

T Helper 1 (Th1) and Th2 Characteristics Start to Develop During T Cell Priming and Are Associated with an Immediate Ability to Induce Immunoglobulin Class Switching

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Summary

The respective production of specific immunoglobulin (Ig)G2a or IgG1 within 5 d of primary immunization with Swiss type mouse mammary tumor virus [MMTV(SW)] or haptenated protein provides a model for the development of T helper 1 (Th1) and Th2 responses. The antibody-producing cells arise from cognate T cell B cell interaction, revealed by the respective induction of C γ 2a and C γ 1 switch transcript production, on the third day after immunization. T cell proliferation and upregulation of mRNA for interferon γ in response to MMTV(SW) and interleukin 4 in response to haptenated protein also starts during this day. It follows that there is minimal delay in these responses between T cell priming and the onset of cognate interaction between T and B cells leading to class switching and exponential growth. The Th1 or Th2 profile is at least partially established at the time of the first cognate T cell interaction with B cells in the T zone.

The addition of killed *Bordetella pertussis* to the hapten-protein induces nonhapten-specific IgG2a and IgG1 plasma cells, whereas the anti-hapten response continues to be IgG1 dominated. This indicates that a Th2 response to hapten-protein can proceed in a node where there is substantial Th1 activity.

After infection with pathogens such as *Leishmania*, *Listeria*, mycobacteria, or helminths, the T cell response is strongly polarized into either Th1 or Th2 type cytokine secretion; in other responses, however, T cells producing mixed patterns of cytokines are observed (for review see references 1–4). In mice, IFN- γ and IL-4 are characteristic of secretions in Th1 and Th2 responses, respectively (5). IL-4 classically induces sequential switching to IgG1 and then to IgE (6–8), whereas IFN- γ is associated with switching to IgG2a (9). The relative amounts of the different antibody isotypes is governed by signals that control Ig class switch recombination (10, 11). During T cell-dependent antibody responses, switching is heavily dependent upon CD40 ligation (12–15). Although sustained CD40 ligation *in vitro* has been reported to induce class switching by itself (16), cytokines influence extent of switching and the class of antibody produced (5–9).

Ig isotype switching is heralded by the production of switch (germline) transcripts of the C_H region. These start

from an I exon upstream of the appropriate switch region and proceed into the constant region (for review see reference 17). It has been shown that expression of switch transcripts *in vivo* is essential for Ig class switching (18–22), although this is not necessarily sufficient (23).

In a previous study the sites of immunoglobulin class switching were assessed during secondary immune responses to hapten-protein conjugates in the spleen (24). Within 12 h of secondary challenge, antigen-specific memory B cells migrate to the T zones, interact with memory T cells, and start to produce C γ 1 switch transcript. Although these transcripts have also been shown to be produced during the germinal center reaction (25), our studies indicate that the amount of C γ 1 switch transcript recovered per antigen-specific germinal center B cell is <1% of that during the cognate interaction between T and B cells in the T zone (24).

Recently, we have described the primary lymph node response of BALB/c mice to footpad injection with either

haptened protein (4-hydroxy-3-nitrophenyl)acetyl chicken γ globulin (NP-CGG)¹ or Swiss type mouse mammary tumor virus [MMTV(SW)] (26). MMTV(SW) induces both a polyclonal and envelope-specific superantigen-dependent antibody response (27, for review see reference 28). Superantigen-specific T cells start to show signs of activation within 65 h of virus injection (29). T cell proliferation also starts on the third day after immunization in the response to NP-CGG; in both responses this proliferation occurs first in association with interdigitating dendritic cells. This is followed, with minimal delay, by cognate interaction of T cells with B cells and subsequent exponential growth of B cells in the medullary cords (26). Ig class switching in the MMTV(SW) response is predominantly to IgG2a (30, 31); similar early IgG2a production has been seen in response to other viruses (32). In contrast, switching in the response to NP-CGG and similar hapten-protein conjugates is mainly to IgG1 (33–35). In this study the stage of lymphocyte activation is compared with the relative amounts of $C\gamma 1$ and $C\gamma 2a$ switch transcript and IL-4 and IFN- γ mRNA produced in lymph nodes during the responses to MMTV(SW) and NP-CGG. The findings confirm the earlier observation that Th2 cytokine profiles are established at an early stage during immune responses to protein antigens (36–38). This study indicates that Th1 and Th2 cytokine profiles start to be established during T cell priming and that immunoglobulin class switching starts when virgin B cells that have taken up antigen make cognate interaction with primed T cells in the outer T zone.

Materials and Methods

Mice and Immunizations. BALB/c mice were purchased from HO Harlan OLAC Ltd. (Bicester, UK) and kept in isolators under sterile conditions. Immunogens NP-CGG and MMTV(SW) were prepared as described elsewhere (26). Adult mice (6–12 wk) were injected into one hind footpad with MMTV(SW) ($\sim 10^8$ virus particles) and into the other hind footpad with 25 μ g alum-precipitated NP-CGG with or without 5×10^8 chemically killed *Bordetella pertussis* (Evans Medical, Liverpool, UK) or *B. pertussis* alone. Mice received 5-bromo-2'-deoxyuridine (BrdU) 2 h before killing as described (26).

Tissue Preparation. Mice were killed by CO₂ asphyxiation and draining popliteal lymph nodes and spleens were removed. The lymph nodes were put on aluminum foil in a defined orientation, embedded in OCT compound (Miles Inc., Kankakee, IL), and frozen by sequential dipping in liquid N₂. Spleens were put on aluminum foil and snap-frozen by sequential dipping in liquid N₂. Tissues were stored in sealed polythene bags at -70°C until use. 5- μm cryostat sections of the tissue were mounted on four-spot glass slides for immunohistology. After cutting the first eight sections, which were used for immunohistology, one 5- μm section of spleen or three 24- μm sections of lymph node were cut, placed in a polypropylene microfuge tube, and stored at -70°C

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.

Immunohistological Staining. Immunohistological reagents and staining was as described earlier (26). Tissue sections were triple stained for CD3 with IgD and BrdU, double stained for MHC II or syndecan-1 together with BrdU, or double stained for NP-specific cells together with IgM, IgG1, or IgG2a. Additional antibodies used were rat mAbs anti-IgM (LO-MM-9), anti-IgG1 (LO-MG1-2), and anti-IgG2a (LO-MG2a-3; all from Serotec Ltd., Kidlington, Oxford, UK). The primary rat antibodies were detected using biotinylated rabbit anti-rat Ig (Dako Ltd., High Wycombe, UK). NP-binding cells were detected with NP conjugated to sheep anti-human IL-2 IgG (The Binding Site, Birmingham, UK). This antiserum does not react unspecifically with cells of unimmunized mouse lymph nodes (see Fig. 5) or lymph nodes immunized with an unrelated antigen (data not shown). Sheep IgG was detected using biotinylated rabbit anti-goat Ig (Dako Ltd.).

