immunohistology on these sections has not proved practicable, and cells with a particular phenotype have to be identified in a serial section, reducing the precision of the technique. Nevertheless, it is hoped that it will be possible by these means to analyze switching at different stages in the development of germinal centers and to distinguish follicular from extrafollicular switching. By analysis of the phenotype of cells at the sites of switch recombination, it may be possible to obtain more precise information about the signals that are inducing this process in different situations in vivo.

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