The number of T cells proliferating in the T zone was assessed from sections that were double stained for Ia and BrdU. The Ia staining identifies both interdigitating dendritic cells and B cells. BrdU⁺ nuclei not surrounded by Ia were taken to be proliferating T cells. The location of the T zone was determined on an adjacent section stained for CD3 and IgD; this section could not be used to count proliferating T cells as BrdU⁺ B cells in the T zone surrounded by CD3⁺ cells can not be identified as being CD3⁻ cells. Proliferation of plasmablasts and plasma cells was assessed after double staining for syndecan-1 and BrdU and counting the numbers of BrdU-positive and -negative cells expressing syndecan-1. Switched B cells and plasma cells were identified as cells with cytoplasmic IgG1 or IgG2a in sections double stained with anti-IgG1 or anti-IgG2a and anti-IgD. Switching in NP-specific cells was determined by double staining with NP-conjugated sheep IgG and anti-IgM, anti-IgG1, or anti-IgG2a and counting IgM-, IgG1-, or IgG2a-positive and NP-binding cells. Germinal center sizes were determined on sections triple stained for CD3, IgD and BrdU and measuring of IgD⁻ areas within the IgD⁺ follicles as described in Toellner et al. (24).

Semiquantitative Reverse Transcriptase-PCR. Lymph node sections were allocated random numbers before cDNA preparation and PCR to avoid systematic errors or bias. cDNA from tissues was prepared as previously described (24). cDNAs were diluted to 100 μl with H₂O and stored at 4°C . Mouse β -actin-specific primer sequences were obtained from Stratagene (Cambridge, UK). IL-4- and IFN- γ -specific primers were as described by Svetc et al. (37). Other intron-spanning primers were designed using OLIGO version 5.0 (National Biosciences, Plymouth, MA). $C\gamma 1$ switch transcript-specific primers were (CCTCCTA-GACAAGCACAGGCATGTAGA) and (ACCATGGAGTTA-GTTTGGCAGCAG) specific for the first exon of $C\gamma 1$ switch transcript and the first exon of $C\gamma 1$ constant region (these data are available from GenBank/EMBL/DBJ under accession numbers M12389 and J00453). Primers specific for $C\gamma 2a$ switch transcript were (GTGCCTACCTGCAGCCTGGGAT) located in exon 1 upstream of the first splice site of the $C\gamma 2a$ switch transcript (39) and (CACTGACCACCCGGAGAGTACTGTTG) located in the $C\gamma 2a$ constant region (these data are available from GenBank/EMBL/DBJ under accession number J00470). Primers were synthesized by Life Technologies (Paisley, UK).

To quantitate cDNAs by Southern blot analysis, ~ 10 fewer cycles than would be required to detect the PCR product using

¹Abbreviations used in this paper: BrdU, 5-bromo-2'-deoxyuridine; CCG, chicken γ globulin; MMTV(SW), Swiss type mouse mammary tumor virus; NP, (4-hydroxy-3-nitrophenyl)acetyl.

ethidium bromide gels were done, this was determined in preliminary experiments. For each cDNA sample three amplifications at different cycle numbers were made to improve precision and check that amplification was logarithmic in the range of cycle numbers used. PCR was performed with 2 μ l cDNA template in 20 μ l vol. For amplification of β -actin and C γ 1 switch transcript 0.1 μ l Taq-Polymerase (Promega Corp., Madison, WI) per reaction was mixed with TaqStart antibody (CLONTECH Laboratories, Inc., Palo Alto, CA) 1:1 5 min before use. All other cDNAs were amplified using 0.1 μ l AmpliTaq Gold (Perkin Elmer, Langen, Germany) per reaction. Buffers were used as supplied with the enzymes, plus 1 μ M of each primer, 200 μ M of each dNTP and 2.0 mM MgCl₂ for C γ 1 switch transcript, 2.75 mM MgCl₂ for C γ 2a switch transcript, or 1.5 mM MgCl₂ for the other cDNAs. The reaction mix was overlaid with one drop of mineral oil (Sigma Chemical Co., Poole, England). The three PCRs were performed in 0.2-ml 96-well polypropylene plates in the three blocks of a Touch Down thermal cycler (Hybaid, Middlesex, UK). Cycling was done with an initial denaturation step of 2 min for Taq-Polymerase or 9 min for AmpliTaq Gold, followed by 30 s at 94°C, 30 s at annealing temperature (60°C for β -actin, 65°C for switch transcripts, and 50°C for cytokine mRNA) and 2 min plus 2 s for every cycle at 72°C (3 min for C γ 2a switch transcript). Cycle numbers were 16 \pm 2 for β -actin, 24 \pm 2 for both switch transcripts, 28 \pm 2 for IL-4 mRNA and 31 \pm 2 for IFN- γ mRNA. The PCR product was separated on a 1.5% agarose gel and transferred onto a prewetted Hybond-N+ membrane (Amersham International, Little Chalfont, UK) by capillary transfer

under alkaline conditions (40). The membrane was hybridized with a ³²P-labeled purified PCR product from an earlier PCR as a probe as described earlier (24) and imaged using a PhosphorImager (Molecular Dynamics, Kent, UK).

Using the ImageQuant software (Molecular Dynamics) a grid was laid over the PCR bands, with individual fields covering the central 50% of a band. The signal in each field was calculated and these figures transferred to a spreadsheet software to sort the randomized figures to the correct order. The average of the three PCRs with different cycle number for each gene was taken and divided by the average of the three corresponding β -actin PCRs. These values are equivalent to the relative amount of mRNA for a gene per cell. This was multiplied with the section area, determined by microscopy on adjacent sections using the point counting technique (41), to give the mRNA amount per section.

PCR specific for β -actin, C γ 1 switch transcript, IL-4, and IFN- γ mRNA resulted in single bands and PCR for C γ 2a switch transcript in three bands of the expected size (39). Identity of the PCR products was confirmed by DNA sequencing (Alta Biosciences, Birmingham, UK), using the PCR primers as sequencing primers. The data were controlled for any signs of saturation of the PCR reaction between different cycle numbers. This was done by plotting PCR data from the three PCRs for a particular gene and checking for variations in the efficiency of the PCR amplification over different cycle numbers in samples containing a high amount of target cDNA with samples having low content of target cDNA. The amplification was shown to be logarithmic within the range of cycle numbers used.

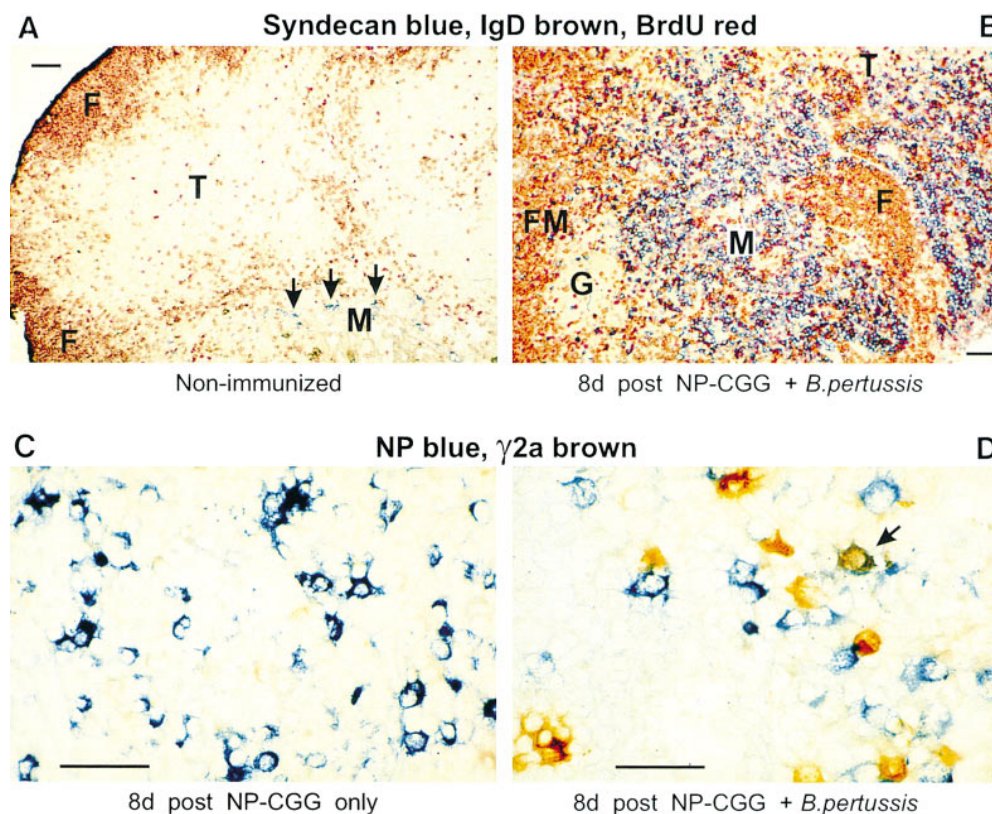


Figure 1. Photomicrographs showing the extrafollicular plasma cells in the response to NP-CGG with or without *B. pertussis*. (A) A typical popliteal lymph node from a nonimmunized, isolator bred, and maintained specific pathogen-free mouse. There are no germinal centers, minimal T zone proliferation, and only occasional plasma cells (arrowed) are seen in the undeveloped medullary cords: F, primary follicles; T, T zone; M, medulla. (B) Section of a mouse popliteal lymph node 8 d after immunization with NP-CGG plus *B. pertussis*. Well-developed medullary cords are present, which are filled with syndeacan⁺ plasma cells (blue). The red nuclear staining marks cells that have taken up BrdU given in the 2 h before the node was taken: FM, follicular mantle; G, germinal center. (C and D) Sections through medullary cords stained to show NP-binding in blue and IgG2a in gold; (C) from a node 8 d after immunization with NP-CGG only; NP-specific plasma cells are present but no IgG2a⁺ cells are seen; (D) from a node 8 d after immunization with NP-CGG with *B. pertussis*; NP-specific cells and non-NP-specific IgG2a⁺ plasma cells are seen; one IgG2a⁺ NP-specific cell is stained dark brown (arrowed). Quantitative data corresponding to the cells shown in C and D are given in Table 1. Bars: (A and B) 100 μ m; (C and D) 50 μ m.

sific cells and non-NP-specific IgG2a⁺ plasma cells are seen; one IgG2a⁺ NP-specific cell is stained dark brown (arrowed). Quantitative data corresponding to the cells shown in C and D are given in Table 1. Bars: (A and B) 100 μ m; (C and D) 50 μ m.

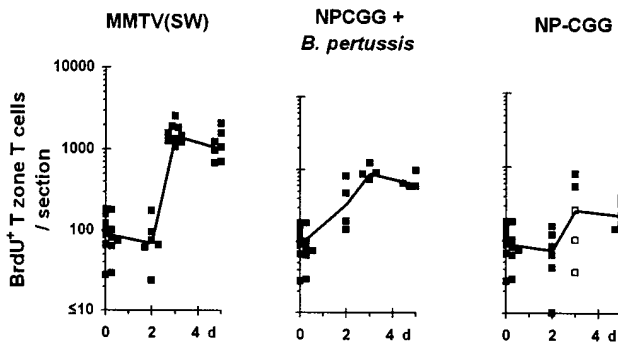


Figure 2. The onset and extent of T cell proliferation in the T zone response to MMTV(SW), alum-precipitated NP-CGG with *B. pertussis*, and alum-precipitated NP-CGG alone. Number of T cells in the T zone are shown that had taken up BrdU during a 2-h pulse before the lymph node was taken. Each point shown represents the value for one mouse. Three of the five mice immunized with alum-precipitated NP-CGG alone 3 d earlier showed T zone T cell proliferation levels that were at or near background levels. The values for these three mice in this and subsequent figures are shown in open squares (\square). Mice were given an i.p. injection of 1 mg BrdU 2 h before the lymph nodes were taken. Cell identification and the counting procedures are described in Materials and Methods. The lines are drawn through the median values at each time point.

Results

T Cell Priming Begins on the Third Day After Immunization. The background level of T and B cell proliferation in popliteal lymph nodes from the isolator bred and maintained mice studied was low (Figs. 1 A and 2). The onset of

T cell priming in the popliteal lymph node response to footpad injection with MMTV(SW) or NP-CGG was assessed by the time when T cell proliferation was first noted in the T zone. The data shown in Fig. 2 confirm our previously published observation (26) that in the response to MMTV(SW) and to alum-precipitated NP-CGG given with *B. pertussis* T cell proliferation starts during the third day after immunization. In that paper flow cytometry studies of lymph node cells in the response to MMTV showed some 60,000 CD4⁺ T cells in cell cycle per lymph node on day 3 and twice this on day 5. After this there was a dramatic fall in the number of proliferating cells. In the NP-CGG response 20,000 CD4⁺ T cells were in cell cycle on day 3 and 60,000 on day 5. After this T cell proliferation stopped in the T zone but some continued in germinal centers. This switch from T zone to follicular T cell proliferation, which coincides with the onset of antibody production, is documented in detail in Gulbranson-Judge et al. (42). In the absence of *B. pertussis* the start of the T cell response to NP-CGG was more variable (Fig. 2); strong T cell proliferation was present in 2 out of 5 animals after 3 d, it was well established in all animals by 5 d.

Plasma Cell Production and Immunoglobulin Class Switching. Very few plasma cells or plasmablasts (Figs. 1 A and 3) were present in the popliteal lymph nodes of nonimmunized control mice. In all responses local plasma cell production was associated with exponential growth of antigen-specific B blasts in the medullary cords. These blasts were most apparent 5 d after immunization (Fig. 3); at this

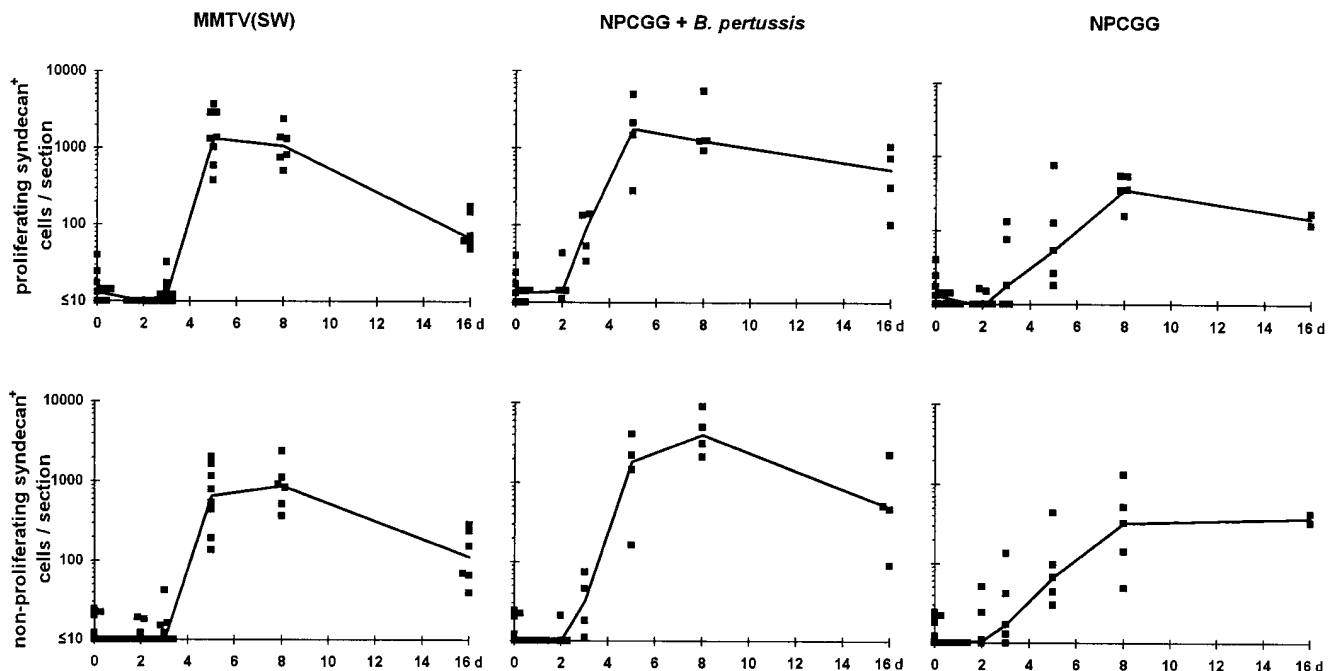


Figure 3. The onset and extent of B cell proliferation and plasma cell formation in response to MMTV(SW), alum-precipitated NP-CGG with *B. pertussis*, and alum-precipitated NP-CGG alone. The upper panels show the number of plasmablasts (syndecan-1⁺, BrdU⁺ cells) in the medullary cords. The lower panels show the number of plasma cells (syndecan-1⁺, BrdU⁻ cells) in the medullary cords. Each square shown represents the value for one mouse. Mice were given an i.p. injection of 1 mg BrdU 2 h before the lymph nodes were taken. Cell identification and the counting procedures are described in Materials and Methods. The lines are drawn through the median values at each time point.

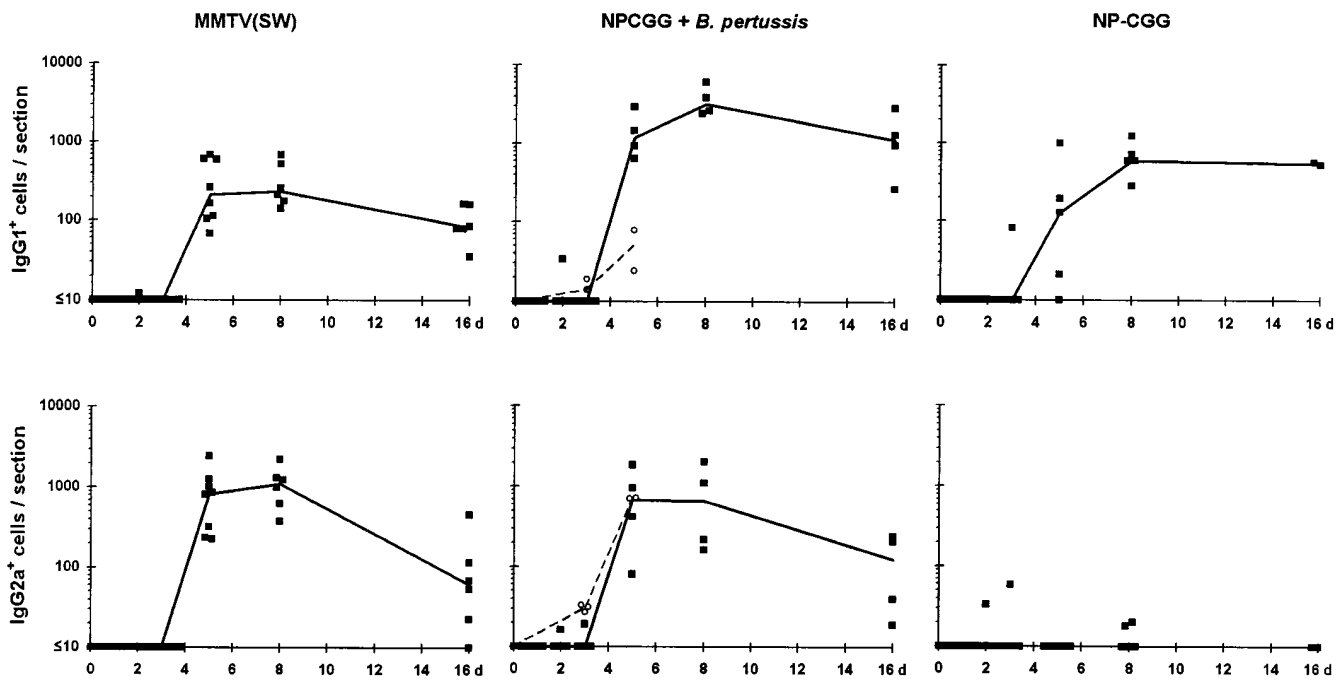


Figure 4. The number of IgG1 and IgG2a containing cells in lymph nodes from mice immunized with: MMTV(SW), alum-precipitated NP-CGG with *B. pertussis* and alum-precipitated NP-CGG alone. The lines are drawn through the median values at each time point. Open circles (○) show the response to *B. pertussis* alone.

stage the commitment of B cells to differentiation to plasma cells was already apparent by their expression of syndecan-1 and specific IgM and IgG antibody is detectable in the serum (26). By 8 d many of these cells had come out of cell cycle and had become fully differentiated plasma cells, which filled the distended medullary cords (Fig. 1 B). Again the response in animals immunized with protein without adjuvant was slightly delayed (Fig. 3).

The response to MMTV(SW) is associated with a bias to IgG2a production with four to five times more IgG2a than IgG1 plasma cells (Fig. 4). In the response to NP-CGG alone switching was almost exclusively to IgG1 (Fig. 4). When *B. pertussis* was added to NP-CGG the amount of IgG1 increased and substantial numbers of IgG2a were produced (Fig. 4). Control immunizations with *B. pertussis* alone showed relatively little IgG1 production, but numbers of IgG2a plasma cells equivalent to those seen in mice immunized with NP-CGG with *B. pertussis* (Fig. 4). Whereas 23–60% of the NP-specific cells were IgG1⁺ on d 5 of the response to NP-CGG with *B. pertussis*, only 0.2–1.8% of the NP-specific cells had switched to IgG2a (Table 1). By day 8 the respective ranges were 80–95% for IgG1 and 0.4–10.6% for IgG2a (Fig. 1, C and D). Thus, factors inducing non-NP-specific plasma cells to switch to IgG2a were only affecting small numbers of the NP-specific cells activated in the same node.

In the response to NP-CGG, antigen-specific B cells and plasma cells were detected and the relative numbers of IgG and IgM NP-specific plasma cells were assessed (Fig. 5). By day 5 up to 40% of the cells containing NP-specific anti-

body had switched in the responses to NP-CGG with or without *B. pertussis*. There was a further increase in the number of switched NP-specific plasma cells with 95% of these being switched in the response with *B. pertussis*.

Switch Transcript Production, Like T Cell Proliferation, Starts During the Third Day After Immunization. Semiquantitative values for the amount of Cγ1 switch transcript and Cγ2a produced in mouse popliteal lymph nodes at intervals after

Table 1. *Ig Class Switching in NP-specific Plasma Cells*

Day after NP-CGG+ <i>B. pertussis</i>	Total number of plasma cells and percentage of these expressing different Ig isotypes			Number of NP-specific plasma cells and percentage of these expressing different Ig isotypes				
	number	μ ⁺	γ1 ⁺	γ2a ⁺	number	μ ⁺	γ1 ⁺	γ2a ⁺
5	3210	26%	45%	30%	328	75%	23%	1.8%
5	1078	34%	59%	7%	157	61%	38%	0.6%
5	5057	45%	18%	37%	270	59%	40%	0.7%
5	4755	30%	61%	9%	968	40%	60%	0.2%
8	2796	9%	86%	6%	525	6%	93%	0.4%
8	7920	11%	76%	14%	1,397	9%	87%	3.5%
8	3060	8%	85%	7%	500	4%	95%	1.2%
8	6645	12%	58%	31%	1,118	10%	80%	10.6%

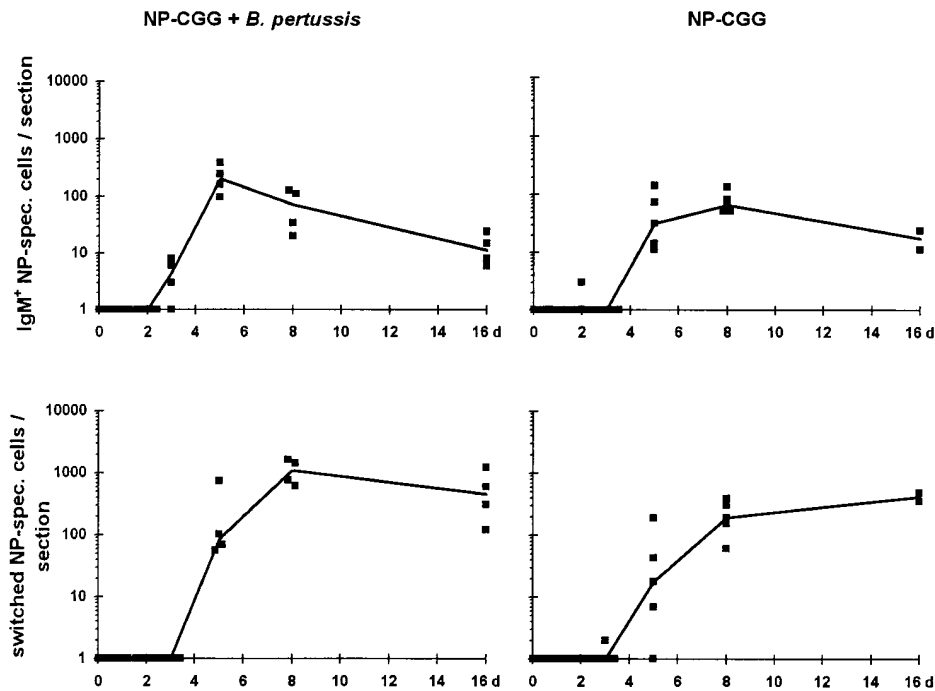


Figure 5. The number of NP-specific IgM⁺ and IgG⁺ cells produced in response to alum-precipitated NP-CGG given with (*left*) or without *B. pertussis*. The lines are drawn through the median values at each time point.

immunization are shown in Fig. 6. Switch transcripts were already easily detectable 3 d after primary immunization and correspond to the immunoglobulin isotype profile of plasmablasts that are seen two days later (Fig. 4). At this stage, in the response to NP-CGG plus *B. pertussis*, NP-specific B cells were identifiable in the T zone. These amounted to <10 cells per section (Fig. 5). The number of nonswitched NP-specific cells increased ~50-fold over the next 2 d through exponential growth in the medullary cords. This growth is reflected in the appearance and increase in proliferating syndecan⁺ cells. These increase some 20-fold between day 3 and 5 in the response to NP-CGG with *B. pertussis* (Fig. 3 and reference 26). Importantly the level of C γ 1 or C γ 2a switch transcripts less than doubles over that period (Fig. 6), indicating that the main production of switch transcripts is at the time of cognate B cell T cell interaction as opposed to the period of B cell growth in the medullary cords. Three of the five mice given alum-precipitated NP-CGG without *B. pertussis* did not show T cell proliferation 72 h after immunization; C γ 1 switch transcript in these mice was at background levels. On the other hand, in the two mice where T cell proliferation in the T zone had started switch transcript production was apparent. A consistent finding is that switch transcript levels only rise above background levels in nodes where T cell proliferation has already started. Equally no mice were found where T cell proliferation had started in the absence of detectable switch transcript levels. These two processes evidently start well within 24 h of each other.

mRNA for Cytokines Associated with Class Switching Is Upregulated as T Cells Start to Proliferate. IL-4 message was upregulated from the day T cell proliferation was first noted in nodes draining the site of immunization with

alum-precipitated NP-CGG with or without *B. pertussis* (Fig. 7). By contrast IL-4 message increased later and to a lesser extent in the nodes draining the site of MMTV(SW) injection. This correlated with relatively modest production of C γ 1 switch transcript in this response.

The upregulation of IFN- γ message was less impressive than that for IL-4. Nevertheless significant elevation of IFN- γ message occurred in the responses associated with switching to IgG2a, MMTV(SW) and NP-CGG with *B. pertussis*. By contrast in the response to NP-CGG alone where IgG2a plasma cells did not appear IFN- γ message levels remained in the control range. The time of upregulation of cytokine message is sufficiently early in the response to be consistent with the concept that Ig class switching is induced by cognate T cell interaction in the T zone and is influenced by the production of these cytokines at that stage.

Germinal Center Formation. Developing germinal centers were identifiable by day 5 in the NP-CGG responses but their development was delayed by some days in the MMTV(SW) response (Fig. 8). In all groups the germinal center size at d 16 was similar to or greater than that at d 8. The onset of switch transcript production antedates germinal center formation and the time when centrocytes are selected in germinal centers. Nevertheless the persistence of switch transcripts through day 16 suggests that there is continued switching in germinal centers.

Control Immunizations. Footpad immunization with *B. pertussis* alone induced C γ 2a switch transcripts (Fig. 6) and IFN- γ mRNA (Fig. 7) at the times and levels comparable to those induced by immunization with NP-CGG plus *B. pertussis*. Mice immunized with *B. pertussis* alone showed some increase in C γ 1 switch transcript levels and IL-4

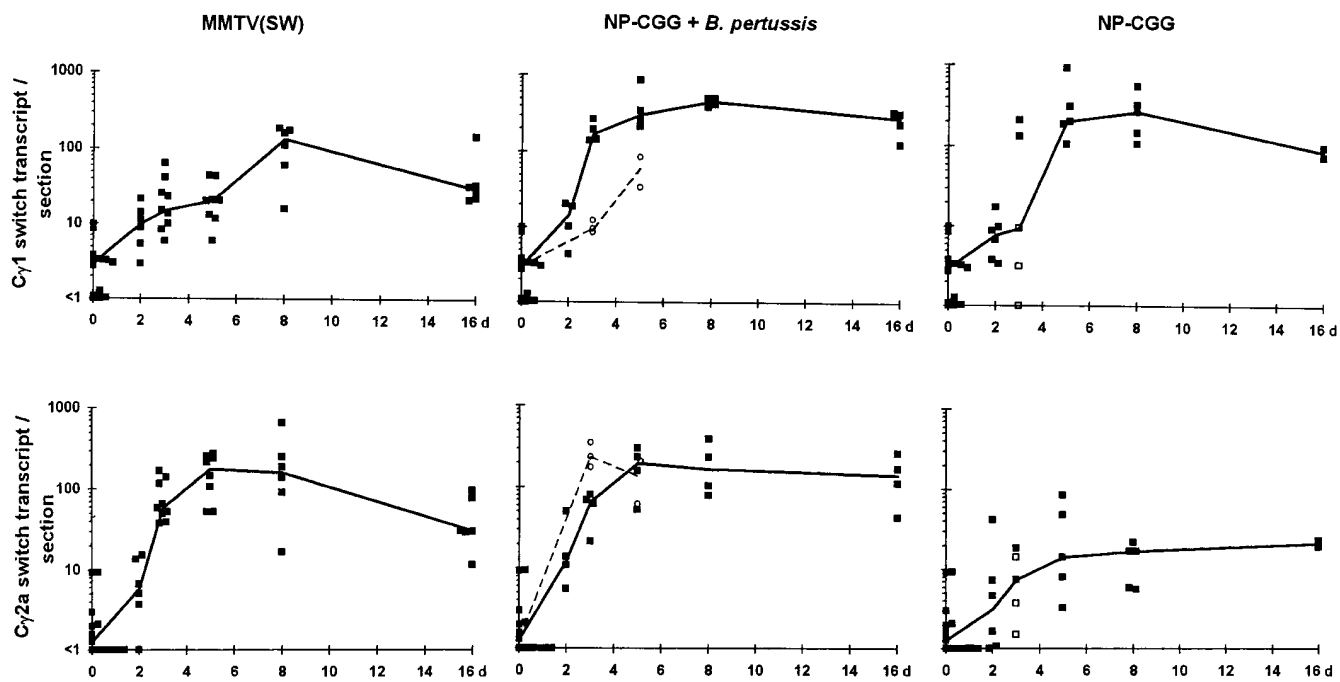


Figure 6. Differential induction of C γ 1 and C γ 2a switch transcript in groups of mice immunized with alum-precipitated NP-CGG with or without *B. pertussis* or MMTV(SW). Symbols show the amount of switch transcript per lymph node section of individual mice determined by semiquantitative reverse transcriptase-PCR (see Materials and Methods). Three of the five mice immunized with alum-precipitated NP-CGG alone 3 d earlier showed T zone T cell proliferation levels that were at or near background levels, the values for these mice are shown in open squares (□) as in Fig. 2. Open circles (○) show the response to *B. pertussis* alone. Lines are drawn through the median values at each time point.

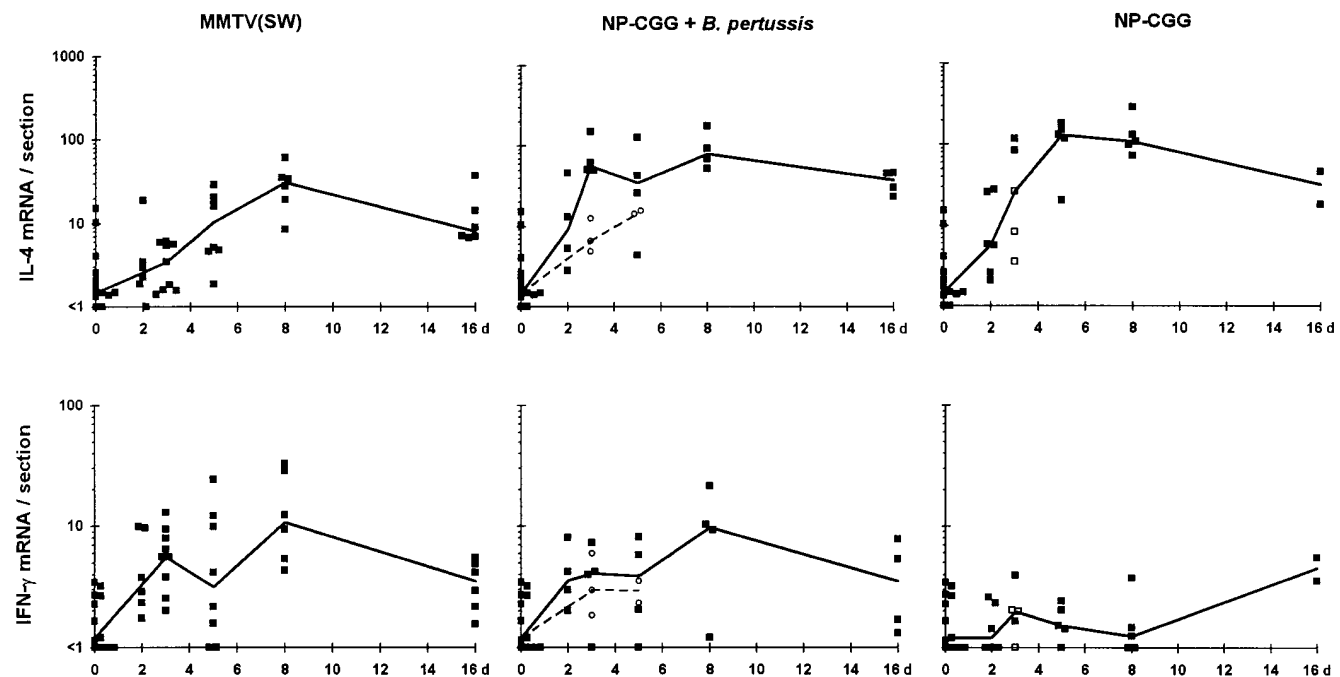


Figure 7. Differential induction of IL-4 and IFN- γ mRNA in groups of mice immunized with alum-precipitated NP-CGG with or without *B. pertussis* or MMTV(SW). Amount of cytokine mRNA per lymph node section determined by semiquantitative reverse transcriptase-PCR. Open squares (□) indicate mice which had not shown T cell proliferation on day 3 (Fig. 2). Open circles (○) show the response to *B. pertussis* alone. The lines are drawn through the median values at each time point.

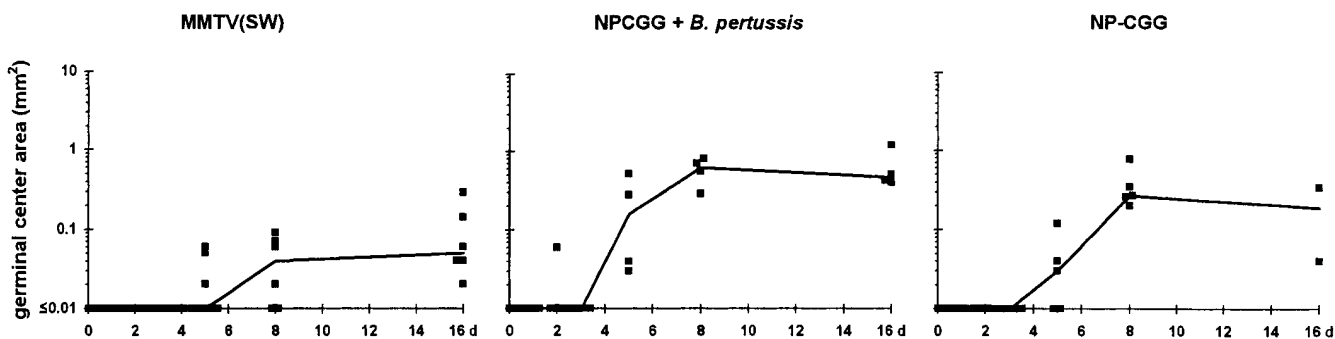


Figure 8. The development and size of germinal centers during primary responses to: MMTV(SW), alum-precipitated NP-CGG with *B. pertussis*, and alum-precipitated NP-CGG alone. Germinal centers were identified in sections triple stained for IgD, CD3, and BrdU. Germinal centers are IgD⁻ areas surrounded by IgD⁺ follicular mantle B cells and contain proliferating BrdU⁺ cells and only scattered T cells. The lines are drawn through the median values at each time point.

mRNA levels on d 5, but no significant increase above background of either of these on d 3 (Fig. 6 and 7).

Spleens taken 3 and 5 d after footpad immunization with MMTV(SW) and NP-CGG with *B. pertussis* were analyzed for NP-specific cells, switch transcripts, and cytokine mRNA. NP-specific cells B cell numbers in the spleen did not increase above the extremely low levels seen in nonimmunized mice and were also not found in the popliteal lymph node draining the site of MMTV(SW) injection (data not shown). Levels of C γ 1 and C γ 2a switch transcript and IFN- γ and IL-4 message from the d 3 and 5 spleens of these isolator reared mice remained in the range seen in nonimmunized mice (data not shown).

Discussion

Early Commitment to Th1 and Th2 Patterns of Response. The time when the Th1 and Th2 cytokine profiles, respectively, start to develop in response to MMTV(SW) and NP-CGG has been determined by assessing the time when IL-4 and IFN- γ message increases in primary immune responses. This occurs during the third day after immunization when T cells first start to proliferate in association with interdigitating dendritic cells (26). Cognate interaction between T and B cells leading to B cell growth and the production of switch transcripts occurs on the same day, presumably shortly after the onset of T cell priming. The functional significance of the switch transcripts observed is indicated by the development of switched plasmablasts by day 5 after immunization. In the response to NP-CGG it is theoretically possible that T cell priming started before the onset of a detectable increase in T cell proliferation. This could apply if the number of antigen-reactive T cells initially was very low. This reservation does not apply to the response to MMTV(SW) where 10% of T cells are superantigen-reactive through their expression of V β 6 (26). The availability of so many antigen-specific T cells allows the timing of the onset of the priming process to be predicted with confidence.

The different switch transcript and cytokine profiles that appear during the responses to MMTV(SW), NP-CGG

with *B. pertussis* and NP-CGG alone indicate that Th1 and Th2 characteristics start to develop as T cells are primed or very shortly after this. These observations indicate that Th1 or Th2 characteristics can be exhibited by cells that have not gone through an uncommitted Th0 phase of proliferation with the production of both IL-4 and IFN- γ (43, 44). The Th cells functioning early in the primary response may undergo subsequent alteration, but as the Th1 and Th2 cytokines are self-reinforcing (44) the initial pattern is likely to have an important impact on the way a response becomes established.

Studies of the primary responses in lymph nodes to KLH have reported the early development of Th2 cells. Upregulation of IL-4 mRNA was noted on the third day after immunization and IL-4 producing cells could be cultured from this time (36, 37). In a recent study, Nakamura et al. (45) reported that naïve T cells cultured with Con A initially upregulate both IFN- γ and IL-4 message, but when IL-4 is present in the culture the IL-4 message was selectively retained while the IFN- γ message is lost within 48 h. The converse is the case when IL-12 is added to cultures. The move to IL-4 production has been associated with continued expression of the transcription factor GATA-3 while this is not expressed in Th1 cells (46). It would be of interest to assess the expression of this transcription factor in T cells proliferating in the T zone on the third day of the test responses analyzed in this study.

The early differentiation of Th1 and Th2 characteristics raises the possibility that this behavior initially is established by signals delivered by the interdigitating dendritic cells in the T zone and that their precursor Langerhans cells in turn acquire the ability to deliver these signals in the site where they are induced to take up and process antigen. Thus, tissue Langerhans cells are induced to take up and process antigen following local tissue injury (47); LPS, IL-1, and TNF- α induce this behavior and the migration of the activated cells to lymphoid tissues (48, 49). The perturbation that leads to the activation of Langerhans cells may also induce local cytokine production that influences the way differentiation to interdigitating dendritic cells occurs. Mast cells may release IL-4 after mechanical disruption or C3a-

or IgE-induced degranulation, CGG might have induced complement fixation and IFN- γ may be released by a range of cells including NK cells.

De Smedt et al. (50) found that interdigitating dendritic cells induced to differentiate from Langerhans cells in the presence of IL-10 failed to prime T cells to differentiate into Th2 cells. Differential CD80 and CD86 induction during Langerhans cell maturation has been described under the influence of Th1 and Th2 cytokines (51). Both CD80 and CD86 were found to be upregulated in the presence of IL-4; CD80 was seen to be downregulated by IL-10 or IFN- γ and CD86 expression reduced by IL-10 but not IFN- γ . The level of IL-12 produced and released by IDC may also be influenced by the conditions of Langerhans cell activation (52).

Cells other than interdigitating dendritic cells that might influence the very early differentiation of Th cells in primary responses include bystander CD4 or CD8 T cells, NK cells, NK1.1 T cells, B cells, macrophages, or mast cells. In the response to *Leishmania major* V β 4V α 8-expressing CD4 T cells produce large amounts of IL-4 within 90 min of injection of LACK protein or after infection in susceptible but not resistant mice (53). *Staphylococcal enterotoxin* superantigens have been found to activate CD8 T cells expressing the appropriate V β despite the association of the superantigen with MHC class II molecules (54, 55). This stimulation would be likely to induce IFN- γ release, but in MMTV(SW) infection the superantigen has not been seen to activate V β 6-expressing CD8 T cells (28, 29).

The finding that *B. pertussis* induces a substantial level of switching to IgG2a without markedly deviating the overwhelming IgG1 predominance of the response to NP-CGG suggests that bystander effects were, at best, small in this study. This observation may reflect a very short range effect of cytokines. Cytokines have been shown to be preferentially released at the site of contact between T and B cells during cognate interactions. In this situation there is likely to be a highly selective influence on the cell that is recognized specifically (56, 57). It will be important in future studies to attempt to visualize cytokine protein production and release in vivo in relation to cognate T cell B cell interactions. Although this is possible in intact cell conjugates formed in vitro it remains a technical challenge to reproduce these studies consistently in tissue sections.

NK1.1 T cell (58, 59) and NK cell (60) activity in lymph nodes is generally low, but even rare cells could have a marked effect locally. Mast cells in lymph nodes are generally confined to the medulla. In this study no direct information about the cells that are producing cytokine message is available. Although this is a technically difficult area of investigation, information is required about the cytokines that are produced in the series of microenvironments that provide the theater for immune responses: (a) the site of immunization, (b) the T zone during T cell priming, (c) at the edge of the T zone during cognate interaction between T and B cells, and (d) in follicles and extrafollicular sites of exponential B cell growth and differentiation.

Class Switching During Primary Cognate Interaction between T Cells and B Cells. When the numbers of antigen-spe-

cific B cells found in lymph node sections are compared to the amount of switch transcript in adjacent sections, it is seen that C γ switch transcript levels per cell are greatest while B cells undergo their first cognate interaction with T cells in the T zone on day 3 after immunization. This correlates with findings from studies in vitro, where signals from T cells like CD40 ligation on B cells can induce Ig class switching efficiently (15, 61). It is also similar to findings in secondary immune responses, where the highest amount of switch transcript per antigen-specific B cell is found during the first cognate interaction in the T zone (24). Switching during cognate interaction in the T zone inevitably has a major impact on the Ig classes and subclasses produced during a primary or secondary immune response because these B cells subsequently undergo exponential growth both within and outside follicles (24, 62, 63).

Ig class switching has also been shown to occur in germinal centers (25). Continued switch transcript production at 16 d in the primary responses reported here is likely to be occurring as centrocytes are selected in germinal centers (25). There is also less switch transcript found on day 16 in the MMTV(SW) response where germinal centers are much smaller and the increase of switching to IgG1 in this response occurs as germinal centers are formed. Although the number of antigen-specific cells in germinal centers at day 16 is much greater than the number present in the lymph nodes 3 d after immunization, the amount of switch transcript recovered at these two times is comparable. B cells of human tonsil undergoing T-B interaction in the T zone have not been isolated and there is no information about whether they undergo switch recombination (25). The impact of switching in centrocytes may be lower, as B cells that have been positively selected in germinal centers are only likely to undergo further proliferation if they are reactivated by antigen.

Ig class switching has also been reported to occur in sites of inflammation. This is exemplified by switching to IgE in the nasal mucosa in patients with allergic rhinitis (64). Switching to IgE is likely to be a secondary switching event after previous switch to IgG1 (6-8).

In a previous study of the primary splenic response to intraperitoneal NP-CGG (24), markedly lower levels of switch transcript production were detected per unit amount of β -actin than in the primary lymph node response. This may reflect in part a higher proportion of cells involved with class switching in lymph nodes, which lack the large red pulp component of the spleen. Ig class switching in the spleen is occurring later after primary immunization with NP-CGG (63) than in lymph nodes. The slower rate of T cell priming that occurs in the spleen (65) compared to lymph nodes (66) may be associated with a lower number of cognate T cell B cell interactions occurring at any one time.

It is perhaps surprising that there is little change in the level of switch transcript production between days 3, 5, and 8 after immunization, for there is massive exponential growth of B cells in the medullary cords and follicles during this period and Ig class switching has been associated to

B cell proliferation (10, 67, 68). New antigen-specific virgin B cells are likely to continue to arrive in the node for several days after immunization. Some of these will be derived from recirculating cells arriving from distant lymphoid tissues (69) and others will be newly produced virgin B cells (70). These cells are likely to make cognate interaction with primed T cells in the node draining the site of immunization. The rate of these cognate interactions might be expected to be relatively constant in the first 5 d after immunization until sufficient amounts of antibody are pro-

duced to bind free antigen. Most of the switching observed during this period may be occurring in B cells during these cognate interactions rather than in their proliferating progeny. This tentative conclusion suggests that the dual signals provided by CD40 ligation and cytokines are delivered by the interacting primed T cell. Other cytokines produced by the responding B cells and adjacent interdigitating dendritic cells may also contribute, but cytokine influence during the subsequent growth phase of the B cells may have a relatively small effect on Ig class switching.

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